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N-acetylcysteine supplementation improves endocrine-metabolism profiles and ovulation induction efficacy in polycystic ovary syndrome

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Abstract

Background Polycystic ovary syndrome (PCOS) affects 6–20% of women worldwide, with insulin resistance and hyperinsulinemia occurring in 50-70% of patients. Hyperinsulinemia exacerbates oxidative stress, contributing to PCOS pathogenesis. N-acetylcysteine (NAC) is an antioxidant and insulin sensitizer that shows promise as a therapeutic for PCOS. Our current study aimed to investigate the effects of NAC supplementation on endocrine-metabolic parameters in PCOS mice and its effect on ovulation induction (OI) efficacy in women with PCOS.

Methods Female C57BL/6 mice were orally administered letrozole (LE) to induce PCOS and then randomly divided into groups receiving daily oral administration of 160 mg/kg NAC (PCOS + NAC group), 200 mg/kg metformin (PCOS + Met group), or 0.5% carboxymethyl cellulose (drug solvent) (pure PCOS group) for 12 days. Healthy female mice served as pure controls. Estrous cycles were monitored during the intervention. Metabolic and hormone levels, ovarian phenotypes, antioxidant activity in ovarian tissues, and oxidative stress levels in oocytes were assessed post-intervention. Furthermore, a pragmatic, randomized, controlled clinical study was conducted with 230 PCOS women, randomly assigned to the NAC group (1.8 g/day oral NAC, $n = 115$) or the control group ($n = 115$). Patients in both groups underwent ≤ 3 cycles of OI with sequential LE and urinary follicle-stimulating hormone (uFSH). Cycle characteristics and pregnancy outcomes were compared between groups.

Results Similar to metformin, NAC supplementation significantly improved the estrous cycles and ovarian phenotypes of PCOS mice; reduced the LH concentration, LH/FSH ratio, and T level; and increased glucose clearance and insulin sensitivity. Notably, NAC significantly reduced oocyte ROS levels and increased the mitochondrial membrane potential in PCOS mice. Additionally, NAC significantly enhanced enzymatic and nonenzymatic antioxidant activities in PCOS mouse ovaries, whereas metformin had no such effect. In the clinical trial, compared to women in the control group, women receiving NAC had significantly lower average uFSH dosage and duration ($p < 0.005$) and significantly greater clinical pregnancy rates per OI cycle and cumulative clinical pregnancy rates per patient ($p < 0.005$).

Conclusion NAC supplementation improved endocrine-metabolic parameters in PCOS mice and significantly enhanced OI efficacy with sequential LE and uFSH in women with PCOS. Therefore, NAC could be a valuable adjuvant in OI for women with PCOS.

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Keywords Polycystic ovary syndrome, N-acetylcysteine, Insulin resistance, Metformin, Oxidative stress, Ovulation induction, Pregnancy rate

Background

Polycystic ovary syndrome (PCOS) is a heterogeneous familial disorder [1]. Depending on different diagnostic criteria, the prevalence among reproductive-age women fluctuates between 6% and 20% [2]. From an evolutionary medicine perspective, the overlap of risk alleles for PCOS between European and Chinese women suggests that PCOS is an ancient disorder [3]. Although PCOS has been studied extensively over the years, its pathogenesis remains largely unclear. Recent research suggests that insulin resistance, oxidative stress (OS) and mitochondrial dysfunction play crucial roles in the development of the disorder [4, 5].

Oxidative stress (OS), defined as an imbalance between oxidants and antioxidants, is commonly linked to the overproduction of reactive oxygen species (ROS). Numerous studies have shown that markers of oxidative damage are significantly elevated in the serum of PCOS patients compared to healthy subjects [6, 7]. Excessive ROS levels can damage mitochondrial DNA (mtDNA), resulting in mitochondrial dysfunction and impaired cellular energy production, which contribute to the metabolic and hormonal imbalances characteristic of PCOS. In turn, dysfunctional mitochondria generate even more ROS, perpetuating a vicious cycle. Additionally, oxidative stress and mitochondrial dysfunction further disrupt energy and metabolic regulation, leading to insulin resistance and hyperandrogenism, which are key pathological mechanisms in PCOS development [4, 5]. Consequently, similar to the use of insulin sensitizers and anti-androgen therapies, the exploration of antioxidants for the treatment of PCOS has become a significant area of current research.

While Metformin is currently the most frequently recommended insulin sensitizer for PCOS treatment [8], it is associated with notable side effects. Over 30% of patients experience noticeable gastrointestinal side effects during the initial treatment period, including bloating, diarrhea, constipation, nausea, vomiting, and an increase in serum homocysteine levels, which may lead to intolerance and discontinuation of the drug [9]. Therefore, clinicians have been actively exploring new drugs with improved tolerability and multiple therapeutic effects for treating PCOS. Among them, N-acetylcysteine (NAC) is considered a promising drug with potential applications [10]. NAC is the acetylated precursor of L-cysteine and reduced glutathione. It has been established as a potent cell-permeable antioxidant that effectively prevents cell apoptosis and promotes cell survival through antioxidant stress [11, 12]. The antioxidant activity of NAC is attributed to its thiol group, which enhances the activity of glutathione S-transferase, thereby protecting target cells and cell membranes [13]. In animal experiments, Fan et al. showed that NAC protects against oxidative stress toxicity and mitochondrial functional damage induced by repeated ovulation stimulation [14]. Furthermore, in vitro studies have demonstrated that NAC activates insulin secretion in pancreatic cells [15] and modulates insulin receptors in human erythrocytes [16]. However, there is limited research on the effects of NAC on endocrine-metabolic profiles and ovarian antioxidant enzymatic systems in PCOS animal models.

In addition, several clinical studies have explored the effects of NAC supplementation in various induction ovulation (OI) protocols for women with PCOS. In a randomized controlled trial (RCT) involving 150 PCOS women with clomiphene citrate (CC)-resistant, Rizk AY et al. [17] reported that the use of NAC (1.2 g/day) as an adjuvant to CC significantly increased ovulation and pregnancy rates compared to the use of a placebo (1.3% vs. 49.3%, 0 vs. 21.3%) [17]. However, in a 2017 RCT, Behrouzi Lak et al. reported that NAC supplementation did not improve clinical pregnancy rates in CC combined with letrozole (LE) induction of ovulation followed by intrauterine insemination. However, it is important to note that the study had a small sample size (only 97 participants), and the NAC supplementation group already showed a trend toward higher clinical pregnancy rates (32.7% vs. 18.8%) [18]. Therefore, further clinical studies with sufficient sample sizes are needed to explore the application of NAC supplementation in OI for women with PCOS.

In this study, we initially investigated the effects of NAC supplementation on endocrine-metabolic parameters and ovarian/oocyte oxidative stress responses in LE-induced PCOS mice through in vivo experiments. Furthermore, a pragmatic clinical trial (PCT) was conducted to assess the impact of NAC supplementation on ovulation induction (OI) cycles and clinical pregnancy outcomes in PCOS patients with anovulation or oligo-ovulation, following sequential LE and urinary follicle-stimulating hormone (uFSH) treatment. The aim of the current study was to evaluate the effects of NAC supplementation on endocrine-metabolic parameters in PCOS mice and its efficacy in improving OI outcomes in women with PCOS.

Materials and methods

PCOS mouse model and grouping intervention

Three-week-old SPF-grade C57BL/6 female mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and housed in the Laboratory Animal Center of Huazhong University of Science and Technology. The mice were maintained at a constant temperature of 26 °C with a 12-hour light/dark cycle (light period: 07:00 to 19:00) and had ad libitum access to food and water. After one week of acclimatization, 15 mice were randomly selected to constitute the control group. The mice were administered 0.5% carboxymethyl cellulose (CMC; Shanghai Biochemical Technology Co., Ltd., Shanghai, China) solution (1 mg/kg) via gavage for 21 consecutive days, followed by no further treatment for the next 12 days. The remaining mice were used to construct the PCOS model using LE, following methods reported in previous literature [19, 20]. Briefly, LE (Furui, Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) was dissolved in a 0.5% CMC solution and administered by gavage at a dosage of 1 mg/kg/day for 21 consecutive days to induce the PCOS model. During the last 10 days of LE administration, the estrous cycles of the mice were monitored daily. After 21 days of LE treatment, three mice from the control group and three from the PCOS model group were randomly selected at the diestrus stage and anesthetized with isoflurane, and blood samples were collected from the retro-orbital plexus. The mice were then euthanized by cervical dislocation, and ovarian tissues were collected to evaluate the success of the PCOS model.

The successfully generated PCOS model mice were then randomly divided into three groups: a pure PCOS group (model control), a PCOS + metformin (Met) group, and a PCOS + NAC group. Starting on day 22, the three groups of PCOS mice were administered 0.5% CMC, 200 mg/kg/day metformin (Shanghai Squibb Pharmaceutical Co., Ltd.), or 160 mg/kg/day NAC via gavage for 12 days. In this study, a dose of 160 mg/kg of NAC was selected based on the dosing regimens reported in previous studies by Rafiee et al. [21] and Mahmoodi et al. [22]. During the intervention period, estrous cycle monitoring continued. After the intervention, six mice from each group were euthanized in the same manner as described above, and blood samples and ovarian tissues were collected. The remaining six mice in each group were treated with pregnant mare serum gonadotropin (PMSG) to obtain oocytes. Due to the inability to determine the standard deviation and effect size, the sample size for this study was determined using Mead's method for variance analysis [23]. The experiment involved four groups: Control, PCOS + NAC, PCOS + Met, and PCOS. According to Mead's principle, the error degrees of freedom (E) should be between 10 and 20 to ensure reliable statistical analysis. This calculation yielded a sample size of 4 to 6 animals per group. However, since both blood sample collection and oocyte retrieval require sacrificing animals in two phases post-intervention, the sample size was determined to be 12 animals per group. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (approval number: 2585) and were conducted in accordance with international ethical guidelines and the relevant requirements of the ethics committee of Huazhong University of Science and Technology.

Mouse estrous cycle assessment

Vaginal secretions were collected daily between 12:00 and 13:00 from each group of mice and smeared onto slides. These slides were then examined under an optical microscope to determine the stage of the estrous cycle for each mouse. Diestrus was characterized by predominantly leukocytic vaginal smears, while proestrus exhibited a high concentration of nucleated cells. The estrus was typified by vaginal smears primarily composed of cornified epithelial cells, whereas metestrus displayed the presence of both cornified epithelial cells and leukocytes.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

Prior to the glucose tolerance test, the mice were fasted for 16 h (from 17:00 to 09:00 the next day) with ad libitum access to water. Blood samples were drawn from the tail tips of the mice before and 30–60, 90, and 120 min after intraperitoneal administration of D-glucose (2.0 g/kg body weight). The blood glucose levels were then tested with an Accu-Chek glucose monitoring system (Roche Diagnostics). In the insulin tolerance test (ITT), mice were fasted for four hours (with ad libitum access to water) before receiving an intraperitoneal injection of insulin (1 U/kg body weight). Blood glucose levels were monitored at 0, 15, 30, and 45 min after insulin infusion.

Infrared thermography and core body temperature measurement

Infrared thermography and core body temperature measurement are commonly used methods for studying energy metabolism and metabolic phenotypes in animals. These indicators can be employed to assess the energy expenditure of mice [24]. A brief outline of the procedures for both methods is provided below. Mice were individually housed in cages and subjected to a 4 °C cold chamber for a maximum of 4 h, with continuous access to food and water. Images were captured using an infrared digital thermal camera (E60: compact infrared thermal imaging camera; FLIR), and the data were analyzed

using FLIR Quick Report software (FLIR ResearchIR Max 3.4; FLIR). The core body temperature of each group of mice was assessed using rectal probes connected to digital thermometers.

Assessment of hormone levels in mouse blood

Isoflurane was administered to anesthetize the mice before blood samples were collected from the retro-orbital plexus. After blood collection, the samples were left at room temperature for no more than 2 h, followed by centrifugation at 3000 rpm for 15 min. The resulting supernatant (plasma) was collected and stored at -80 °C for subsequent analysis. Plasma concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (T) were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Quanzhou RuiXin Biotechnology Co., Ltd., Fujian, China). The sensitivities of the ELISA kits for LH, FSH, and T were 0.1 mIU/L, 0.1 mIU/mL, and 0.1 ng/mL, respectively. The intra- and interassay coefficients of variation for all three kits were less than 10%.

Mouse ovarian tissue sectioning and staining analysis

Ovarian tissues from each group of mice were preserved overnight in 4% formaldehyde at room temperature. Following embedding in paraffin, the tissues were sectioned serially and then stained with hematoxylin and eosin (H&E). H&E staining was carried out as previously described [25]. Briefly, the mouse ovaries were halved and fixed in 4% paraformaldehyde. Following embedding in paraffin wax, the tissue was sliced into 5 μm thick sections and mounted onto glass slides. These sections then underwent a series of steps, including deparaffinization, hydration, H&E staining, dehydration, and mounting. Subsequently, images were captured under a microscope, and the number of follicles and corpora lutea at each stage was determined. Ovarian follicles and corpora lutea were classified using Pederson's classification system [26]. Briefly, primordial follicles are described as having a compact oocyte surrounded by a single layer of flattened granulosa cells (GCs). Primary follicles are identified by an enlarged oocyte encircled by a single layer of cuboidal GCs. Secondary follicles are defined by an enlarged oocyte surrounded by at least a partial or complete second layer of cuboidal GCs. Antral follicles are characterized by the presence of areas of follicular fluid or a single large antral space. To avoid double counting, primordial, primary, and secondary follicles were counted once every 10 consecutive sections, while antral follicles were counted once every 40 consecutive Sect. [27].

Mouse oocyte collection

After intraperitoneal injection of 10 IU PMSG, mice from each group received an intraperitoneal injection of 5 IU human chorionic gonadotropin (HCG) 48 h later. Approximately 14–16 h post-HCG administration, the mice were euthanized by cervical dislocation. Cumulus-oocyte complexes (COCs) were then retrieved from the ampullary region of the fallopian tubes. The collected COCs were transferred to a growth medium containing hyaluronidase (Solarbia, catalog number H8030) to remove the surrounding GCs. Mature mouse oocytes at the metaphase II (MII) stage were obtained, washed, and placed in M16 medium (Sigma–Aldrich). Subsequently, the oocytes were cultured at 37 °C in a 5% CO2 incubator.

Quantification of mitochondrial membrane potential (MMP)

The oocyte MMP was measured as described in previous studies [28]. Oocytes were exposed to a JC-1 working solution (Beyotime Biotech, Shanghai, China) and maintained at 37 °C for 20 min. After two washes with JC-1 staining buffer (Beyotime Biotech, Shanghai, China), the oocytes were examined using a fluorescence microscope. In mitochondria with low membrane potential, the JC-1 probe exists in its monomeric form, emitting green fluorescence. Conversely, in mitochondria with high membrane potential, the JC-1 probe underwent J-aggregation, emitting red fluorescence. The degree of mitochondrial depolarization was assessed by the ratio of red to green fluorescence.

Quantification of ROS levels

Oocytes were incubated in a dark environment with a diluted solution of the fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Beyotime Biotech, Shanghai, China) at 37 °C for 20 min. After being washed with M2 media, images were captured using a fluorescence microscope. Upon exposure to reactive oxygen species (ROS), DCFH emits green fluorescence, and the intensity of the fluorescence directly correlates with the ROS level.

Biochemical analysis

Mouse ovarian tissue was rapidly homogenized in an ice-cold 0.9% NaCl solution (10% w/v). The supernatant from centrifugation (3500 rpm, 15 min, 25 °C) was utilized for biochemical analysis. The tissue protein concentration was determined using bovine serum albumin as a standard. The activities of reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) in mouse ovarian tissue

homogenates were measured using assay kits (Beyotime Biotech, Shanghai, China) following the protocols provided by the manufacturer.

Pragmatic clinical trial and participants

PCT was conducted at Union Hospital, Tongji Medical College, Huazhong University of Science and Technology to evaluate the effects of NAC supplementation in OI for PCOS patients with anovulation or oligo-ovulation. Participants were responsible for covering the costs of examinations and medications, with no additional compensation provided. The study received approval from the medical ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval number 2023 − 0353; approval date June 6, 2023) and was registered on the Chinese Clinical Trial Registry (www.chictr.org.cn; trial registration number: ChiCTR2300077709, registration date: July 1, 2023.). All participants provided informed consent before enrolling in the study. Detailed information about the trial protocol is available in the Chinese Clinical Trial Registry (https://www.chictr.org.cn/bin/project/edit?pid=205777).

The study recruited PCOS patients suffering from anovulation or oligo-ovulation. The diagnosis of PCOS was based on the modified Rotterdam criteria [29], requiring the fulfillment of any two of the following three criteria: oligo-ovulation or anovulation, clinical or biochemical signs of hyperandrogenism, and polycystic ovaries, while excluding diseases such as congenital adrenal hyperplasia or tumors, folliculogenesis abnormalities, Cushing's syndrome, and androgen-secreting ovarian tumors. Additionally, the subjects met the following inclusion criteria: ① aged ≥ 21 years and ≤ 38 years. ② Observation of at least one patent fallopian tube during hysterosalpingography or laparoscopy. ③ A BMI < 35 kg/m^2. ④ A male partner must have a minimum sperm concentration of 15 million per milliliter, based on the World Health Organization's criteria. ⑤ Neither the patients nor their male partners experienced any sexual dysfunction, and both agreed to engage in regular intercourse to achieve pregnancy. The exclusion criteria were as follows: ① Infertility due to causes other than PCOS-related ovulatory disorders. ② Abnormal thyroid-stimulating hormone levels. ③ Use of oral contraceptives, metformin, inositol, N-acetylcysteine, or other medications in the past three months. ④ Presence of large ovarian cysts (≥ 5 cm), uterine malformations, intrauterine adhesions, submucosal fibroids, etc. ⑤ Presence of concurrent pregnancy or surgical or medical conditions, including but not limited to uncontrolled diabetes, hypertension, liver disease, kidney disease, thyroid disease, adrenal disorders, autoimmune diseases, etc. PCOS women who met the inclusion criteria and did not have the exclusion criteria were provided with detailed information about the study's objectives, procedures, potential benefits, and risks. After providing fully informed consent, they were enrolled in the study by signing an informed consent form.

Patient interventions

Using a stratified randomization method, eligible participants were assigned in a 1:1 ratio to either the control group or the NAC group. Both groups underwent a sequential LE and urinary follicle-stimulating hormone (uFSH) protocol for OI as described below. In the control group, all patients underwent baseline ultrasound examination starting from the 2nd to the 4th day of spontaneous or induced menstruation. Those meeting the criteria for OI initiated LE (Furui, Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) at a dose of 2.5 mg daily for 5 consecutive days. The day after completing LE, they received intramuscular injections of uFSH (Urofollitropin, Lizhu Pharmaceutical Factory, Zhuhai, China) at a dosage of 75–150 IU daily. Regular vaginal ultrasound examinations were conducted to monitor follicular growth. Upon the attainment of at least one follicle with a diameter of 18–20 mm, recombinant human chorionic gonadotropin (rhCG) (Ovidrel, Merck Serono, Aubonne, Switzerland) was administered at a dose of 250 μg to induce ovulation, with instructions for timed intercourse 12–36 h after rhCG injection. Subsequent vaginal ultrasounds were performed every other day postrhCG injection to assess ovulation. Following ovulation, patients were prescribed oral progesterone capsules (Laiting, Zhejiang Medicine Co., Ltd., Xinchang, China) for 14 days. Urine pregnancy testing was subsequently conducted to confirm pregnancy. In the NAC group, women with PCOS were administered oral NAC (Jinkangsuli, Zhejiang Jinhua Kang'enbei Biopharmaceutical Co., Ltd., Jinhua, China) at a dose of 1.8 g/day (dosage: 0.6 g, orally, three times a day) from the 2nd to the 4th day of the menstrual cycle for five consecutive days. The dosage selection was based on the drug's instructions and findings from previous clinical studies [30]. The remaining OI procedures mirrored those of the control group. For both groups, pregnancy outcomes were documented after each OI cycle, and the intervention study was considered complete upon clinical pregnancy. The follow-up period for both groups was limited to ≤ 3 OI cycles.

Randomization and masking in the clinical trial

The study utilized block randomization with a block size of 4. Randomized sequences were generated using SPSS software (Version 26, IBM, Armonk, NY) and were securely stored in sealed opaque envelopes. Eligible participants who met the inclusion and exclusion criteria

and provided informed consent were assigned by a third party (nurse) in a 1:1 ratio to either the NAC group or the control group based on their enrollment order. Ultrasound examiners, statisticians, and outcome assessors remained blinded to the allocation, while treating physicians and participants were not blinded.

Calculation of the sample size for the clinical trial

The current study is a pragmatic, randomized, parallel, noncontrolled superiority trial. Sample size calculations for both groups were performed using PASS software version 11.0 (NCSS, LLC. Kaysville, Utah, USA). We aimed to investigate whether NAC supplementation enhances the clinical pregnancy rate in women with PCOS who have undergone OI compared to women in the control group. Based on prior clinical research conducted at our center, the clinical pregnancy rate per OI cycle for PCOS-related ovulatory disorders using sequential LE + uFSH therapy was determined to be 23% [31]. Our objective was to assess the difference in pregnancy rates between the two groups at a significance level of 10%, with $\alpha = 0.05$ (one-sided) and a test power of 0.8, resulting in a minimum required induction cycle count of 219 per group. This calculation accounts for a dropout rate of 10%. Consequently, 239 cycles of patients were planned for each group in this study.

Outcome measures of the clinical trial

The primary outcome measure was the clinical pregnancy rate per OI cycle. Clinical pregnancy was defined as the presence of one or more gestational sacs observed via ultrasound, including intrauterine pregnancy, ectopic pregnancy, and combined intra- and extrauterine pregnancy, with or without visible fetal heartbeats. Multiple gestational sacs were counted as one clinical pregnancy. Secondary outcome measures included the ongoing pregnancy rate, early miscarriage rate, biochemical pregnancy rate, ovulation rate, the incidence of ovarian hyperstimulation syndrome (OHSS) and treatment discontinuation rate. Ongoing pregnancy is defined as an intrauterine pregnancy with at least one visible fetal heartbeat observed after 12 weeks of gestation. Biochemical pregnancy was defined as a serum level of human chorionic gonadotropin greater than 10 IU/L. OHSS was defined by the Golan criteria [32]. Mild OHSS involves abdominal distension and discomfort, with possible nausea, vomiting, or diarrhea. Moderate OHSS includes these symptoms plus ultrasonographic ascites. Severe OHSS features clinical ascites and/or hydrothorax, breathing difficulties, and may involve hemoconcentration, coagulation issues, and reduced renal function.

Statistical analysis

In animal experiments, comparisons between groups were conducted using independent samples t tests or one-way analysis of variance (ANOVA) with Tukey's post hoc test (for continuous variables) or the chi-square test (for categorical variables).

In clinical PCT studies, comparisons of outcome measures between the two study groups were conducted using intention-to-treat (ITT) analyses, with per-protocol (PP) analysis also performed as a sensitivity analysis (Supplementary Tables S1 and S2). Descriptive analysis was employed to compare baseline data between the two groups, while balance analysis between the two groups was assessed by analyzing different baseline data. For continuous variables, the normality of the distribution was initially estimated using frequency histograms and the Kolmogorov–Smirnov test. Normally distributed continuous variables are presented as the means ± SDs, and statistical comparisons were made using Student's t test. Alternatively, if continuous variables did not follow a normal distribution, they are presented as the median and interquartile range (IQR), and differences between groups were analyzed using the Mann–Whitney U test. Categorical variables were described in terms of n (%), with the chi-square test used for difference testing and the Fisher exact probability test employed when expected frequencies were less than 5. Statistical significance was determined using one-sided tests, and the primary outcome measure was reported using superiority tests.

All the statistical analyses were conducted using the statistical software package SPSS V.25.0 (SPSS). Statistical significance was defined as $p < 0.05$.

Results

NAC supplementation reverses estrous cycle irregularities and the ovarian phenotype in PCOS mice

An irregular menstrual cycle is one of the primary diagnostic criteria for PCOS [33]. In the present study, vaginal cytology of female mice after 21 days of LE intervention revealed a lack of estrous cycle regularity. Compared with those in the control group, the serum LH levels and LH/FSH ratios were considerably greater in the LE intervention mice. H&E staining of ovarian tissue sections revealed that the LE intervention mice (PCOS model mice) had considerably more follicles and fewer corpora lutea (Supplementary Figure S1), confirming the successful modeling of PCOS in mice induced by LE intervention.

Following a 12-day random group intervention in the PCOS mice (Fig. 1A), vaginal cytology revealed that 83.33% (9/12) of the mice in the PCOS + NAC group and 75.00% (9/12) of the mice in the PCOS + Met group had

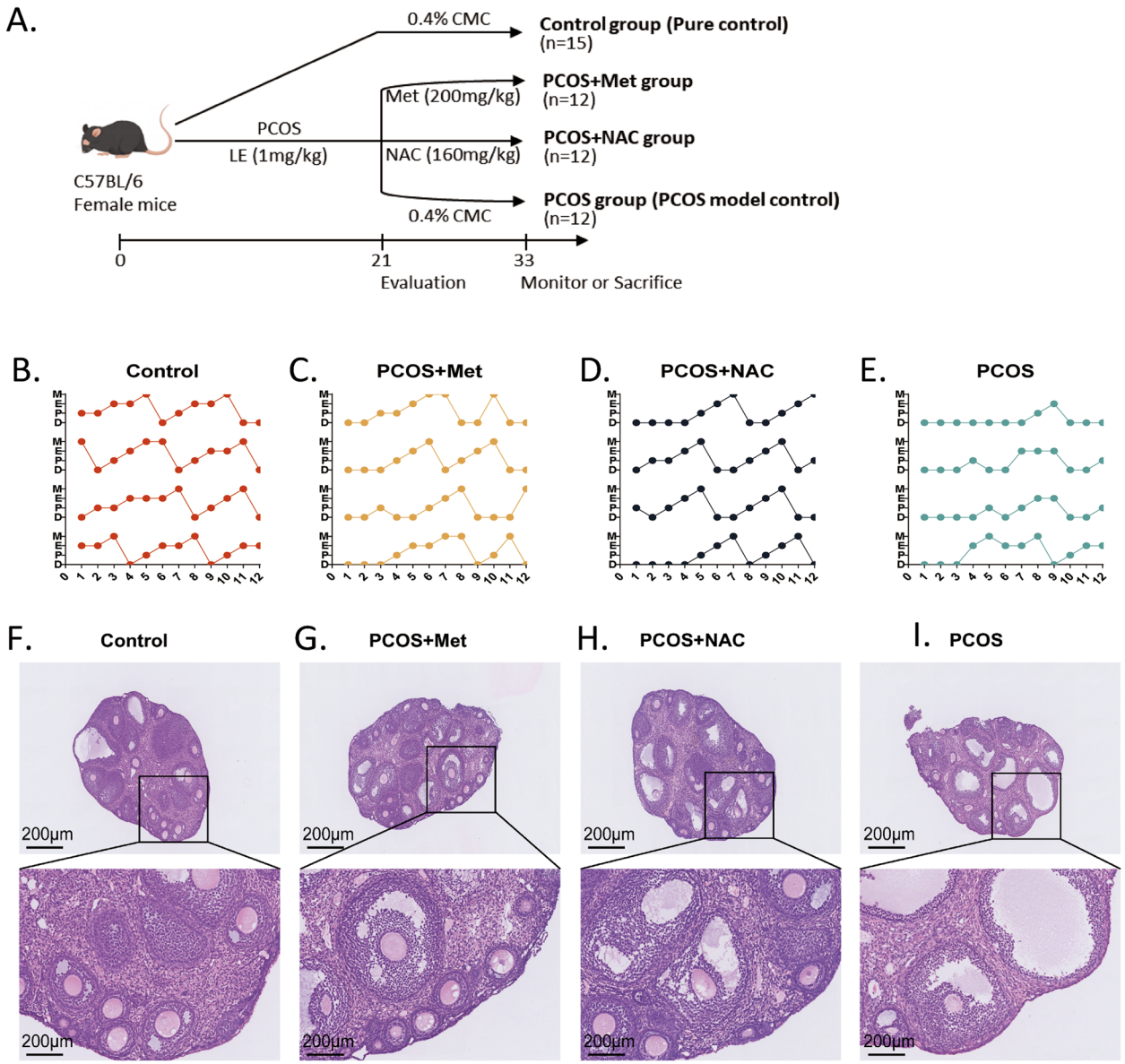

Fig. 1 NAC supplementation reverses estrous cycle irregularities and ovarian phenotypes in PCOS mice. **A** Schematic diagram of the animal study, where C57BL/6 female mice were administered letrozole (LE) by gavage to establish a PCOS model, and subsequently randomly divided into intervention groups. **B**-**E** Representative estrous cycle curves for the pure control group, PCOS + Met, PCOS + NAC, and the pure PCOS group during the intervention period (*n* = 12). **F**-**I** Representative ovarian histopathological images for the pure control group, PCOS + Met, PCOS + NAC, and the pure PCOS group after 12 days of intervention (n=6). Scale bar = 200 μm. LE, letrozole; Met, metformin; NAC, N-acetylcysteine; CMC, carboxymethyl cellulose

normalized estrous cycles. In contrast, the pure PCOS group showed no cyclicity throughout the 12-day intervention period, but the 12 control mice had a normal estrous cycle (Fig. 1B-E). Notably, H&E staining of ovarian tissue sections revealed that, compared to the pure PCOS group, the PCOS + NAC and PCOS + Met groups had considerably more corpora lutea and fewer follicles (Fig. 1F-I). This finding suggested that NAC intervention can dramatically improve estrous cycle abnormalities and the ovarian phenotype in PCOS mice, with effects similar to those of Met.

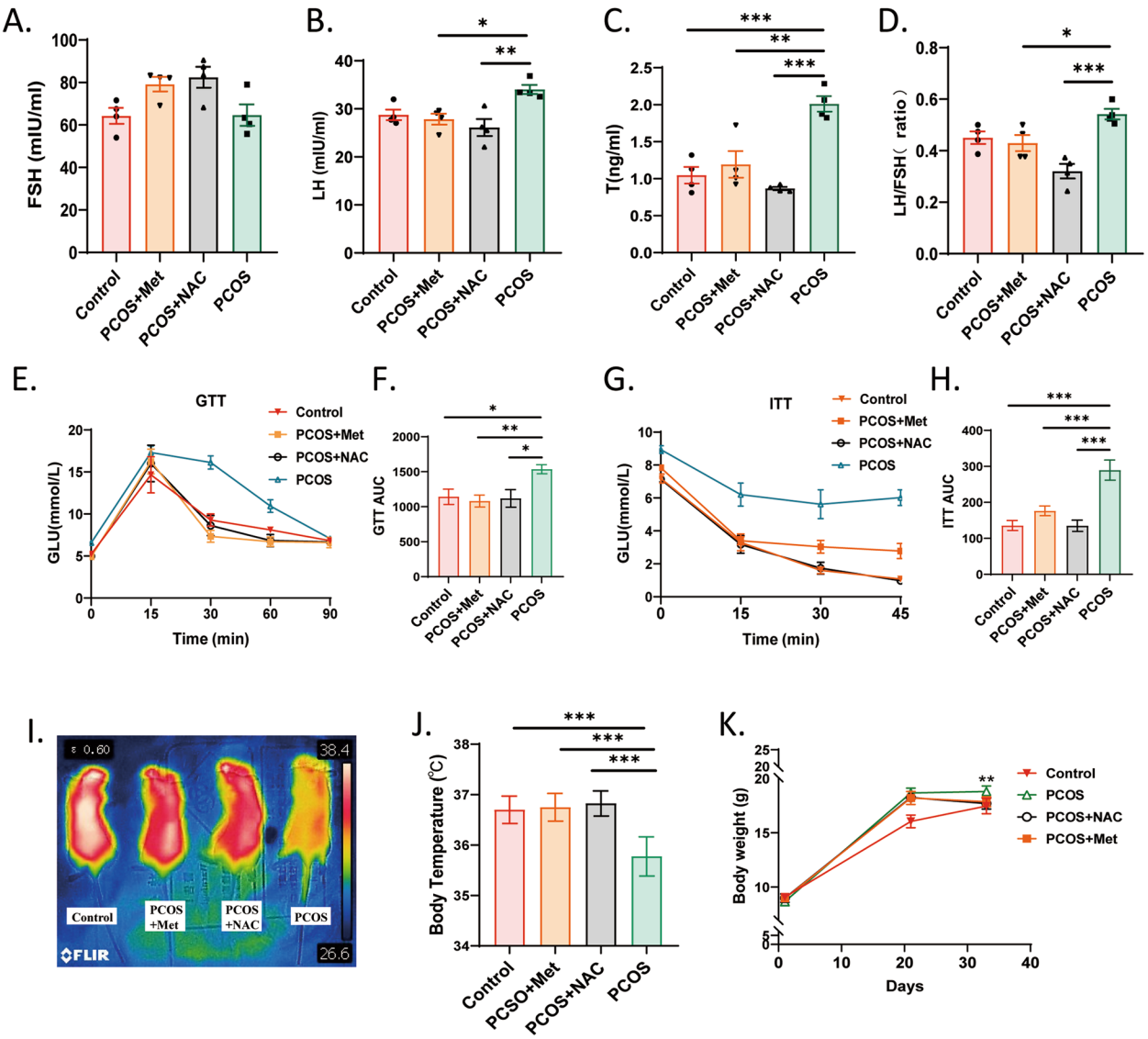

Fig. 2 Effects of NAC supplementation on endocrine and metabolic profiles in LE-induced PCOS mice. Serum levels of (**A**) follicle-stimulating hormone (FSH), (**B**) luteinizing hormone (LH), (**C**) testosterone (T), and (**D**) LH/FSH ratio were compared among different groups of mice after 12 days of intervention. Glucose tolerance test (**E**) and insulin tolerance test (**G**) results were also compared at the end of the intervention, with (**F**) and (**H**) representing the area under the curve for GTT and ITT, respectively. Infrared thermographic images (**I**) and core body temperature (**J**) for each group of mice at the conclusion of the study, along with body weight change curves (**K**) for each group throughout the study. Data are presented as mean ± SD and analyzed using one-way ANOVA with Tukey's post hoc test (n = 4-6). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Met, metformin; NAC, N-acetylcysteine

NAC supplementation alleviates endocrine and metabolic disorders in LE-induced PCOS mice

PCOS patients often have endocrine and metabolic disorders. To determine the effect of NAC intervention on the endocrine status of PCOS mice, we measured sex hormone levels in each group after the intervention. Although the serum FSH levels did not differ significantly among the groups, the serum LH levels and LH/FSH ratios were significantly lower in the PCOS + NAC and PCOS + Met groups than in the pure PCOS group, returning to levels similar to those in the pure control group (Fig. 2A-C). Furthermore, the serum T levels in the PCOS + NAC and PCOS + Met groups were considerably lower than those in the PCOS group following the intervention (Fig. 2D). These results suggest that NAC supplementation can alleviate endocrine disorders in LE-induced PCOS mice.

To investigate whether NAC intervention affects glucose metabolism in LE-induced PCOS mice, we conducted GTTs and ITTs. After 12 days of intervention, the PCOS + NAC and PCOS + Met groups had significantly lower fasting blood glucose (4.98 ± 0.39, 4.92 ± 0.71 vs. 6.59 ± 0.64; both $p < 0.01$) and fasting insulin (7.20 ± 0.42, 7.84 ± 0.31 vs. 8.94 ± 0.52; both $p < 0.05$) levels than the pure PCOS group and were similar to the pure control group. The area under the GTT/ITT curve indicated that the pure PCOS group exhibited significant insulin resistance compared to the pure control group, while NAC and Met intervention improved insulin sensitivity and enhanced plasma glucose clearance in LE-induced PCOS mice (Fig. 2E-H).

Studies have reported that adaptive thermogenesis in response to stimuli such as postprandial or cold exposure is reduced in PCOS patients [34]. In mice, most energy expenditure is used to maintain core body temperature, and measuring core body temperature can directly reflect energy expenditure [35]. In this study, compared with the control group, the pure PCOS group exhibited a significant decrease in body temperature under cold stimulation. However, NAC and Met significantly stimulated thermogenesis in PCOS mice, as indicated by a significant increase in body temperature in these two groups (Fig. 2I-J).

Due to the glucose metabolism and thermogenesis-inducing effects observed with NAC intervention, the average body weight of the PCOS + NAC group was significantly lower than that of the pure PCOS group after 12 days of intervention and was comparable to the average body weight of the PCOS + Met and pure control groups (Fig. 2K).

NAC supplementation improves the oxidative stress response in oocytes of LE-induced PCOS mice

To evaluate the effect of NAC supplementation on the oxidative stress response in the oocytes of LE-induced PCOS mice, we used JCI and DCFH-DA fluorescent probes to analyze the MMP and ROS levels in the oocytes from each group after the intervention. Compared to those in the pure control group, oocytes from the pure PCOS group had significantly lower MMP and significantly higher intracellular ROS levels at the end of the intervention. However, NAC intervention, similar to Met intervention, significantly increased the MMP and decreased intracellular ROS levels in oocytes from LE-induced PCOS mice. These findings suggest that NAC has the potential to counteract the excessive oxidative stress response in the oocytes of PCOS mice, possibly improving oocyte quality and pregnancy outcomes. (Fig. 3A-D).

NAC supplementation increased the activity levels of antioxidant enzymes in mouse ovarian tissue

Compared to those in the pure control group, the ovarian tissues of mice in the LE-induced PCOS group exhibited significantly lower activity of the enzymatic antioxidants SOD, GSH-Px, and CAT, as well as the nonenzymatic antioxidant GSH. In contrast, the activities of GSH-Px, SOD, and GSH in the ovarian tissue of the PCOS + NAC group were significantly greater than those in the ovarian tissue of the PCOS group. However, the activity levels of these enzymatic and nonenzymatic antioxidants in the ovarian tissue of the PCOS + Met group did not differ significantly from those in the ovarian tissue of the PCOS group (Fig. 3E-H). This shows that NAC treatment can boost the activity of the antioxidant system in ovarian tissue.

Comparisons of baseline characteristics between the NAC group and control group

Based on promising results from in vivo animal studies, we designed a pragmatic randomized, parallel-group controlled clinical study to determine the therapeutic efficacy of NAC supplementation in improving pregnancy outcomes for PCOS-related infertility patients. In the current PCT, 252 women with PCOS were recruited and screened, 230 of whom eventually enrolled in the research. Among them, 115 PCOS women were randomly assigned to the NAC group, with the remaining 115 PCOS women allocated to the control group. The patients' baseline characteristics were similar across the two study groups (see Table 1). During the intervention period, the NAC group had two incidences of natural conception, while the control group had three (these five participants were omitted from the final statistical analysis). Overall, the NAC group had 113 subjects and 284 complete OI cycles, whereas 112 patients in the control group had 279 complete OI cycles. (Fig. 4)

NAC supplementation shortened the days of uFSH stimulation and the total days of sequential OI with LE/uFSH

There were no significant differences between the two groups in terms of ovulation rate per OI cycle (96.13% vs. 96.77%, $p = 0.678$), mono-ovulation rate (82.04% vs. 81.36%, $p = 0.835$), or multiple-ovulation rate (14.08% vs. 13.62%, $p = 0.109$). However, patients in the NAC group required fewer days of uFSH injection per OI cycle [5.00 (4.00, 6.75) vs. 6.00 (5.00, 7.00), $p = 0.017$] and had a significantly shorter total duration of OI [10.00 (9.00, 12.00) vs. 11.00 (10.00, 13.00), $p = 0.032$] than did patients in the control group. Furthermore, there were no significant differences between the two groups in terms of OHSS or endometrial thickness on the trigger day. (See Table 2).

NAC Supplementation Improves Clinical Pregnancy Rates in LE/uFSH sequential therapy

In the comparison of clinical pregnancy rates, both per OI cycle and cumulative per patient, the rates in the NAC group were significantly greater than those in the control group (30.99% vs. 23.30%, $p=0.040$; and 77.88% vs. 58.04%, $p=0.001$, respectively). The ongoing pregnancy rate per patient was also significantly greater in the NAC group than in the control group (69.91% vs. 53.57%, $p=0.012$). However, there were no significant differences between the two groups in terms of multiple pregnancy rate, early miscarriage rate, or ectopic pregnancy rate (all $p>0.05$). (Table 3).

Our current PCT included 225 participants, 49.78% (112/225) of whom were overweight or obese. Overweight or obesity negatively impacts both natural and assisted conception cycles in women with PCOS [36]. Notably, our stratified analysis revealed that among participants with a BMI ≥ 24, the cumulative clinical pregnancy rate was significantly greater in the NAC supplementation group than in the control group (73.08% vs. 45.00%, $p=0.003$). These pregnancy outcomes suggest that NAC supplementation can improve clinical pregnancy rates after subsequent LE/uFSH therapy, with particularly pronounced benefits for overweight or obese women.

Discussion

PCOS, a highly prevalent and incurable disease affecting female reproductive, endocrine, metabolic, and mental health, demands the urgent exploration of more effective, safer, and cost-effective drugs or therapies to improve symptoms and enhance fertility. Insulin resistance, hyperandrogenemia, and oxidative stress imbalance are the three critical pathological mechanisms involved in the development of PCOS, forming a vicious cycle [37, 38] that perpetuates the disease's progression. NAC, an effective antioxidant, has recently shown potent insulin-sensitizing effects in vitro [15, 16] and in animal models of obesity [39] and diabetes mellitus [40]. Several studies [17, 18, 38, 39], including our current study, have explored the use of NAC as a daily medication for PCOS management or as an adjuvant for fertility treatments. In the present study, we are the first to verify that NAC intervention reverses endocrine-metabolic parameters and ovarian pathological phenotypes in LE-induced PCOS mouse models. We demonstrated that NAC has the ability to counteract oxidative stress damage to oocytes and enhance the activity of enzymatic and non-enzymatic antioxidants in ovarian tissues. Furthermore, through a clinical PCT study, we revealed that NAC supplementation significantly improved the efficacy of OI with sequential LE/uFSH. The current study not only includes multilayered in vitro and in vivo validation but also incorporates clinical studies, ultimately confirming that NAC supplementation can significantly enhance OI efficacy with sequential LE and uFSH in women with PCOS. Therefore, NAC could be a valuable adjuvant in OI for women with PCOS.

Insulin resistance is the core pathological mechanism involved in the development of PCOS [41]. Treating patients with insulin sensitizers is essential for alleviating symptoms, signs, and concomitant complications [8]. Currently, metformin is the most commonly used insulin sensitizer for treating PCOS. However, its gastrointestinal side effects limit its use in some patients [9]. Therefore, there is a need for alternate drugs with greater acceptance and fewer side effects. NAC has recently gained attention as a novel insulin sensitizer for PCOS [30]. In the present study, we first confirmed that NAC reversed the abnormal estrous cycles and ovarian phenotypes in LE-induced PCOS mice. NAC also significantly improved glucose homeostasis, insulin sensitivity and energy expenditure in PCOS mice. A recent stereopathological study on the uterus and ovaries of LE-induced PCOS mice also demonstrated that NAC, similar to metformin, could significantly reverse the pathological phenotypes to those of normal mice [42]. However, there are still limited studies on the effects of NAC on insulin sensitivity and endocrine-metabolic parameters in PCOS model mice.

As early as 2002, Fulghesu et al. [43] reported that oral administration of NAC at 1.8 g/day (or 3.0 g/day for a BMI > 30) improved insulin sensitivity, testosterone levels, and lipid profiles in women with PCOS. Subsequent studies have shown that daily oral intake of 1.8 g of

(See figure on next page.)

Fig. 3 NAC supplementation enhances the oxidative stress response in oocytes and increases antioxidant enzyme activity levels in the ovaries. After group intervention, MII oocytes were collected from each group of mice. Representative images of mitochondrial membrane potential (MMP) measured with a JC-1 fluorescent probe (**A**) and reactive oxygen species (ROS) levels measured with a DCFH-DA fluorescent probe (**C**), along with statistical analysis of MMP (red/green) (**B**) and ROS signal (**D**). Comparison of nonenzymatic antioxidant glutathione (GSH) levels (**E**) and enzymatic antioxidants superoxide dismutase (SOD) (**F**), glutathione peroxidase (GSH-Px) (**G**), and catalase (CAT) (**H**) in the ovarian tissues of each group after 12 days of intervention using the corresponding assay kits. Data are presented as mean ± SD and analyzed by one-way ANOVA with Tukey's post hoc test (n = 4-6). *, $p < 0.05$; ***, $p < 0.001$. Scale bar = 200 μm. BF, bright field; ROS, reactive oxygen species; Met, metformin; NAC, N-acetylcysteine

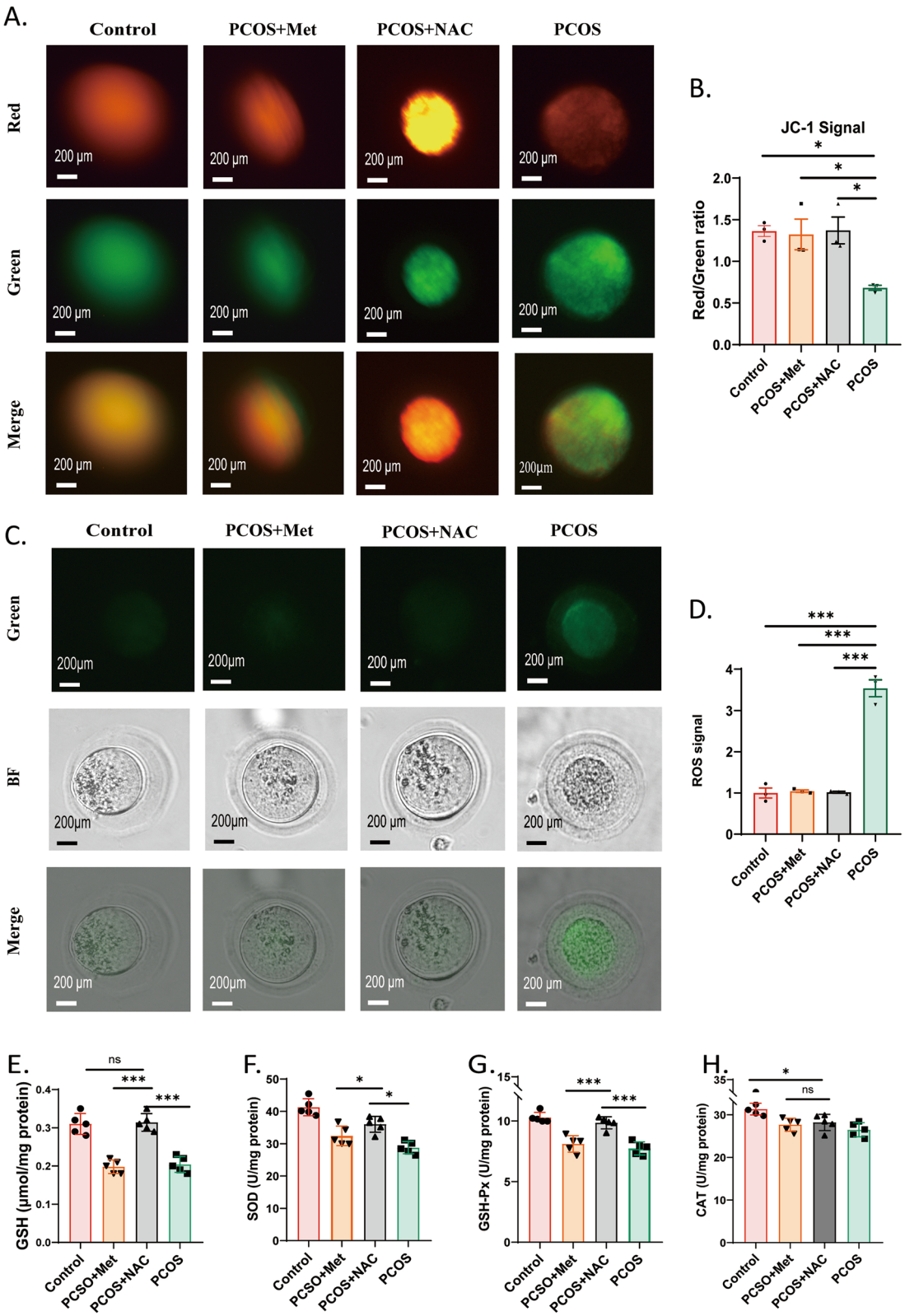

Fig. 3 (See legend on previous page.)

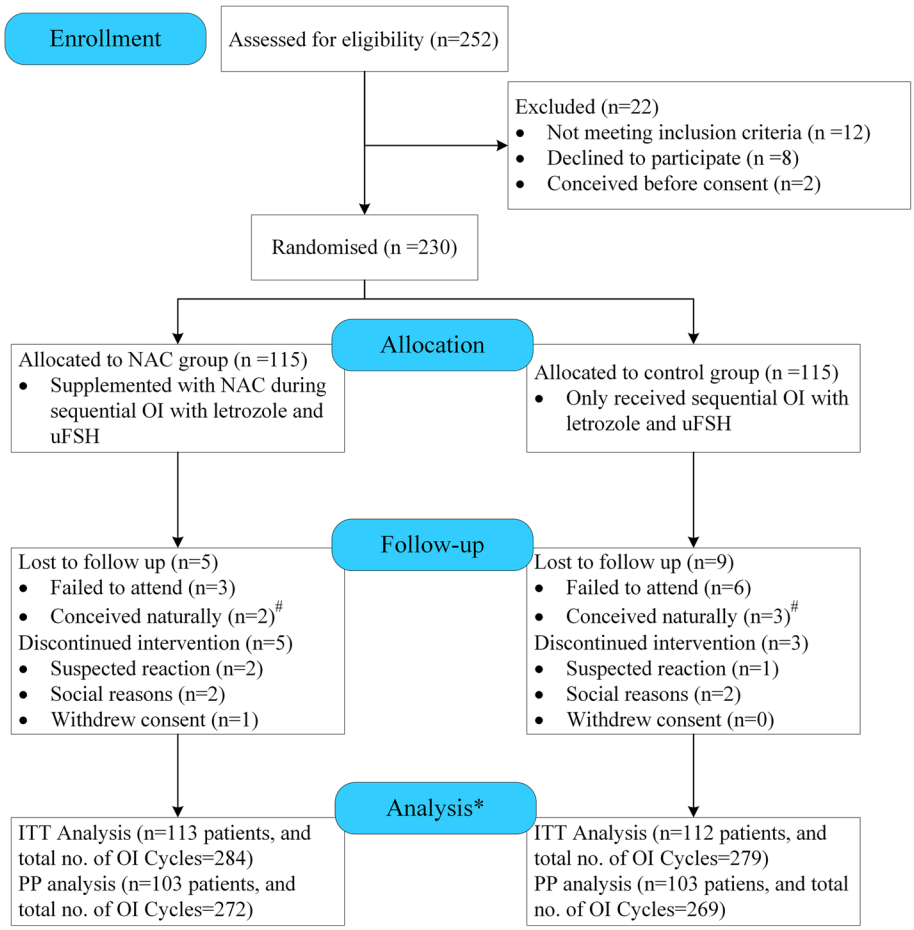

Fig. 4 Enrollment, allocation, and follow-up of participants and the CONSORT diagram of the pragmatic clinical trial. CONSORT (Consolidated Standards of Reporting Trials). # During the intervention stage, patients who conceived naturally were excluded from the final statistical analyses. *Incomplete ovulation induction cycles were not included in the final statistics. NAC, N-acetyl-L-cysteine; OI, ovulation induction; ITT, intention-to-treat; PP, per-protocol

NAC had similar [44] or superior [45] effects compared to daily oral intake of 1.5 g of metformin in improving BMI, fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR), and menstrual irregularities. A meta-analysis in 2020 also demonstrated that NAC had comparable effects to metformin in improving serum LH levels, the LH/FSH ratio, and fasting insulin in women with PCOS [46]. In the current study, we also found that NAC supplementation had effects similar to those of metformin in enhancing the glucose clearance rate and insulin sensitivity. Although many researchers have studied the insulin-sensitizing effects of NAC, the mechanisms by which NAC reverses insulin resistance in individuals with PCOS remain unclear. These mechanisms may be closely related to its antioxidant and anti-inflammatory properties [47] and its ability to improve mitochondrial function [48].

In this study, we found that the activities of enzymatic antioxidants (SOD, GSH-Px, and CAT) and nonenzymatic antioxidants (GSH) were significantly decreased in the ovarian tissues of LE-induced PCOS mice. Additionally, these mice exhibited reduced MMP and increased ROS levels in their oocytes, indicating mitochondrial dysfunction and oxidative stress imbalance. This observation is consistent with that observed in clinical PCOS patients [49, 50]. Oxidative stress can impact various physiological and pathological processes, leading to reproductive disorders such as PCOS, endometriosis, and recurrent spontaneous abortion [51]. Oxidative stress disrupts follicular development and maturation by damaging oocytes and granulosa cells. It also causes mitochondrial dysfunction, impairing insulin signaling and disrupting glucose metabolism, leading to insulin resistance [50]. The oxidative stress imbalance in PCOS primarily stems from impaired antioxidant capacity and the resulting overproduction of ROS. Our study demonstrated a decrease in the enzyme and nonenzyme antioxidants SOD, GSH-Px, CAT, and GSH

Table 1 Baseline characteristics of PCOS women in the NAC group and control groups

Characteristic	NAC group $n=113$	Control group $n=112$	*p* value	t or U value /Z value	95% CI /Risk ratio (95% CI)	Effect size* (Cohen's d/r/ φ/ Cramér's V)
Age (years)	28.50 (2.81)	28.02(2.99)	0.2092	1.259	-1.25-0.27	0.18
BMI (kg/m^2)	23.36(20.70, 26.24)	24.17(22.04, 27.04)	0.1748	5665	-0.35-1.70	-0.09
Infertility duration (months)	12.00(6.00, 23.50)	12.00(6.00, 23.50)	0.6573	6113	-3.00-1.00	-0.03
Serum basal FSH (mIU/ml)	6.05(1.50)	5.79(1.63)	0.2284	1.208	-1.59-0.66	0.16
Serum basal LH(mIU/ml)	8.12(4.75, 12.08)	9.61(6.13, 13.79)	0.0724	5451	-0.12-2.69	-0.12
LH/FSH ratio	1.66(1.25, 2.45)	1.64(1.10, 2.41)	0.3366	5805	-0.33-0.12	-0.17
Serum basal E2(pg/ml)	33.71(26.00, 45.33)	35.35(26.00, 46.08)	0.4381	5841	-2.19-4.92	-0.05
Testosterone (ng/mL)	0.54(0.40, 0.78)	0.58(0.42, 0.84)	0.3098	5516	-0.04-0.11	-0.07
Prolactin (ng/ml)	13.89(10.96, 19.04)	13.19(10.45, 19.35)	0.4698	5186	-2.16-1.00	-0.05
The proportion of hyperprolactinemia (> 30 ng/ml) %(n)[#]	7.96% (9/113)	6.25% (7/112)	0.617	0.25	1.30(0.47–3.62)	0.02
AMH (ng/ml)	8.76(6.17, 11.76)	8.40(6.04, 11.13)	0.7751	6188	-1.13-0.86	-0.02
HOMA-IR	3.27(2.42, 4.73)	3.28(2.21, 4.83)	0.8925	6262	-0.47-0.40	-0.01
The proportion of women with insulin resistance (HOMA-IR > 2.69)	67.26% (76/113)	65.18% (73/112)	0.742	0.109	1.10(0.63–1.91)	0.01
Fasting insulin (mIU/L)	14.14(10.87, 20.58)	14.02(10.20, 21.00)	0.8633	6188	-1.87-1.57	-0.01
Fasting glucose (mmol/L)	5.20 (4.90, 5.50)	5.20 (4.90, 5.50)	0.7856	6195	-0.10-0.10	-0.02
Menses, %(n)						
Oligomenorrhoea	40.71% (46/113)	42.86% (48/112)	0.353	-0.062	-	0.004
Amenorrhoea	7.08% (8/113)	10.71% (12/112)				
Irregular	41.59% (47/113)	41.07% (46/112)				
Regular	10.61% (12/113)	5.36% (6/112)				
Primary infertility, %(n)	80.53% (91/113)	75.89% (85/112)	0.399	0.71	1.31(0.70–2.48)	0.05
Primiparity, %(n)	92.04% (104/113)	91.96% (103/112)	0.984	0	1.01(0.39–2.65)	0

Data are presented as the means ± SDs, medians (25th and 75th percentiles) or percentages (numbers). Student's t tests or Mann–Whitney U tests were used for continuous variables, and chi-square tests were used for categorical variables

NAC N-acetyl-L-cysteine, *AMH* anti-Mullerian hormone, *HOMA-IR* homeostasis model assessment insulin resistance index, *FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *E2* estradiol

*Effect size measures the magnitude of the relationship or difference between groups. Cohen's d was used for comparing means, r was for rank correlations, Phi(φ) was for 2 × 2 chi-square tests, and Cramér's V is for larger contingency tables

[#] All patients with hyperprolactinemia had already received medication and achieved normal serum prolactin levels before ovulation induction

in the ovarian tissues of pure PCOS mice, thus confirming this hypothesis. In our research, supplementation with NAC significantly increased the activities of GSH-Px, SOD, and GSH in the ovarian tissues of PCOS mice. Concurrently, the MMP of oocytes was enhanced, and ROS levels were reduced, indicating that NAC treatment enhanced antioxidant system activity in ovarian tissues, improved mitochondrial function, and ameliorated oxidative stress (OS) in PCOS mice. Cai et al. demonstrated in vitro that adding 0.5 mg/mL N-acetylcysteine (NAC) to a rabbit granulosa cell damage model induced by D-galactose (D-gal) significantly inhibited granulosa cell apoptosis and promoted proliferation. Moreover, NAC intervention in vitro was observed to suppress the release of cytochrome C, a marker of oxidative stress, while significantly increasing the activities of antioxidants such as CAT, GSH, and SOD [52]. The mechanism by which NAC enhances antioxidant activity in cells and tissues is considered to be the result of multiple pathways. First, as a precursor of glutathione (GSH), NAC transforms into GSH, which exhibits significant antioxidant effects; second, under conditions of significant depletion of endogenous cysteine (Cys) and GSH, NAC can also act as a direct antioxidant against certain oxidants (nitrogen dioxide and hypochlorous acid); third, NAC has the ability to decompose disulfide proteins, thereby releasing free thiols and reducing proteins to exert antioxidant effects [53].

The above findings collectively suggest that NAC supplementation has the potential to improve oocyte quality and fertility in women with PCOS. To validate this hypothesis, we further conducted a PCT study. The results showed that NAC supplementation reduced the total dose and duration of gonadotropins required for

Table 2 Cycle characteristics for NAC group versus control group—intention-to-treat analysis

Outcome	NAC group *N* = 113	Control group *N* = 112	*p*	u value/ Z value	95% CI/ Risk ratio (95% CI)	Effect size* (r/φ/Cramér's V)
Underwent single-cycle OI	10.62% (12/113)	8.92% (10/112)	0.067	-0.017	-	0.001
Underwent two-cycle OI	27.43% (31/113)	33.04% (37/112)				
Underwent three-cycle OI	61.95% (70/113)	58.04% (65/112)				
Total no. of OI cycles	284	279	-	-	-	-
OI cycle characteristics						
Ovulation per cycle, %(n)	94.37% (268/284)	93.55% (261/279)	0.684	0.166	1.16(0.58, 2.31)	0.01
No. of anovulatory and follicular atresia cycles, %(n)	3.87% (11/284)	5.02% (14/279)	0.510	0.435	0.76 (0.34, 1.71)	0.02
No of Mono-ovulation cycles, %(n)	82.04% (233/284)	81.36% (227/279)	0.835	0.044	1.05 (0.68, 1.61)	0.002
No of Multiple-ovulation cycles, %(n)	14.08% (40/284)	13.62% (38/279)	0.109	2.576	1.47 (0.92, 2.36)	0.11
Days of uFSH stimulation, (d)	5.00 (4.00, 6.75)	6.00 (5.00, 7.00)	0.017	2748	0.00–2.00	0.82
Total days of ovulation induction, (d)	10.00 (9.00, 12.00)	11.00 (10.00, 13.00)	0.032	2409	-0.00-2.00	0.81
Endometrial thickness on hCG trigger day (mm)	9.00 (8.00, 9.00)	9.00 (8.00, 9.00)	0.856	30,617	-1.00-1.00	0.20
OHSS rate per cycle[#], %(n)	3.53% (10/284)	3.94% (11/279)	0.792	0.070	0.89 (0.37, 2,13)	0.003

Data are presented as medians (25th and 75th percentiles) or percentages (numbers). Mann–Whitney U tests were used for continuous variables, and chi-square tests were used for categorical variables

NAC N-acetyl-L-cysteine, *OI* ovulation induction, *uFSH* urinary follicle-stimulating hormone, *hCG* human chorionic gonadotropin, *OHSS* ovarian hyperstimulation syndrome, *CI* confidence interval

*Effect size measures the magnitude of the relationship or difference between groups. r was used for rank correlations, Phi(φ) was for 2x2 chi-square tests, and Cramér's V was for larger contingency tables. The mono-ovulation cycle is defined as a cycle in which only one follicle measuring 14 mm or larger is ovulated. Multiple ovulation cycles were defined as two or more follicles, each measuring 14 mm or more, ovulating. Total days of ovulation induction refers to the duration from the commencement of oral letrozole administration to the day of hCG trigger

[#] All participants had mild OHSS

OI in women with PCOS. Importantly, NAC supplementation significantly increased the clinical pregnancy rate per OI cycle (30.99% vs. 23.30%) and the cumulative clinical pregnancy rate per enrolled patient (77.88% vs. 58.04%). In a 2018 study involving 130 women with PCOS who underwent OI with LE (5 mg/day), oral administration of NAC (1.2 g/day) for 5 days significantly improved ovulation and pregnancy rates compared to placebo [54]. Notably, the ovulation rates in both groups (16.1% vs. 33.3%) were significantly lower than the typically reported ovulation rates of approximately 90% [55]. A meta-analysis including 15 RCTs with a total of 2,330 women showed that oral NAC at doses of 1200–1600 mg/day tended to increase ovulation rates, clinical pregnancy rates, and live birth rates compared to placebo or no-treatment controls, although the differences did not reach statistical significance [56]. However, it is important to note that among the 15 RCTs included, 10 studies used CCs for OI, one used LE, one used laparoscopic ovarian drilling, one used oral contraceptives, and one did not report the intervention method. Given that the pregnancy rates in women with PCOS who use CCs for OI are significantly lower than those who use CCs for LE [55], this could be a reason for the inconsistency between our study results and this meta-analysis.

NAC supplementation has been shown to improve clinical pregnancy outcomes in women with PCOS undergoing ovulation induction (OI), likely due to its insulin-sensitizing, antioxidant properties, and protective effects on mitochondrial function, as demonstrated in previous studies. In an animal study using 5α-dihydrotestosterone + insulin treatment to simulate a miscarriage model in PCOS patients, NAC supplementation improved fetal survival rates in model mice by protecting mitochondrial function, restoring the balance of SOD1 and the Keap1/Nrf2 antioxidant response, reducing excessive ROS production, and mitigating placental formation defects [57]. Similarly, another study found that NAC supplementation increased GSH-Px4 protein levels in the placenta, inhibiting placental ferroptosis, which was associated with reduced pregnancy loss in PCOS model mice [58]. In older women undergoing IVF, researchers have demonstrated that oral administration of 1.8 g/day NAC, starting from the beginning of the menstrual cycle preceding controlled ovarian hyperstimulation and continuing until the trigger day, significantly reduces the gonadotropin dose required and increases the number of high-quality blastocysts. Additionally, NAC supplementation has been found to significantly increase glutathione (GSH) levels in follicular fluid [59]. Moreover, studies in PCOS patients

Table 3 Pregnancy outcomes for NAC group versus control group—intention-to-treat analysis

Outcome	NAC group $N=113$	Control group $N=112$	*p*	Z value	Risk ratio (95% CI)	Effect size* (φ)
Pregnancy outcomes (per cycle)						
Biochemical pregnancy rate per cycles, %(n)	33.45% (95/284)	25.09% (70/279)	0.029	4.749	1.50(1.04, 2.16)	0.20
Clinical pregnancy rate per cycle, %(n)	30.99% (88/284)	23.30% (65/279)	0.040	4.204	1.48(1.02, 2.15)	0.18
Clinical pregnancy rate per cycle—strata 1 (BMI < 24), %(n)	31.05% (50/161)	25.00% (38/152)	0.234	1.419	1.35(0.82, 2.22)	0.08
Clinical pregnancy rate per cycle—strata 1 (BMI ≥ 24), %(n)	30.89% (38/123)	21.26% (27/127)	0.083	3.015	1.66(0.94, 2.93)	0.19
Cumulative clinical pregnancy (per patient)						
Cumulative clinical pregnancy rate per patient	77.88% (88/113)	58.04% (65/112)	0.001	10.175	2.55(1.42, 4.55)	0.68
Cumulative clinical pregnancy rate—strata 1 (BMI < 24), %(n)	81.97% (50/61)	73.08% (38/52)	0.256	1.288	1.68(0.68, 4.10)	0.12
Cumulative clinical pregnancies—strata 2 (BMI ≥ 24)	73.08% (38/52)	45.00% (27/60)	0.003	9.017	3.32(1.50, 7.36)	0.85
Singleton pregnancy rate, %(n)	82.95% (73/88)	81.54% (53/65)	0.820	0.052	1.10(0.48, 2.55)	0.004
Multiple pregnancy rate, %(n)	14.77% (13/88)	16.92% (11/65)	0.718	0.131	0.85(0.035, 2.04)	0.01
Early miscarriage rate, %(n)	7.95% (7/88)	6.15% (4/65)	0.670	0.182	1.32(0.37, 4.71)	0.002
Ectopic pregnancy rate, %(n)	2.27% (2/88)	1.54% (1/65)	1.000[$]	-	1.49(0.13, 16.77)	-
Cumulative ongoing pregnancy rate per patient	69.91% (79/113)	53.57% (60/112)	0.012	6.36	0.17(0.07, 2.55)	0.42

Data are presented as % (number)

NAC N-acetyl-L-cysteine, *BMI* body mass index, *CI* confidence interval

*Effect size measures the magnitude of the relationship or difference between groups. Phi(φ) was used for 2 × 2 chi-square tests, and Cramér's V was for larger contingency tables. The chi-square test or Fisher's exact probability test was used for categorical variables

treated with NAC (1.8 g/day for 6 weeks) have shown a decrease in receptor tyrosine kinase c-kit protein levels in follicular fluid, while the expression of growth differentiation factor-9 in mature oocytes increases, suggesting that NAC may enhance oocyte maturation and embryo quality [60]. Another study on neutrophils in PCOS demonstrated that NAC reduces oxidative stress, apoptosis, and Ca2 + entry via the transient receptor potential vanilloid 1 (TRPV1) channel, while also lowering serum hormone and inflammatory marker levels, providing further evidence of NAC's therapeutic potential in PCOS [11]. Thus, the mechanisms by which NAC supplementation improves OI outcomes in PCOS patients are likely multifaceted, involving improvements in the follicular environment, oocyte and embryo quality, and implantation rates, and merit additional exploration in future research.

In addition, our current study revealed a more pronounced effect of NAC intervention on increasing the cumulative clinical pregnancy rate per enrolled patient after OI in overweight and obese PCOS women with a BMI $\geq$ 24 kg/m^2 (73.08% vs. 45.00%). This may be related to the fact that overweight and obese PCOS women often exhibit more significant insulin resistance and oxidative stress imbalance [61, 62]. In a meta-analysis that included 15 RCTs involving a total of 2,330 women, the results also indicated that NAC may have a certain efficacy as an adjunct therapy for infertility related to PCOS and unexplained infertility, particularly in women with high BMI, insulin resistance, and oxidative stress [56]. Although these findings still require further validation through rigorously designed randomized controlled trials and the evaluation of clinical outcomes, such as live birth rates, over longer follow-up periods, the outcomes suggest that overweight and obese women with PCOS may be a priority population for NAC supplementation.

This study has several limitations. First, we did not explore in detail the mechanisms by which NAC enhances the activity of antioxidant enzymes, particularly SOD and GSH-Px, in ovarian tissue. Previous research suggests that NAC may promote the nuclear translocation of Nrf2 [14] or activate NF-κB signaling pathways [63], leading to increased expression of SOD and GSH-Px, thereby reducing excessive ROS that damage mitochondria in mouse oocytes. However, whether these molecular pathways contribute to the reduction of oxidative stress by NAC in LE-induced PCOS mouse ovaries in our study, or if other molecular pathways are involved, remains to be investigated. Second, our animal model study did not evaluate the impact of NAC intervention on fertility outcomes in mice. Therefore, we cannot directly conclude that the observed improvements in endocrine-metabolic profiles and oxidative stress in PCOS mouse ovaries following NAC intervention lead to enhanced fertility. However, our clinical PCT study showed that NAC supplementation improved OI efficacy in PCOS patients, indicating a potential benefit of NAC for fertility in PCOS. Third, in the current PCT study, we did not conduct a controlled evaluation of changes in glucose, lipid metabolism, or hormone levels before and after NAC intervention in participants. As a result, we cannot conclusively determine whether short-term NAC intervention improves these metabolic and endocrine parameters in PCOS patients. Future studies should address this gap to provide further clarity on these effects. Fourth, we did not include a Metformin treatment group in our PCT study, so it remains unclear whether differences exist between NAC supplementation and Metformin treatment in OI with sequential LE/uFSH. This warrants further investigation in future studies.

Conclusions

In summary, this study demonstrated that NAC supplementation significantly reversed endocrine-metabolic changes and improved ovarian tissue phenotype in LE-induced PCOS mice. It also highlighted the protective effects of NAC against oxidative stress-induced damage to oocytes, while enhancing both enzymatic and nonenzymatic antioxidant activities in ovarian tissue. These findings suggest that NAC's therapeutic benefits in improving metabolic and endocrine parameters could complement or serve as an alternative to current treatments, such as Metformin, for managing PCOS. Furthermore, through a pragmatic, randomized, parallel-controlled clinical study, we confirmed that NAC supplementation with sequential LE/uFSH significantly improved clinical pregnancy outcomes in patients undergoing OI. Thus, NAC may serve as a valuable adjuvant therapy for OI in women with PCOS.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-024-01528-8.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

YF, HD, TL and YHL performed the experiments. XZ, DL and YHL analysed and interpreted the data. YHL wrote and conceived, provided financial support the manuscript. YL reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology (IACUC approval number: 2585). The pragmatic clinical trial was approved by the medical ethics committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval number 2023 – 0353) and was registered in the Chinese Clinical Trial Registry (www.chictr.org.cn; identifier ChiCTR2300077709). Informed consent was obtained from all individual participants included in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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