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Caffeic acid's role in mitigating polycystic ovary syndrome by countering apoptosis and ER stress triggered by oxidative stress

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder that affects women of reproductive age, characterized by androgen-induced oxidative stress leading to several metabolic disorders. In this study, we investigated the potential therapeutic effect of caffeic acid on PCOS and its underlying molecular mechanism. We used a human ovarian granulosa cell line (KGN cells) induced by hydrogen peroxide (H_2O_2) to examine how caffeic acid influences the protein expression of oxidative stress-induced apoptosis-related markers. Our results indicate that caffeic acid significantly inhibits intracellular reactive oxygen species (ROS) generation and safeguards KGN cells against oxidative stress. For the in vivo aspect of our study, female Sprague-Dawley (SD) rats were utilized to induce the PCOS model using dehydroepiandrosterone (DHEA). Caffeic acid was then administered to the rats for a duration of 6 weeks. The outcomes revealed that caffeic acid effectively improved irregular estrous cycles, fasting blood glucose levels, liver function, and lipid profiles in DHEA-induced PCOS rats. Additionally, it mitigated hyperandrogenism, enhanced steroidogenesis enzyme expression, and modulated apoptosis-related protein expression. Our findings strongly suggest that caffeic acid holds promising potential in reducing oxidative stress-induced damage and ameliorating PCOS-related complications by modulating ER stress.

1. Introduction

According to the National Institutes of Health (NIH) and the 2003 Rotterdam consensus workshop, polycystic ovary syndrome (PCOS), a reproductive system disease that causes metabolic abnormalities and reproductive dysfunction, affects approximately 5–26 % of women [1]. PCOS is commonly accompanied by hirsutism, infertility, overweight, and irregular menstruation, significantly impacting the quality of life for affected patients [2]. Infertility due to ovarian dysfunction is a major hallmark of PCOS diagnosis, along with hyperandrogenemia, decreased ovulation, and the presence of polycystic ovaries [3]. These factors collectively contribute to altered hormone secretion. The dysfunction in the hypothalamic-pituitary-ovarian axis (HPO axis) results in excessive secretion of luteinizing hormone (LH), which increases the LH receptor in theca cells. This activation triggers steroidogenesis, including steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A1), and androgen synthase such as 3β-Hydroxysteroid dehydrogenase (3β-HSD) activity [4], ultimately promoting excessive androgen secretion while reducing estrogen production [5]. Furthermore, hyperandrogenemia and hyperglycemia contribute to the proliferation of theca cells, resulting in the excessive production of reactive oxygen species (ROS) and activation of endoplasmic reticulum (ER) stress in granulosa cells. These processes lead to reduced corpus luteum capacities for estrogen and progesterone

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secretion, as well as increased oxidative stress in the follicles [6,7],ultimately causing irregular menstrual cycles, compromised oocyte health, and infertility [8]. Metformin, a traditional first-line treatment for PCOS and an insulin sensitizer [9], operates by reducing gluconeogenesis and insulin resistance in the liver, improving tissue sensitivity to insulin, and consequently lowering blood sugar levels [10]. Metformin also modulates hormones by reducing insulin concentration through the regulation of gonadotropin-releasing hormone (GnRH) production, which in turn decreases the excessive secretion of androgens, thereby improving menstrual cycles and ovulation in the ovaries [11].

Caffeic acid (CA), a phenolic acid and cinnamic acid derivative, is naturally present in various sources such as fresh vegetables, fruits, tea, and coffee, often as quinic acid [12]. CA has demonstrated antioxidative and anti-inflammatory effects, along with the ability to regulate blood glucose levels and modulate lipid profiles [13,14]. Additionally, CA serves as a scavenger of free radicals and can inhibit hydrogen peroxide (H_2O_2)-induced apoptosis [15,16]. In cases of reperfusion-induced oxidative stress, CA has been found to enhance antioxidative capabilities, thereby improving ovarian morphology [17]. Despite these known effects, the impact of CA on ovarian function, insulin resistance, oxidative stress, and hormone regulation in the context of DHEA-induced PCOS remains unclear. Therefore, the hypothesis of this study was to investigate whether CA possesses antioxidant capacities capable of mitigating the high oxidative stress on human granulosa cells (KGN) and subsequently restoring balance to sex hormone secretion and endocrine disruption in a DHEA-induced PCOS animal model.

2. Experimental section

2.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay

DPPH Antioxidant Assay Kit were purchased from Dojindo (Dojindo, Kumamoto, Japan). Based on manufactuer's instruction, CA (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) (Molecular Devices, San Jose, CA, USA). and different concentrations 10, 25, 75, and 100 μg/mL of CA and DPPH solutions were reacted in the dark for 30 min, and measured the absorbance at 517 nm, using ELSIA reader (Molecular Devices, San Jose, CA, USA). The half maximal inhibitory concentration (IC50) value was calculated as the minimum concentration inhibiting 50 % of DPPH free radicals per sample.

2.2. Experiment of cell culture conditions

Human ovarian granulosa cell line (KGN) was purchased from RIKEN BRC (Bioresource Research Center) (RCB1154; Ibaraki, Japan). KGN cells, adhesion type cell, were cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/Ham's F-12) medium (CAISSON, Smithfield, UT, USA), supplying 10 % fetal bovine serum (FBS)(CORNING (Manassas, VA, USA)) and 1 % antibiotics (100 units/mL penicillin, 0.1 μg/mL streptomycin, 0.25 μg/mL amphotericin (CAISSON)), and incubated in 5 % CO_2 and 37 °C condition.

2.3. MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide was purchased from MedChemExpress (Monmouth Junction, NJ, USA). KGN cells (3000 cells/ well) were seeded in 96-well plate. After cells were attached, they were treated with 1, 10, 50, and 100 μM CA for 24 h and then treated with hydrogen peroxide (H_2O_2). To measure cell proliferation, 1 mg/mL of MTT reagent was added to 96-well plate and incubated at 37 °C for 3 h. Cystal formazan was dissolved by DMSO,. the absorbance was measured at 570 nm and 630 nm, using ELISA reader, and cell viability was calculated.

2.4. Crystal violet staining

KGN cells were seeded in 6-well plate. Treated with 1, 10, 50, and 100 μM CA for 24 h and then treated with 300 μM H_2O_2. Cells were fixed with methanol in each well, then stained with 0.5 % crystal violet (Sigma-Aldrich, St. Louis, MO, USA) solution at room temperature for 20 min and then washed with 1× phosphate buffered saline (PBS) and air-dried. The crystalization was dissolved with DMSO and the absorbance was measured at 570 nm wavelength.

2.5. ROS measurement

H_2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate, Cayman) is a redox fluorescent dye that can freely pass through the cell membrane. Therefore, higher intracellular ROS content will result in stronger fluorescent signal. KGN cells were seeded in 96-well plate (3000 cells/well). After cell got attached, they were treated with 1, 10, 50, and 100 μM CA for 24 h and then treated with H_2O_2. 20 mM of H_2DCFDA was diluted to 20 μM in 1 × PBS and 100 μL/well of diluted H_2DCFDA was added to culture medium at 37 °C for 30 min staining in the dark. The ROS signal was then measured at 492, 515 nm by Thermo Varioskan Flash (Thermo Electron Corporation, Vantaa, Finland) or captured by microscopy (Olympus (Tokyo, Japan)), Image J software (Version 1.52 t, NIH, Bethesda, MD, USA) was used to quantify the ROS density in single cells.

2.6. DHEA-induced PCOS animal model

Three weeks old female Spargue-Dawley (SD) rats were purchased from BioLASCO Taiwan Corporation (Taipei, Taiwan) and were housed in the Animal Center with a 12 h light/12 h dark artificial illumination cycle in temperature-controlled room (24 ± 2 °C; 50–60 % humidity). All animal experimental procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC), Taipei Medical University (Permit number: LAC-2021–0237). During the experiment, the chow diet and water were taken ad libitum by all animals.

After adaptation for one-week, Female rats were randomly divided into 5 groups. The control group rats were treated with 0.2 mL sesame oil (Santa Cruz Biotechnology (CA, USA)) by subcutaneous injection (s.c.) and the model group rats were induced by 6 mg DHEA /100 g body weight (BW) (Sigma-Aldrich) dissolved in seasame oil by s.c. injection once daily for 5 weeks [18]. After the PCOS model was successfully induced and confirmed through vagina smears evaluation, low or high dose of CA [5, 25 mg/kg (0.18, 4 mg/kg in human)] respectively, dissolved in 0.5 % carboxymethylcellulose sodium (CMC-Na, Sigma-Aldrich) was given daily to the rats by oral gavage for 6 weeks. Positive control group was given 300 mg/kg metformin daily by oral gavage.

2.7. Body weight and vaginal smears

Rat BW was measured every three days during the experimental period. The four stages of estrous cycle were evaluated by microscopic examination of vaginal smear following staining with 1 % crystal violet during the last 10 days of induction by DHEA and the intervention period [19].

2.8. Ovarian and uterine index

After euthanasia, the excised ovarian and uterine weights were measured to determine the proportion of ovarian and uterine weight relative to body weight. This was calculated using the following formula:

Ovarian index = (Ovarian weight) / (Body weight)

Uterine index = (Uterine weight) / (Body weight)

2.9. Oral glucose tolerance test (OGTT)

After fasting for 12 h, the rats were given 2 g/kg BW glucose via oral gavage. Blood glucose was measured using a blood glucose meter (ACCU-CHECK Active, ISO15197, USA) via tail vein at time points of 0, 30, 60, and 90 min after the administration of the glucose solution.

2.10. Biochemical parameter analysis

After 6 weeks of intervention, all female rats were fasted for 12 h before sacrifice. Serum insulin levels were detected using the Mercodia ultrasensitive rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden). The Homeostasis Model Assessment of Insulin Resistance index (HOMA-IR) was calculated using the following formula:

HOMA-IR= plasma glucose (mmol/l) × serum insulin (mIU/l)/22·5·

Serum levels of androstenedione, testosterone, estrogen, and progesterone were measured by the Le ZEN Reference laboratory (Taipei, Taiwan). Serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) were measured using UV/NADH (Rate method) on the Beckman AU5800 (Beckman Coulter, Ireland). Serum levels of C-Reactive protein (CRP) were measured using Latex Particle Immunoturbidimetric on the Beckman AU5800 (Beckman Coulter, Ireland). Serum levels of low-density lipoprotein cholesterol (LDL-C) were measured using Liquid Selective Detergent, and serum levels of high-density lipoprotein cholesterol (HDL-C) were measured using Accelerator Selective Detergent, both on the Beckman AU5800 (Beckman Coulter, Ireland).

2.11. Thiobarbituric acid reactive substances (TBARS) assay

The oxidative stress, as indicated through malondialdehyde (MDA) levels, was measured in ovary using Thiobarbituric acid reactive substances assay kit (TBARS, Cayman). Ovarian tissue was placed in microcentrifuge tubes, incubated with sodium dodecyl sulfate (SDS) and color reagent for 1 h in 95°C then centrifuged for 10 minuets in 4200 rpm. The absorbance of pink clear supernatant was measured at 532 nm wavelength.

2.12. Hematoxylin and eosin (H&E) staining of ovary

After rats were sacrificed, the ovarian tissue was fixed in 10 % formalin overnight and embedded in paraffin. The embedded ovarian tissue was cut into 5-μm andstained with hematoxylin and eosin (H&E) by the Bio-CHECK LABORTORIES (Taipei, Taiwan). Images were photographed at 40x and 400x magnifications using microscope.

2.13. Western blot

Cells and ovarian tissue were lysed in RIPA buffer containing protease inhibitors (PI, Roche Diagnostics, Rotkreuz, Switzerland) and phosphatase inhibitors cocktail (PHI, Roche). Protein concentration was analyzed using a bicinchoninic acid (BCA) assay kit (T-Pro Biotechnology, New Taipei City, Taiwan). For cells, 30 μg of protein sample was quantified, and for ovarian tissue, 50 μg of protein sample was quantified. Protein samples were separated using 10–15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked in a 5 % bovine serum albumin (BSA) (BioShop, Burlington, ON, Canada) solution for 1 h and then incubated with primary antibodies, including cytochrome C (1:1000, Cell Signaling, Boston, MA, USA), poly ADP-ribose polymerase (PARP, 1:1000, Cell signaling), caspase-3 (1:1000, Cell signaling), steroidogenic acute regulatory protein (StAR, 1:1000, GeneTex (Irvine, CA, USA)), cholesterol side-chain cleavage enzyme (CYP11A1, 1:1000, Proteintech (Rehovot, Israel)), 3β-Hydroxysteroid dehydrogenase (3β-HSD, 1:500, Santa cruz), C/EBP homologous protein (CHOP, 1:1000, Proteintech), activating transcription factor 4 (ATF4, Proteintech) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10000, Proteintech), and incubated overnight at 4°C. The next day, the membranes were washed and incubated with either anti-rabbit antibody IgG (1:10000, Jackson ImmunoResearch Laboratories (West Grove, PA, USA)) or anti-mouse antibody IgG (1:10000, Jackson) for 2 h at room temperature. The membranes were reacted with electrochemiluminescence (ECL, T-pro Biotechnology) immunoassay reagent, and the visual signals were detected using the eBlot Touch Imager (eBlot Photoelectric Technology, Shanghai, China). The bands of target protein were analyzed using the Image-J software program (NIH, Bethesda, MD, USA).

3. Results

3.1. Caffeic acid exerts a protective effect of on KGN cell viability under H_2O_2-induced oxidative stress

To evaluate the effect of CA on KGN cell viability, a range of concentrations (1, 10, 50, and 100 μM) of CA were employed in this study. The results demonstrated that these concentrations did not exhibit any toxicity towards KGN cell viability after a 24-hour exposure period (Fig. 1A). In order to validate the antioxidative potential of our experimental setup and considering quercetin's well-established reputation for its antioxidant activity in natural foods [20], quercetin was used as a positive control (Fig. 1B). Preconditioning KGN cells with 1, 10, 50, or 100 μM CA, or alternatively, 10 μM quercetin, for a duration of 24 h, followed by H_2O_2 treatment, revealed a significant enhancement in KGN cell viability for CA doses of 1, 10, 50, and 100 μM in comparison to the H2O2-treated group (Fig. 1C).

To validate the influence of cell density in the H_2O_2-induced model, we escalated the dosage of H_2O_2 to ensure consistent inhibition, as previously described [21]. Furthermore, we conducted microscopic observation of cell morphology and employed crystal violet staining to corroborate cell viability. The findings revealed that in comparison to the control group, cells in the H_2O_2 -treated group displayed a fractured and detached morphology. However, preincubation with 10, 50, or 100 μM CA for 24 h significantly preserved cellular morphology following H_2O_2 exposure (Fig. 1D). Additionally, the results obtained from crystal violet staining demonstrated that CA contributed to the restoration of cell viability in contrast to the H_2O_2-treated group (Fig. 1E). Cumulatively, our data strongly suggest that CA exerts a protective effect against the deleterious impact of H_2O_2-induced oxidative stress on cellular integrity.

3.2. Caffeic acid reduced H_2O_2-induced reactive oxygen species generation in KGN cell

As CA has been demonstrated to possess notable antioxidant activity (Fig. 2A) and is widely abundant in various natural foods, we proceeded to assess its antioxidant potential through the utilization of the DPPH free radical scavenging assay. The evaluation of CA's free radical scavenging ability was conducted at concentrations of 10, 25, 75, and 100 μg/mL. Gallic acid, employed as a standard, exhibited an IC50 value of 23.7 μg/mL, while CA displayed an IC50 value of 66.2 μg/mL (Fig. 2B). In light of these findings, the intracellular ROS fluorescence density in KGN cells was substantially elevated by H2O2 exposure compared to the control group (Fig. 2C). However, preincubation with 10, 50, and 100 μM CA yielded a noteworthy reduction in ROS fluorescence density within KGN cells (Fig. 2D). This compellingly suggests that CA exerts its antioxidant effects through the mitigation of ROS accumulation in KGN cells.

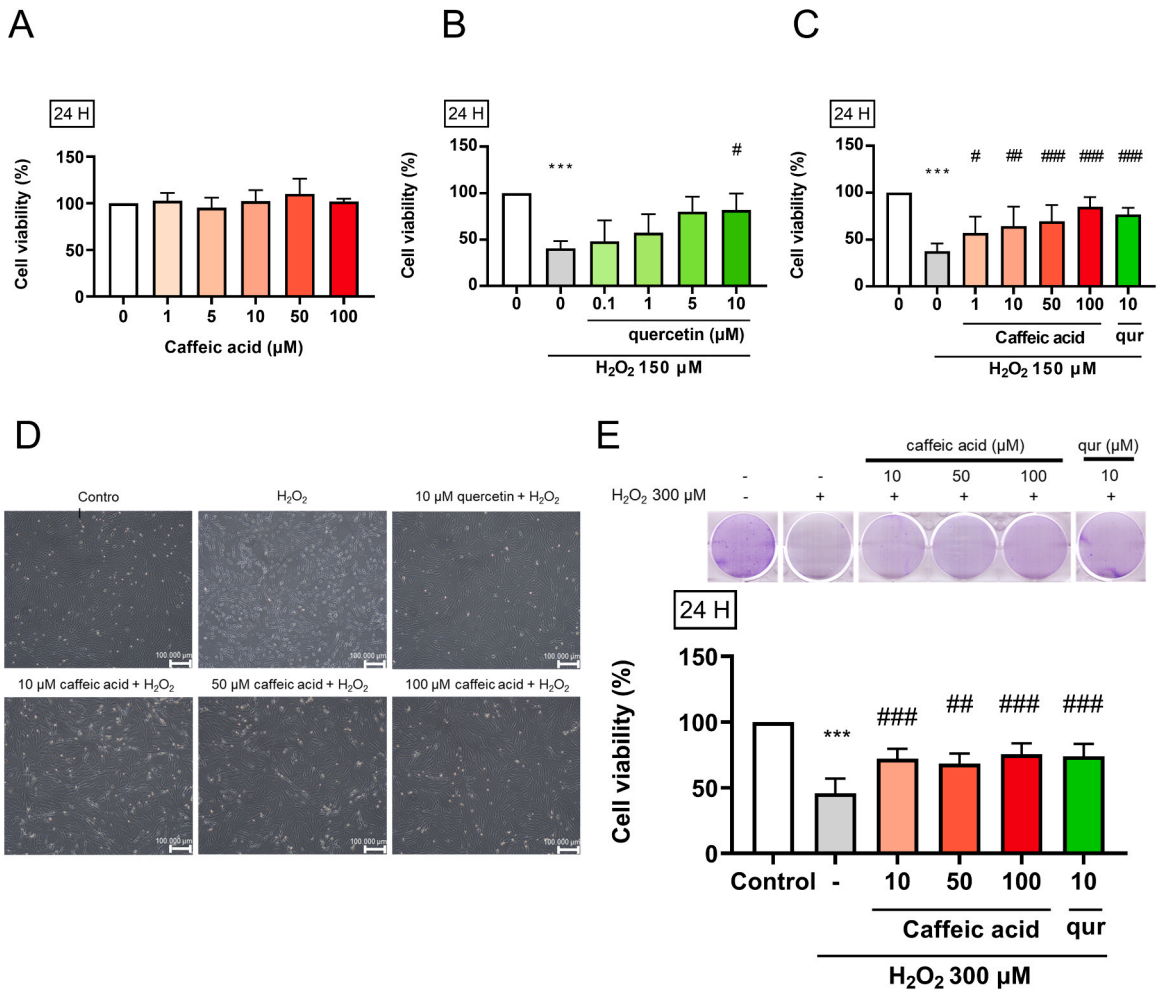

Fig. 1. Protective effect of caffeic acid on KGN cell from H_2O_2-induced oxidative stress. KGN cells were cultured in 96 well plate (3000 cells/well), treated with (A) 1, 5, 10, 50, and 100 μM caffeic acid in 10 % FBS DMEM-F12 for 24 h. (B) Pretreated with 0.1, 1, 5, and 10 μM and treated with H_2O_2. (C) Pretreated with 1, 10, 50, and 100 μM caffeic acid and quercetin as positive control in 10 % FBS DMEM-F12 for 24 h, and then induced by H_2O_2. Cell viability was measured by MTT assay. (D) The morphology of caffeic acid protected H_2O_2-induced on KGN. Scale bar = 100 μm (E) The effects of caffeic acid on the cell survival were detected using the 0.5 % crystal violet stain assay. Data were expressed in mean ± standard deviation (SD) of five independent experiments. ***, $p < 0.001$ compared to control group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared to vehicle group· Abbreviations: H_2O_2, hydrogen peroxide; qur, quercetin; KGN, human ovarian granulosa cell line.

3.3. Caffeic acid reduces H_2O_2-induced apoptosis and ER stress-related protein expression

Apoptosis is a highly regulated process orchestrated by specific signals that orchestrate programmed cell death. In the context of oxidative stress, such as induced by H_2O_2, this process is often accelerated. Essentially, upon exposure to oxidative stress, mitochondrial membrane permeability and potential are reduced, facilitating the release of cytochrome C. Subsequently, this event triggers the activation of caspase-3 and PARP, culminating in cell apoptosis [22]. In this study, we employed Western blotting to scrutinize the impact of CA on the protein expression of apoptosis-related markers induced by H_2O_2. The levels of apoptosis-associated proteins, including cleaved PARP, cleaved caspase-3, and cytochrome C, exhibited a notable increase in the H_2O_2-treated group. However, intriguingly, pre-treatment of cells with 10, 50, and 100 μM CA resulted in a discernible reduction in the expression of cleaved PARP (Fig. 3A), cleaved caspase-3 (Fig. 3B), and cytochrome C (Fig. 3C). Furthermore, considering the pivotal role of endoplasmic reticulum (ER) stress in governing physiological processes within granulosa cells [23], it is pertinent to note its connection to the progression of apoptosis [24]. Remarkably, pretreatment with 10, 50, and 100 μM CA elicited a reduction in the expression of ATF4 (Fig. 3D) and CHOP (Fig. 3E), both recognized participants in ER stress. In aggregate, these findings compellingly underscore the anti-apoptotic potential of CA, as evidenced by its capacity to mitigate the expression of apoptosis-related proteins and alleviate ER stress, ultimately ameliorating H_2O_2-induced cell death.

3.4. Effect of caffeic acid on body weight change and tissue index in DHEA-induced PCOS rats

To investigate the potential impact of CA on DHEA-induced PCOS in rats, female SD rats were subjected to daily subcutaneous injections of 6 mg/100 g BW DHEA for a duration of 5 weeks. Subsequently, these rats were administered either 5 or 25 mg/kg BW of CA (treatment groups), and a positive control group received 300 mg/kg BW of metformin. This experimental setup spanned an additional 6 weeks (Fig. 4A). Throughout the study period, body weights were meticulously recorded at 3-day intervals, revealing no statistically significant differences among the various groups (Fig. 4B).

The volume of polycystic ovaries was quantified using a vernier scale. A conspicuous diminishment in ovarian volume was observed in the DHEA-exposed group compared to the control group. However, strikingly, the CA and metformin treatment groups exhibited

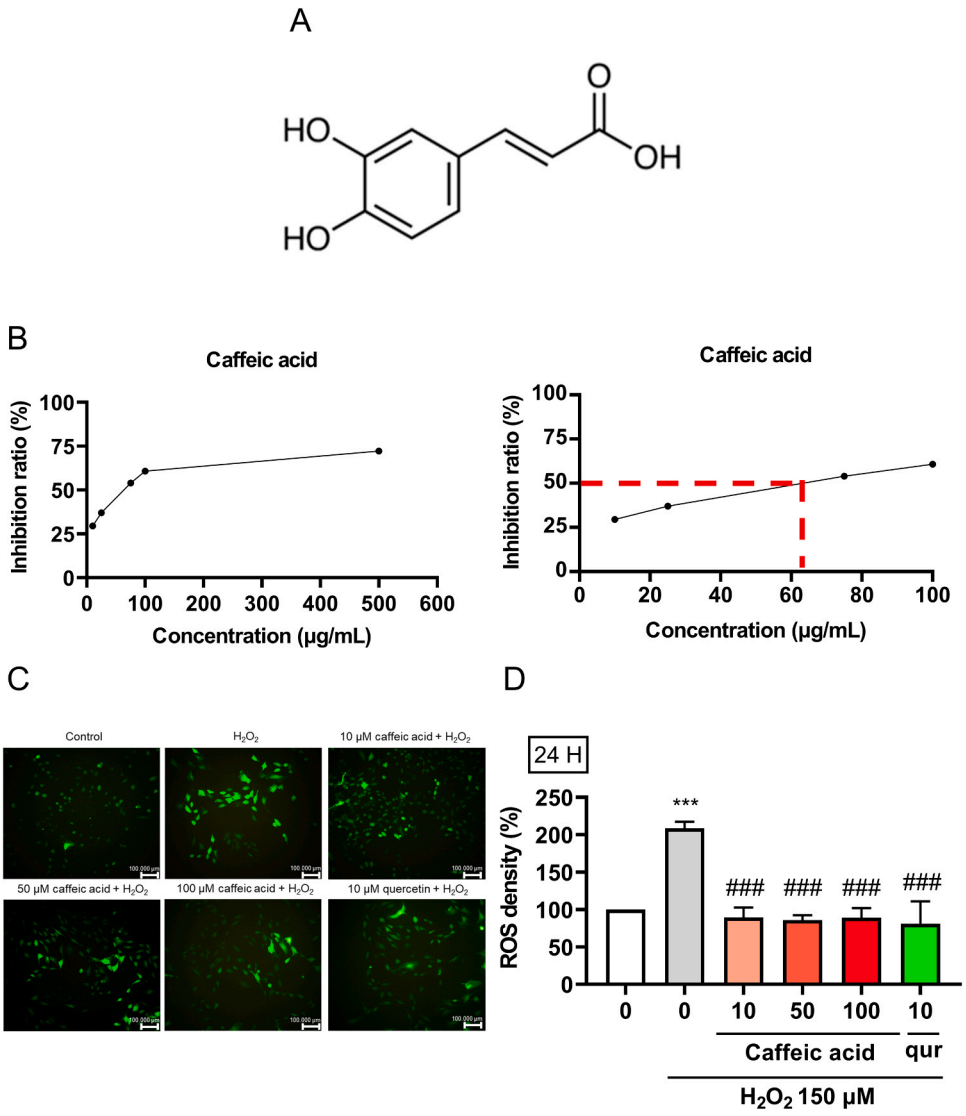

Fig. 2. Antioxidant Efficacy of Caffeic Acid against H_2O_2-Induced Generation of Reactive Oxygen Species in KGN Cells. (A) Structural representation of caffeic acid. (B) The antioxidant capability of caffeic acid was evaluated through its scavenging activity against DPPH radicals. (C) Fluorescence microscope images depicting the impact of caffeic acid on H_2O_2-induced ROS production, visualized using the H_2DCFDA dye. KGN cells were cultured in a 96-well plate, pre-treated with 10, 50, and 100 μM caffeic acid, and subsequently induced by H_2O_2. (D) Caffeic acid effectively mitigated H_2O_2-induced ROS generation in KGN cells. The quantification of fluorescence was conducted using Image J software. Data represent the mean ± standard deviation (SD) from four independent experiments. ***, $p < 0.001$ compared to the control group. ###, $p < 0.001$ compared to the vehicle group. Abbreviations: H_2O_2, hydrogen peroxide; KGN, human ovarian granulosa cell line; qur, quercetin.

significantly augmented ovarian volumes in comparison to the untreated DHEA group (Fig. 4C, D). Furthermore, noteworthy changes were detected in the ovarian and uterine indices. Specifically, the ovarian index displayed a decrease, while the uterine index exhibited an increase in the DHEA group when contrasted with the control group. Encouragingly, intervention with either CA or metformin elicited a marked elevation in the ovarian index and a corresponding reduction in the uterine index (Fig. 4 E, F).

These collective findings prompt the hypothesis that CA possesses the capability to counteract the DHEA-induced reduction in ovarian volume/weight, while concurrently promoting an increase in uterine weight.

3.5. Effect of caffeic acid on the estrous cycle in DHEA-induced PCOS rat model

A regular estrous cycle serves as a key indicator of robust ovarian function. The estrous cycle in female rats unfolds across four distinct stages: proestrous (P), estrous (E), post-estrus (metestrous, M), and post-estrus (diestrous, D), each cycle spanning 4–5 days [18] (Fig. 5A). However, the imposition of excessive androgen levels can induce PCOS, thereby disrupting and causing a standstill in the natural rhythm of the estrous cycle. In light of this, our objective was to investigate the potential of CA in reinstating a regular and synchronized estrous cycle pattern.

The obtained results revealed that, when compared to the control group, the estrous cycle of the DHEA-exposed group experienced disruption and stagnation. Encouragingly, the introduction of 5 or 25 mg/kg BW CA, or 300 mg/kg BW metformin, exhibited a noteworthy enhancement in restoring the rhythmicity of the estrous cycle in comparison to the DHEA group (Fig. 5B, C). Additionally, we undertook an assessment of the efficacy of inducing the PCOS rat model through excessive DHEA injection, as well as the subsequent recovery rates following CA and metformin interventions. Our findings unveiled that the administration of excess DHEA indeed achieved the successful induction of a PCOS model, evidenced by a 100 % success rate in the DHEA group. Intriguingly, when contrasted with the DHEA-induced disruption, the application of CA and metformin exhibited a substantial restoration of the estrous cycle, yielding recovery rates of 66.7 %, 80 %, and 80 % for the low and high doses of CA, and metformin, respectively. Consequently, it becomes apparent that caffeic acid holds the potential to ameliorate the estrous cycle stagnation stemming from heightened androgen exposure in female rats.

3.6. Effect of caffeic acid on the histopathology of the ovaries, theca cell and granulosa cell layers in DHEA-induced PCOS rats

Microscopic examination of ovarian tissue sections from each

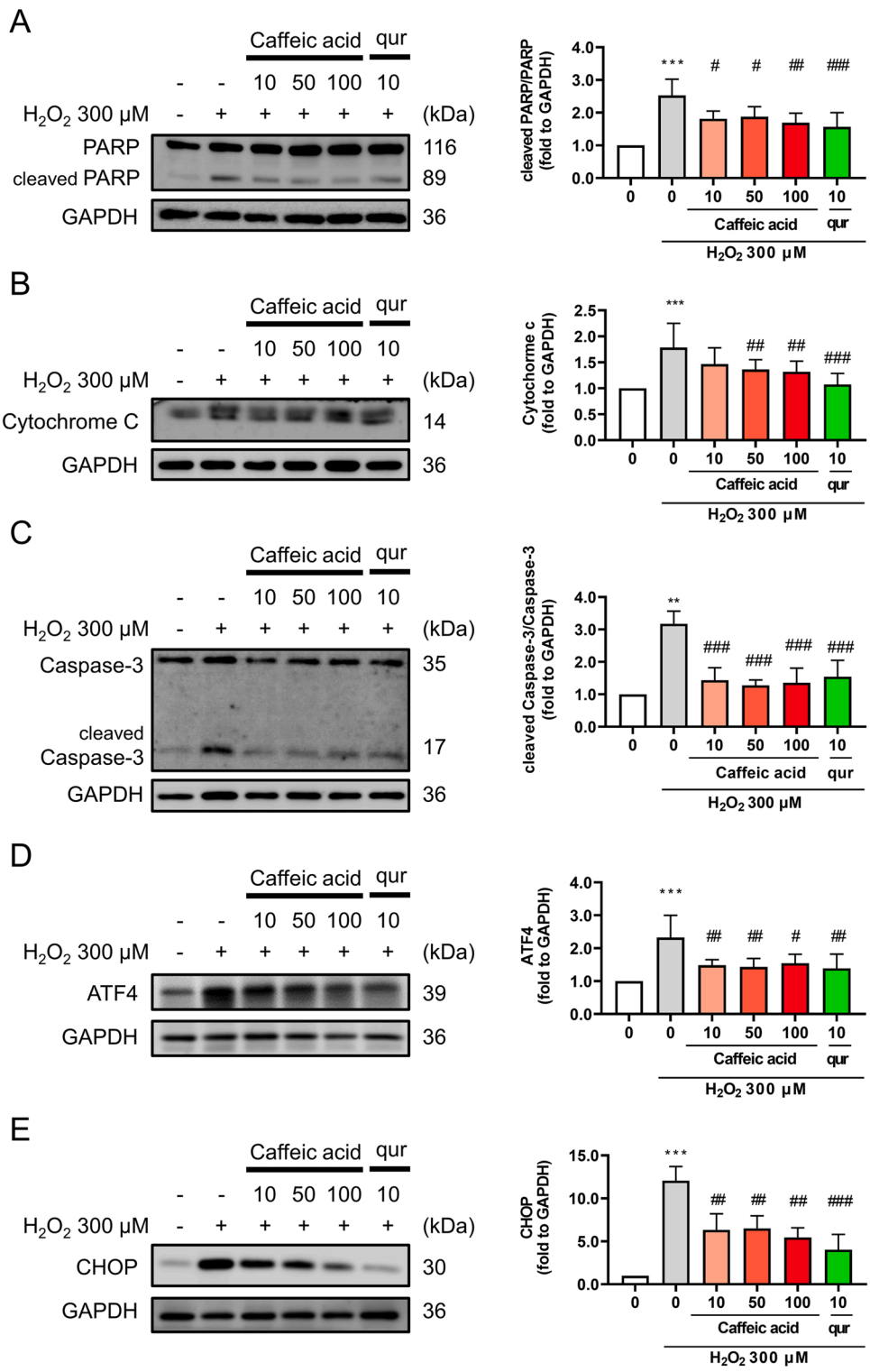

Fig. 3. Effect of caffeic acid on H_2O_2-induced cell-apoptosis-related protein expression in KGN cell. KGN cells were pretreated with 10, 50, and 100 μM caffeic acid and 10 μM quercetin in 10 % FBS DMEM-F12 for 24 h, and then induced by H_2O_2. Then, determined by Western blot. The protein expression of (A) PARP, (B) caspase-3, (C) cytochrome C, (D) ATF4, and (E) CHOP were assayed with Western blotting analysis. The value was quantified and followed by normalizing with internal control GAPDH. The densitometric estimation were quantified by Image J software. Data were represented as mean ± standard deviation (SD) of four independent experiments. ***, $p < 0.001$ compared to control group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared to vehicle group. Abbreviations: PARP, poly ADP-ribose polymerase; H_2O_2, hydrogen peroxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qur, quercetin; KGN, human ovarian granulosa cell line.

experimental group (Fig. 6A) revealed distinct structural variations. The control group exhibited normative ovarian architecture, characterized by the presence of multiple corpus luteum structures, well-defined granulosa cell layers, and diverse stages of folliculogenesis. Conversely, the DHEA-exposed group displayed diminished corpus luteum count, a preponderance of cystic follicles, augmented theca cell layer thickness, and reduced granulosa cell layer width. Intriguingly, administration of CA or metformin induced a restoration of folliculogenesis, accompanied by a notable augmentation in corpus luteum count and granulosa cell layer thickness, concurrently with a reduction in cystic follicles and theca cell layer thickness (Fig. 6B, C, D, E).

These insightful outcomes collectively underscore the inhibitory potential of CA against the formation of cystic follicles and its capability to mitigate the thickness of the theca cell layer within the ovarian tissue. Simultaneously, CA exhibited an encouraging propensity to elevate the number of corpus luteum structures and bolster the thickness of the granulosa cell layer.

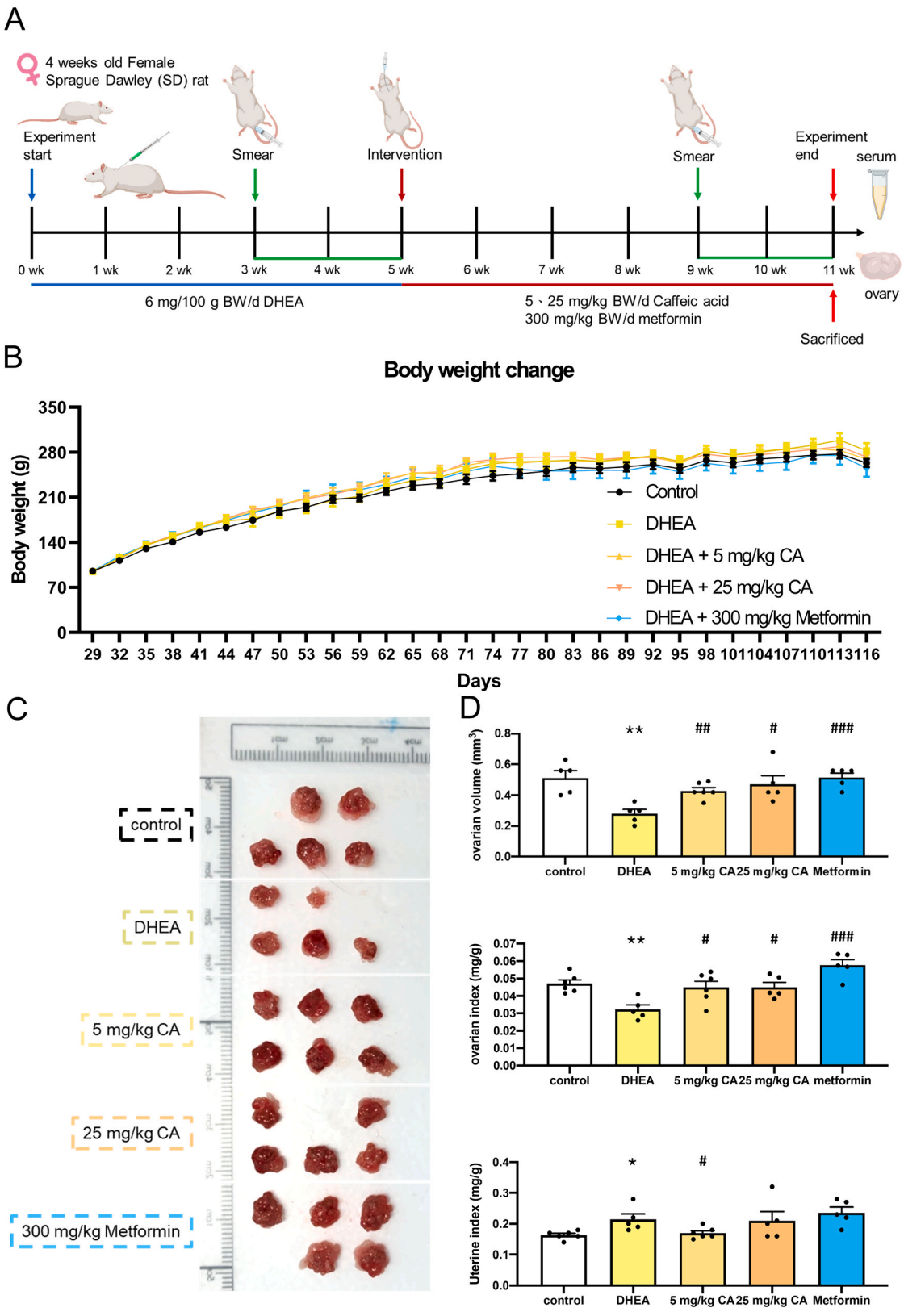

Fig. 4. Effect of caffeic acid on body weight change and tissue index in DHEA-induced PCOS rats. (A) Experimental design for PCOS model via DHEA and caffeic acid treatment daily. (B) The body weight in each group was measured every 3 days during the experimental period. (C, D) The ovarian morphology and ovary volume. (E, F) The ovarian and uterine index of SD rats in control, DHEA, low, and high dosage of caffeic acid, and metformin groups. Data represent mean ± standard error of the mean (SEM). n = 5–6. Different groups indicate significant difference at $p < 0.05$ by using Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$ compared to control group. #, $p < 0.05$; ###, $p < 0.001$ compared to DHEA group. Abbreviations: DHEA, dehydroepiandrosterone; CA, caffeic acid; PCOS, polycystic ovarian syndrome.

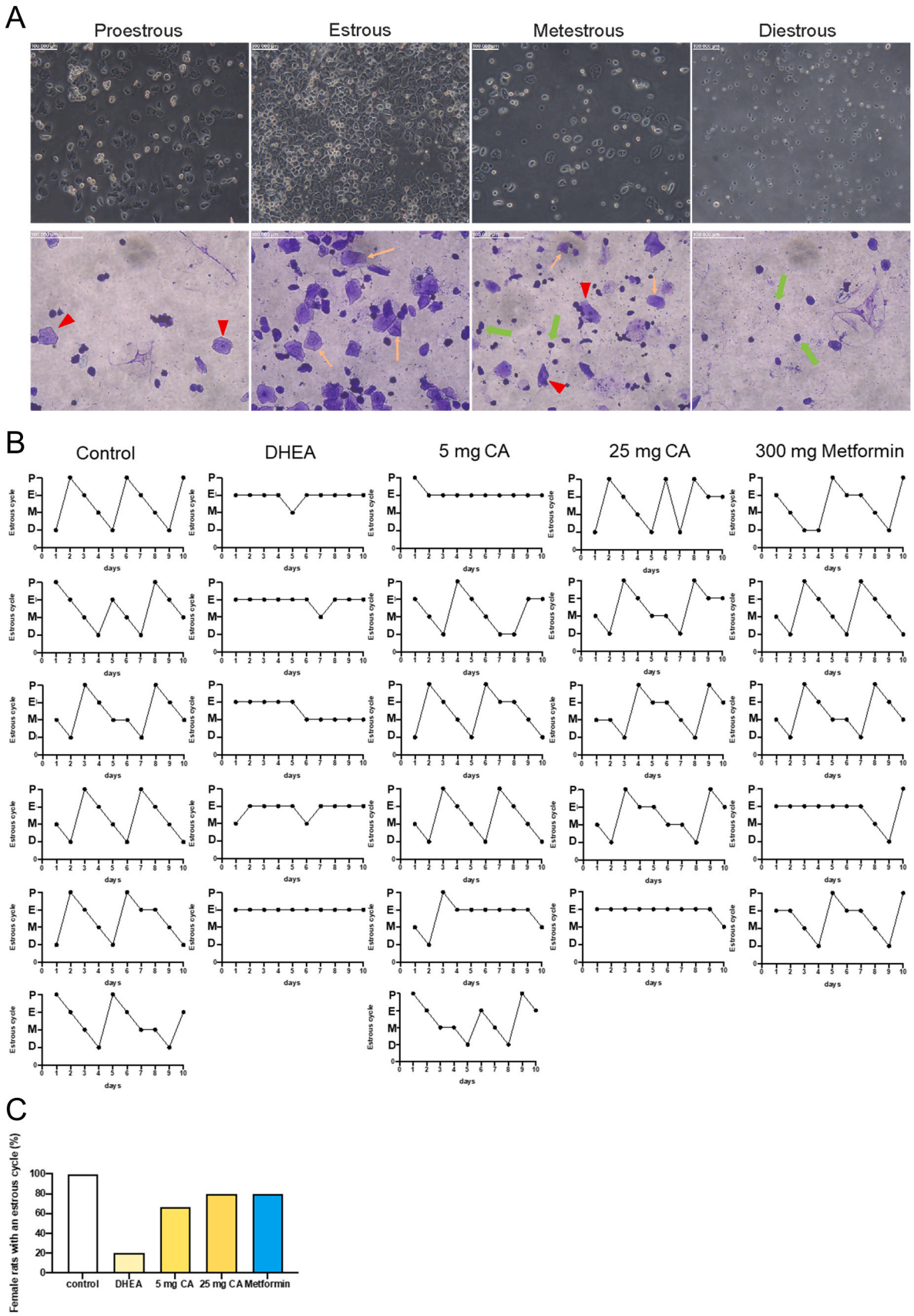

Fig. 5. Effects of caffeic acid on estrous cycle in a rat model of DHEA-induced PCOS rats. (A) The typical cell type of the estrous cycle was photographed and stained by 0.1 % crystal violet. P, proestrus; E, estrus; M, metestrus; D, diestrus. Red arrow indicates nucleated epithelial cells; orange arrow indicates non- nucleated cornified cells; green arrow indicates leukocytes. Scale bar = 200 μm and 400 μm. (B) Ten continuous days of the estrous cycle were monitored by vaginal smear. (C) Estrous cycle restore. n = 5–6. Abbreviations: DHEA, dehydroepiandrosterone; CA, caffeic acid; PCOS, polycystic ovarian syndrome.

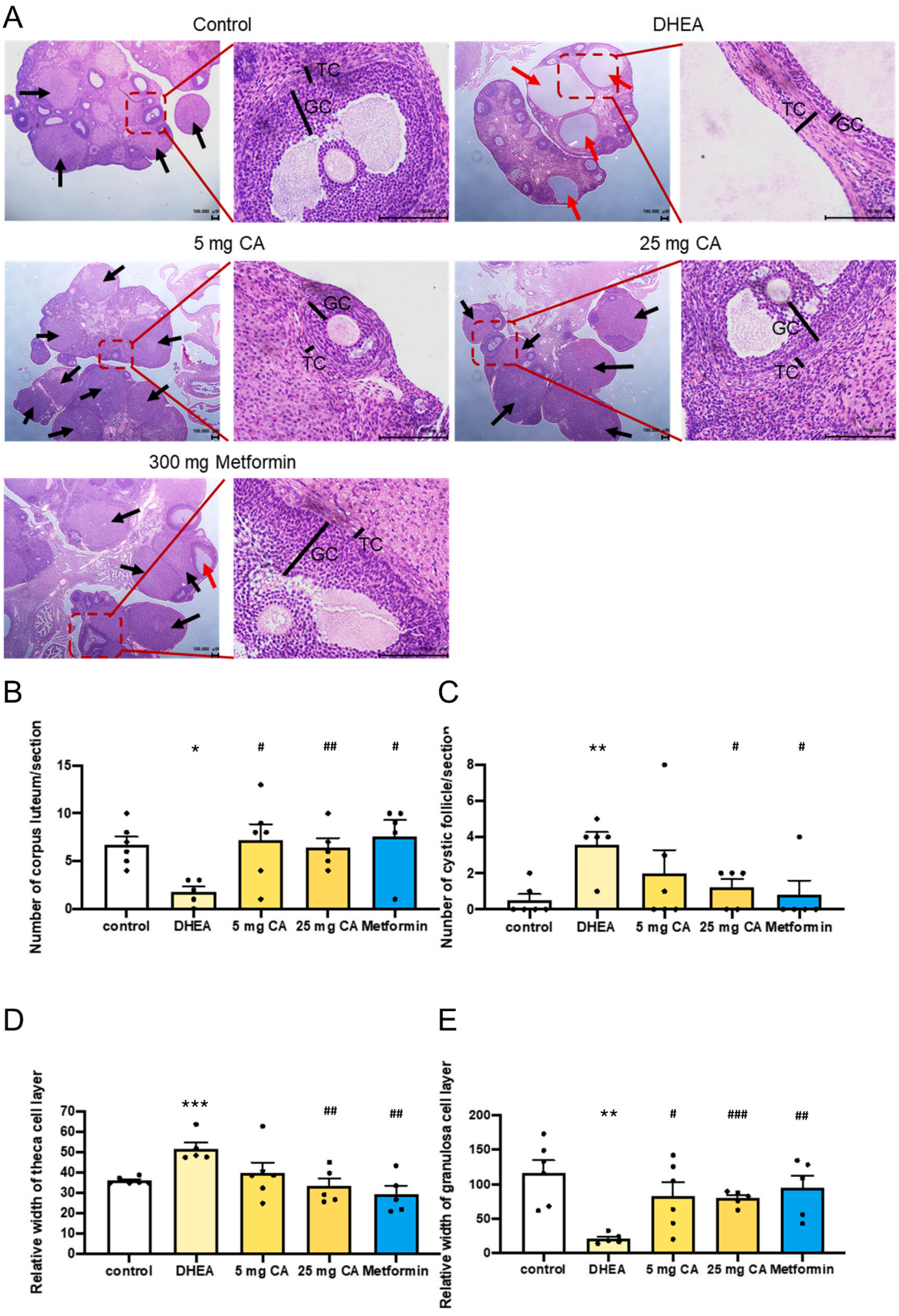

Fig. 6. Effect of caffeic acid on histopathological observations of ovaries, theca cell and granulosa cell layer in DHEA-induced PCOS rats. (A) Histopathologic images of ovarian sections from rats with DHEA-induced PCOS. Scale bar = 40 μm and 400 μm. Number of (B) corpus luteum and (C) cystic follicle. Quantitative analysis of the width of the (D) theca cell and (E) granulosa cell layer in a cross-section. Data represent mean ± standard error of the mean (SEM). n = 5–6. Different groups indicate significant difference at $p < 0.05$ by using Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to control group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared to DHEA group. Black arrow indicates corpus luteum. Red arrow indicates cystic follicle. Abbreviations: DHEA, dehydroepiandrosterone; PCOS, polycystic ovarian syndrome; CA, caffeic acid; TC, theca cell; GC, granulosa cell.

3.7. Effect of caffeic acid on androstenedione, testosterone, estrogen, and progesterone levels in DHEA-induced PCOS rats

Elevated serum androgens, exemplified by androstenedione and testosterone, frequently typify the clinical diagnosis of PCOS, often accompanied by aberrant estrogen and progesterone secretion [25]. Our investigation similarly noted a conspicuous reduction in serum androstenedione, testosterone, and estrogen concentrations among DHEA-induced PCOS rats subsequent to treatments with either CA or metformin. Notably, a discernible elevation in progesterone levels was also observed (Fig. 7A).

3.8. Effect of caffeic acid on OGTT, fasting blood glucose, fasting insulin, and HOMA-IR in DHEA-induced PCOS rats

Elevated androgen levels can induce fasting blood glucose elevation

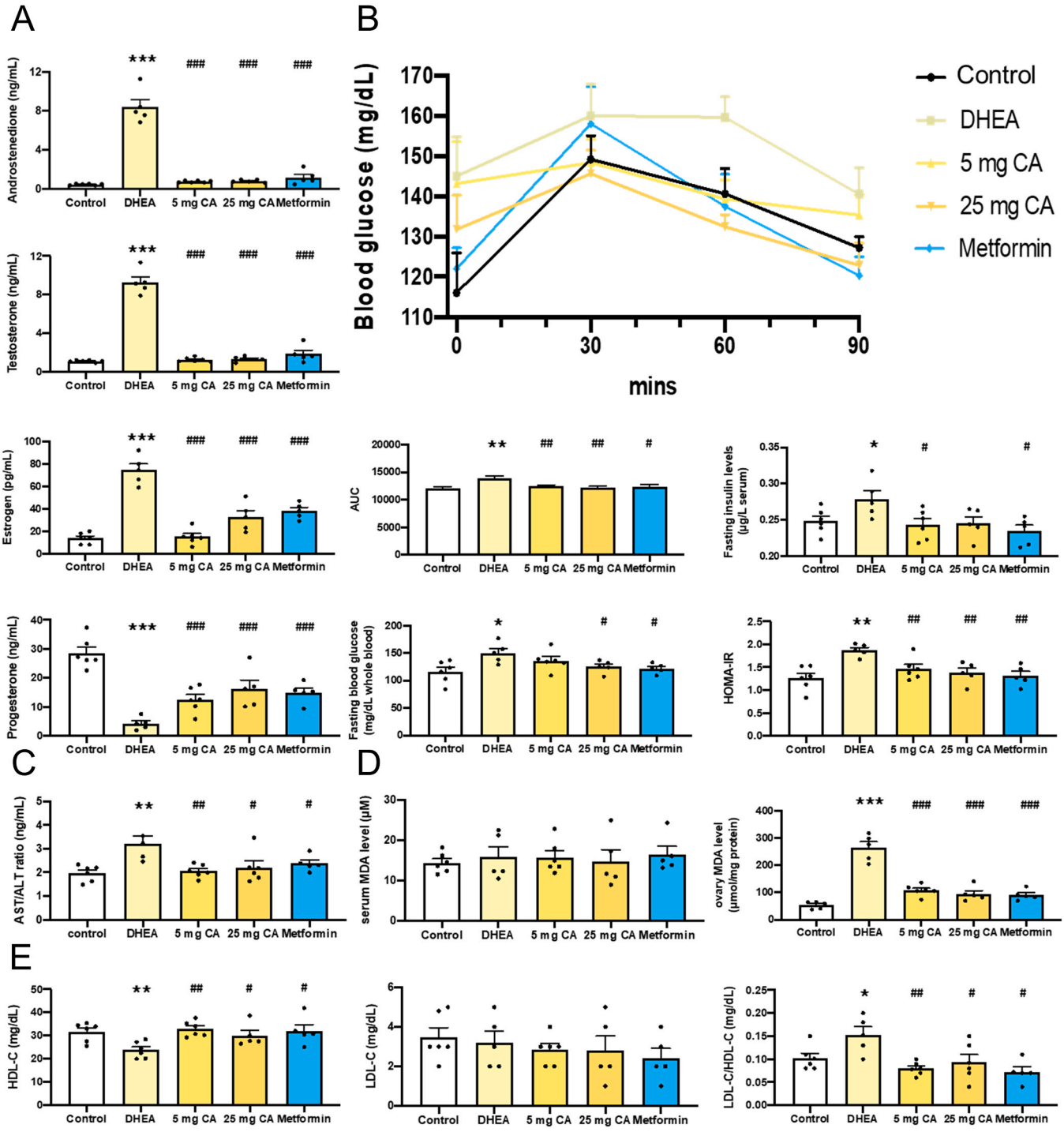

Fig. 7. Effect of caffeic acid on serum biochemical parameters in DHEA-induced PCOS rats. (A) Levels of androstenedione, testosterone, estrogen, and progesterone. After fasting for 12 h, (B) Blood glucose and insulin response were measured. Measured the biochemical parameters (C) Levels of AST/ALT ratio (D) MDA levels in the ovary and serum. (E) Levels lipid profile. Data represent mean ± standard error of the mean (SEM). n = 5–6. Different groups indicate significant difference at $p < 0.05$ by using Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to control group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared to DHEA group. Abbreviations: DHEA, dehydroepiandrosterone; PCOS, polycystic ovarian syndrome; CA, caffeic acid.

and hyperinsulinemia, culminating in insulin resistance [26]. In our study, the administration of DHEA resulted in a substantial increase in fasting blood glucose (FBG) levels and the corresponding area under the curve (AUC) of blood glucose levels. However, treatment with CA or metformin led to a noteworthy reduction in both FBG and AUC compared to the DHEA group. Furthermore, the DHEA group exhibited an elevation in fasting insulin levels, which was effectively mitigated by intervention with 5 mg/kg CA or metformin. Calculations based on the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) revealed a marked escalation in insulin resistance in the DHEA group. In contrast, CA and metformin administration exhibited a significant amelioration of insulin resistance in DHEA-induced PCOS rats (Fig. 7B). Collectively, these results underscore the potential of CA to mitigate elevated fasting blood glucose and enhance insulin sensitivity in DHEA-induced PCOS rats.

3.9. Effect of caffeic acid on liver function, and ovarian oxidative stress in DHEA-induced PCOS rats

The AST/ALT ratio stands as a valuable prognostic indicator utilized to assess liver injury and health [27]. Notably, the AST/ALT ratio exhibited a substantial increase in the DHEA group, while the administration of CA and metformin led to a significant reduction in the levels of the AST/ALT ratio (Fig. 7C), thereby suggesting a potential protective effect on liver health.

The pronounced oxidative stress associated with elevated androgen levels can contribute to various disorders in PCOS [28]. While the serum MDA level exhibited no significant differences among the different experimental groups, a noteworthy increase in MDA levels was observed in the ovary of the DHEA group compared to the control group. Intriguingly, treatment with CA or metformin over a duration of 6 weeks led to a discernible decrease in ovarian MDA levels (Fig. 7D). These findings strongly imply that CA holds the potential to ameliorate liver function and mitigate oxidative stress in the context of DHEA-induced PCOS rats.

3.10. Effect of caffeic acid on lipid profile in DHEA-induced PCOS rats

The levels of LDL-C did not exhibit significant variation among the tested groups. However, a noteworthy decrease was observed in HDL-C levels, consequently leading to a significant increase in the LDL-C/HDL-C ratio within the DHEA group, as compared to the control group. Notably, the administration of CA or metformin effectively led to a significant reduction in the LDL-C/HDL-C ratio when compared to the DHEA group (Fig. 7E).

3.11. Effect of caffeic acid on steroidogenesis enzymes, apoptosis and ER stress related protein expression in DHEA-induced PCOS rats

To evaluate ovarian function, the expression of steroidogenesis enzymes and apoptosis-related proteins was evaluated by Western blot analysis. DHEA group showed increased 3β-HSD protein expression while inhibited the expression of CYP11A1 and StAR proteins, compared to the control group (Fig. 8A–C). The DHEA group also exhibited a noteworthy increase in the expression of apoptosis-related proteins, including cleaved caspase-3 (Fig. 8D), along with an elevation in ER-stress-related protein expression (Fig. 8E, F). Nevertheless, treatment with caffeic acid (CA) or metformin demonstrated a substantial inhibition of cleaved caspase-3, ATF4, CHOP, and 3β-HSD protein expression, while concurrently enhancing the protein expression of CYP11A1 and StAR (Fig. 8). Consequently, it can be inferred that CA potentially possesses the capacity to repress apoptosis and ER stress within ovarian cells, thereby ameliorating the expression of steroidogenesis enzymes.

4. Discussion

In women with PCOS, elevated levels of androgens and blood glucose can lead to oxidative stress within the ovary, resulting in an increased production of ROS and subsequent damage to ovarian tissue [29]. Oxidative stress is a key factor in the pathogenesis of PCOS. The accumulation of oxidative stress due to an imbalance between ROS production and antioxidant defense mechanisms can, in turn, cause severe hyperandrogenemia and insulin resistance. This chronic oxidative stress also triggers inflammation, leading to further damage to the ovaries and exacerbating PCOS symptoms [30]. To mitigate the negative effects of oxidative stress, interventions that enhance ROS clearance may potentially improve PCOS. In the present study, we observed that H_2O_2-induced oxidative stress led to increased damage and apoptosis of KGN cells, along with elevated intracellular ROS levels. Similar results were observed in the DHEA-induced PCOS model, where elevated oxidative stress contributed to ovarian dysfunction, insulin resistance, and abnormal lipid profiles.

Caffeic acid, a phenolic acid, exhibits notable antioxidant properties and has the capability to counteract oxidative stress-induced apoptosis in cells and tissues [31]. Previous studies have shown that CA can increase the expression of the nuclear factor erythroid 2–related factor 2 (Nrf2) gene and the activities of NAD(P)H quinone dehydrogenase 1 (NQO1) and heme oxygenase-1 (HO-1) enzymes, which reduce ROS production and inhibit oxidative stress [32]. Furthermore, CA has been shown to mitigate hepatic steatosis through the regulation of ER stress [33]. In our study, we pretreated KGN cells with CA for 24 h and then exposed them to H_2O_2. Pretreatment with CA significantly inhibited cell damage, reduced ROS fluorescence density, ER stress, and alleviated oxidative stress, thereby improving cell viability. The hyperandrogenism and oxidative stress caused by PCOS may activate ER stress [7] and induce the expression of apoptosis-related markers in ovarian granulosa cells, including caspase-3, PARP, and cytochrome C [34]. Previous studies have shown that treatment with CA can protect human trophoblasts HTR-8/SVneo cells from H_2O_2-induced oxidative stress with subsequent DNA damage and cell apoptosis [35]. In this study, pretreatment with CA followed by exposure to H_2O_2 could inhibit the induction of cleaved PARP, cleaved caspase-3, CHOP, ATF4, and cytochrome C protein expression in KGN cells, thus protecting cells from oxidative stress-induced cell damage (Fig. 4).

The emergence of ER stress results from the accumulation of misfolded or unfolded proteins within the endoplasmic reticulum, a pivotal organelle in charge of protein synthesis and folding processes [36]. Pertinently, the modulation of ER stress is also heightened in PCOS patients [37], a circumstance that can contribute to a spectrum of disruptions including impaired glucose uptake amplifying insulin resistance, perturbations in steroidogenesis, inflammatory reactions, and ovarian dysfunction [23]. Notably, CHOP and ATF4, pivotal players in the induction of ER stress, assume a central role in fostering apoptosis [38], and their activation is instigated by hyperandrogenism in the context of PCOS [7]. Hyperandrogenism, a hallmark feature of PCOS, significantly underpins the manifestation of reproductive and metabolic anomalies observed in affected individuals [39]. Among the agents shaping this androgenic milieu, dehydroepiandrosterone (DHEA) occupies a prominent position. DHEA substantially influences androgen biosynthesis, consequently impacting the formation of polycystic follicles within the ovary. This cascade of events leads to notable outcomes including the thinning of granulosa cells, thickening of theca cells, formation of atretic follicles, perturbed estrous cycles, and diminished corpus luteum [40]. Furthermore, DHEA exerts its influence on the serum levels of various steroid hormones, such as androstenedione and testosterone levels, while concurrently diminishing progesterone levels, thereby culminating in reproductive dysfunction [41]. Notably, DHEA's impact also extends to the concentration of estrogen, which can stem from non-cystic follicles or peripheral ovarian tissues [42,43]. Androstenedione, acting as a precursor of estrogen, holds the potential to drive

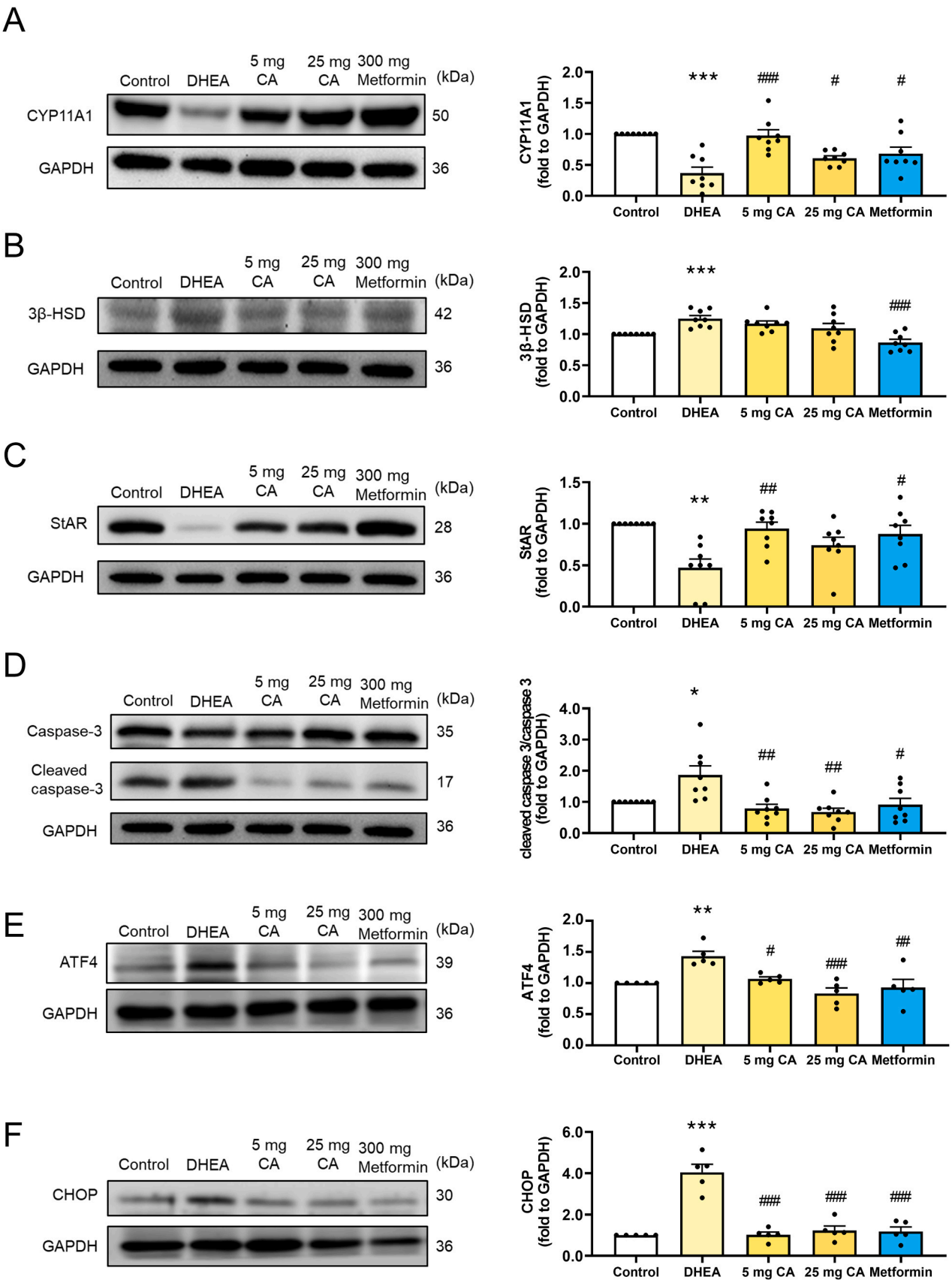

Fig. 8. Effect of caffeic acid on steroidogenesis enzyme and apoptosis related protein expression in DHEA-induced PCOS rats. The protein expression of (A) CYP11A1, (B) 3β-HSD, (C) StAR, (D) cleaved caspase-3, (E) ATF4, and (F) CHOP were assayed by western blot. Different groups indicate significant difference at $p < 0.05$ by using Student's *t*-test. Data represent mean ± standard error of the mean (SEM). n = 5–6. **, $p < 0.01$; ***, $p < 0.001$ compared to control group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared to DHEA group. Abbreviations: DHEA, dehydroepiandrosterone; PCOS, polycystic ovarian syndrome; CA, caffeic acid.

an overproduction of estrogen within granulosa cells when in excess, primarily due to its heightened synthesis by theca cells. Our findings suggest that CA treatment exhibits the capacity to restore ovarian morphology, enhance steroid hormone secretion, and reinstate regular estrous cycles in DHEA-induced PCOS rats.

PCOS patients commonly grapple with a suite of concurrent metabolic irregularities, encompassing hyperglycemia, insulin resistance, inflammation-linked liver complications, and anomalous lipid profiles [44]. Elevated androgen levels can compromise tissue glucose uptake and undermine insulin sensitivity, thereby fueling conditions such as hyperglycemia, heightened fasting blood glucose (FBG), and increased insulin resistance, quantified using the homeostasis model assessment (HOMA-IR). This intricate interplay fosters the emergence of cystic follicles and stimulates excessive androgen synthesis by theca cells, further contributing to erratic estrous cycles [45]. The accumulation of androgen-induced oxidative stress can engender chronic inflammation, which, in turn, gives rise to escalated levels of AST and ALT, in tandem with provoking dyslipidemia [46]. Notably, CA intervention not only rectified ovarian function and disrupted estrous cycles but also bolstered insulin sensitivity, lowered glucose levels, and mitigated oxidative-linked metabolic imbalances.

The formation of cystic follicles and the presence of hyperandrogenism can catalyze apoptosis within granulosa cells of the ovarian milieu [47]. This phenomenon in turn can exert influence on the synthesis of sex hormones and the expression of key proteins, including StAR, CYP11A1, and 3β-HSD, within the theca cells. In the context of PCOS, the protein expression of 3β-HSD, a pivotal enzyme in androstenedione formation, experiences an elevation in theca cells, concomitant with an upregulation in caspase-3 protein expression, thereby fostering excessive androgen secretion while concurrently diminishing progesterone secretion and the granulosa cell layer [48]. Therefore, our results showed that the protein expression of cleaved caspase-3 in the ovary was increased in the DHEA-induced group, promoting ovarian apoptosis. Furthermore, due to the decreased level of progesterone, the protein expression of StAR and CYP11A1 were lower in the DHEA group, while 3β-HSD had a higher expression in the DHEA group. In our study, we found that the intervention of CA could increase the protein expression of StAR and CYP11A1, while inhibiting the protein expression of 3β-HSD in the DHEA-induced PCOS group.

In summary, PCOS characterized by excess androgens and blood glucose leads to oxidative stress within the ovary, triggering inflammation and damaging ovarian tissue. This damage manifests as symptoms of PCOS, including ovarian dysfunction, insulin resistance, and abnormal lipid profiles. Caffeic acid, a phenolic acid with antioxidant properties, effectively inhibits H_2O_2-induced oxidative stress and subsequent apoptosis of ovarian KGN cells. Furthermore, CA treatment restores ovarian morphology, improves steroid hormone secretion, and normalizes irregular estrous cycles in rats with DHEA-induced PCOS. Additionally, caffeic acid enhances insulin sensitivity, reduces glucose levels, ameliorates oxidative-related metabolic disorders, and aids in the recovery of ER stress. These findings collectively highlight the potential of CA as a therapeutic intervention for PCOS-related complications.

5. Conclusion

In conclusion, our findings suggest that CA holds potential as a treatment for PCOS due to its antioxidant capacity, effectively shielding cells from heightened oxidative stress and inhibiting H_2O_2-induced apoptosis. Furthermore, utilizing an in vivo PCOS rat model, CA demonstrated the ability to enhance the estrous cycle in DHEA-induced PCOS rats. This enhancement is accompanied by the regulation of hormone secretion, including androstenedione, testosterone, estrogen, and progesterone, achieved through the modulation of protein expression in steroid hormone synthesis and ER stress recovery. Notably, CA exhibited improvements in fasting blood glucose, fasting insulin levels, and insulin resistance. Additionally, CA elevated HDL-C concentration, rectified dyslipidemia, and mitigated ovarian oxidative stress, thus potentially alleviating complications associated with PCOS.

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CRediT authorship contribution statement

Yi-Fen Chiang: Software, Investigation, Methodology, Visualization, Writing – original draft. **I-Cheng Lin:** Software, Investigation, Methodology, Visualization, Writing – original draft. **Ko-Chieh Huang:** Formal analysis. **Hsin-Yuan Chen:** Formal analysis. **Mohamed Ali:** Writing – review & editing. **Yun-Ju Huang:** Results interpretation. **Shih-Min Hsia:** Conceptualization, Project administration, Resources, Data curation, Supervision, Validation, Writing – review & editing. All authors read and approved the final manuscript.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data Availability

Data will be made available on request.

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