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Original Research

The Effects of Abscisic Acid Administration in PCOS Rat Model and H_2O_2-Induced Human Granulosa Cell Damage: A Preliminary Investigation

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Abstract

Background: Polycystic ovary syndrome (PCOS) is commonly associated with metabolic disorders. Abscisic acid (ABA), a plant hormone found in vegetables and fruits that can be naturally supplied through dietary intake, has previously been studied for its benefits to human health, particularly in people with diabetes. ABA plays a key role in glucose metabolism, inflammation, and tumor growth. The aim of this study was to investigate the therapeutic effect of ABA on letrozole-induced PCOS rats. **Methods**: Wistar rats were implanted with continuous-release letrozole pellets to induce a PCOS-like phenotype, and subsequently treated with ABA or vehicle control. Body weight changes, Testosterone (T) levels, fasting insulin measurements, and glucose tolerance tests were investigated. A cell apoptosis model, induced by hydrogen peroxide (H_2O_2), was established. Cell viability was tested by cell counting Kit-8. Cell apoptosis was tested by flow cytometry, and oxidative stress state of cells was examined via reactive oxygen species (ROS) array. **Results**: Body weight, glucose impairment, and T level were significantly improved in ABA-treated PCOS rats compared to vehicle-treated rats. ABA significantly reduced H_2O_2-induced human ovarian granulosa cell line (KGN) cell apoptosis and ROS levels and increased cell viability. **Conclusions**: The findings suggest that ABA could be an adjunctive treatment candidate for improving the insulin sensitivity and hyperandrogenemia of PCOS patients, and for ameliorating H_2O_2-induced cell apoptosis and oxidative stress.

Keywords: abscisic acid; polycystic ovary syndrome; oxidative stress; cell apoptosis

1. Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting reproductive women, which is associated with reproductive and metabolic alterations, including hyperandrogenism, insulin resistance (IR), polycystic ovaries, infertility, and an increased risk of cardiovascular disease and type 2 diabetes [1]. The prevalence and incidence of metabolic syndrome are much higher in PCOS patients. Hypotheses for this pathological mechanism include genetic abnormalities, hormonal imbalances and environmental and lifestyle factors [2]. Previous studies have reported that PCOS patients who lose weight could benefit from improved ovulation [3,4]. Currently, accessible medication for PCOS is controversial due to the complex nature of this disease.

Abscisic acid (ABA), a phytohormone widely present in fruits and vegetables at varying concentrations, has been shown to behave as an endogenous hormone in mammals. In healthy subjects, it is produced and released from human pancreatic β-cells in response to high glucose concentration [5]. With an insulin-independent mechanism, a low dose of ABA can control the metabolic response to glucose supply by stimulating glucose uptake in skeletal muscle and adipose tissue, as well as increasing energy expenditure in brown and white adipose tissues [6–8]. ABA increases the translocation of the glucose transporter insulin-dependent glucose transporter 4(GLUT-4) to the plasma membrane and GLUT-4-dependent glucose uptake. The mechanism underlying ABA involves the activation of lanthionine synthetase C-like 2 (LANCL2), which is a G protein-coupled peripheral membrane protein [9]. Previous studies found that feeding obese/diabetic db/db (C57BL/KsJ-lepr^{db}/lepr^{db}; db/db) mice with ABA improves insulin sensitivity and suppresses obesity-related inflammation [10]. In diabetic-induced rats, ABA showed 60% of oxytocin-stimulating effects on myometrial and uterine contraction effect, which indicates that ABA may be beneficial as a pre-labor prescription, especially in diabetic women [11]. ABA is generally considered a safe ingredient. In a study of sub-chronic toxicity following 4- and 13-week dietary intervention with varying concentrations of ABA in rats, no adverse toxicological effects were observed for 90 days at intakes of up to 20,000 ppm (around 1500 mg/kg body weight per day) [12]. In addition, the consumption of ABA from fig fruit extracts produced a significant clinical reduction in postprandial glucose and insulin levels in

response to a high- glycemic index (GI) glucose drink in healthy people [13]. Taken together, these studies indicate that this phytohormone may provide a possible biomedical application for PCOS.

Oxidative stress might support IR in PCOS and directly stimulate the overproduction of ovarian androgen. Accumulating researches have demonstrated that oxidative stress induced by reactive oxygen species (ROS) may promote the development of IR and hyperandrogenism, while inflammatory cytokine might be associated with endothelial dysfunction, all of which are key characteristics of PCOS [14,15]. ROS was highly expressed in ovarian tissues of letrozole-induced PCOS rats [16]. Pro-oxidant-antioxidant balance has a pivotal role in folliculogenesis and oocyte maturation of the female reproductive system [14]. ABA is also released by innate immune cells upon physical or chemical stimulation; ABA could stimulate functional activities of these cells, including migration, phagocytosis, and release of ROS and nitric oxide (NO) by inhibiting the nuclear factor kappa B pathway (NF-κB), whose activation is also associated with increased ROS production and modulates various cellular responses, including apoptosis [17,18]. Granulosa cells (GCs) are a type of ovarian cell, and metabolic activity plays a vital role in regulating oocyte quality. Oxidative stress damage in the follicular microenvironment contributes to the impaired oocyte quality in PCOS. ROS represents oxygen-derived molecules, including hydrogen peroxide (H_2O_2), superoxide anions, and hydroxyl radicals. It causes oxidative stress, damage to oocytes, and damage to GCs. To the best of our knowledge, whether ABA can resist human ovarian granulosa cell apoptosis caused by H_2O_2-induced oxidative stress has not yet been confirmed.

Letrozole is believed to recapitulate both the endocrine and metabolic phenotypes of PCOS clinically and is widely used in animal models to induce PCOS experimentally [19]. Previously, we have successfully established a letrozole-induced PCOS rat model with PCOS-like reproductive, endocrine, and metabolic phenotypes [20]. An oxidative stress model of the human ovarian granulosa cell line (KGN) induced by H_2O_2 was established. Herein, we aimed to investigate the possible therapeutic merits of ABA in improving PCOS anomalies in a letrozole-induced rat model and its potential therapeutic efficacy. In addition, *in vitro* experiment was performed to investigate the viability, cell apoptosis, and antioxidant effect of ABA.

2. Material and Methods

2.1 Animals and Experimental Design

Female Wistar rats were obtained from Chengdu Dashuo Experimental Animals Limited Company (Chengdu, Sichuan, China). All animal procedures were conducted in accordance with ethical principles in animal research and approved by the Animal Ethics Committee of West China Second University Hospital of Sichuan University (No. 2018-013). Every effort was made to reduce the number of animals in each experimental group and to ensure that discomfort and pain were kept to a minimum. The Wistar rats were housed five per cage under standard conditions (12:12 h of light-dark cycle; at 23 $\pm$ 2 °C; 55–65% humidity), with *ad libitum* access to a wheat-soybean-meal-based diet food and tap water. At the age of 21 days, with an average body weight of 44 grams, rats were randomly divided into the control group (n = 10), and the PCOS group (n = 30). All PCOS rats were implanted with 90 days of continuous-release letrozole pellets randomly (Innovative Research, Pico Rivera, CA, USA), containing 36 mg of letrozole (daily dose, 400 μg daily, MedChem Express, Princeton, NJ, USA). Control rats were implanted with a placebo lacking the bioactive molecule (Innovative Research, Pico Rivera, CA, USA), as previously described [20]. All implanted pallets were kept until before decapitation. Our earlier study demonstrated that letrozole-treated rats showed increased body weight and PCOS-like reproductive and metabolic phenotypes, including significant alters in serum testosterone (T), 17β-estradiol (E2), progesterone (P), luteinizing hormone (LH), and follicle stimulating hormone; impaired IR; and widespread metabolic abnormalities, as well as disrupted estrous cycles and poly ovaries [20]. Following seven weeks of PCOS induction, rats were randomly divided into ABA treatment and treatment control groups based on baseline fasting blood glucose and weight to provide an equal starting point. 10 mg ABA (862169, Sigma-Aldrich, Darmstadt, Germany) was dissolved in 1 mL ethanol, then diluted it in 1000 mL with tap water. For the controls, only 1 mL of ethanol was added to 1000 mL of tap water. An ABA (0.125 μg/kg) was administered by intragastric route (IG) daily in the PCOS + ABA group as previously reported [12]. The PCOS group and control group were served with ethanol-diluted tape water by IG but with the same feed and rear environment as treatment controls. All rats were sacrificed after two weeks of ABA treatment.

2.2 Tissue Sampling and Histopathology

At 12 weeks of age, blood samples were obtained and stored at –20 °C. The rats were decapitated, ovariectomized, fixed for 24 h in neutral buffered 4% paraformaldehyde, placed in 70% ethanol, dehydrated, and embedded in paraffin. After formalin-fixed ovaries were processed by paraffin embedding, samples were longitudinally and serially cut into 5 μm thick slices, with hematoxylin and eosin (H&E). The ovarian tissue morphology was evaluated by two persons blinded to the origin of the sections under a light microscope.

2.3 Measurement of Body Weight, Testosterone (T) Level, Glucose Tolerance, Fasting Insulin

All rats were weighed weekly on electronic scales during the experiment. After ABA treatment, T level, Glu-

cose tolerance, and fasting insulin were evaluated. T level was determined by gas chromatography and mass spectrum (GC-MS) (Agilent Technologies, Santa Clara, CA, USA). Fasting glucose (FPG) was measured by ACCU-CHEK II (Roche, Basel, Switzerland) as previously mentioned after eight hours of overnight fasting. Rats were then injected intraperitoneally with a bolus of 1 g/kg glucose in 0.9% NaCl. Blood glucose determination was assessed at 0, 15-, 30-, 60-, and 120 minutes post-injection. Fasting insulin (FINS) and fasting glucose (FPG) were measured by a radioimmunoassay kit (EMD Millipore Corporation, Billerica, MA, USA). Additionally, HOMA-IR (homeostasis model assessment of IR) was determined by fasting insulin and glucose values, and calculated as Fasting insulin (mIU/L) $\times$ Fasting glucose (mmol/L)/22.5. All kits contained standard samples for quality control and were used according to manufacturer instructions.

2.4 Cell Culture and ABA Treatment

KGN cells (Procell CL-0603) were obtained from Procell Life Science&Technology Co., Ltd (Wuhan, Hubei, China) in 2021. The cell line had been authenticated using short tandem repeat (STR) markers, and mycoplasma testing has been done. KGN cells were cultured in Dulbecco's modified eagle's medium/nutrient mixture F-12 (DMEM/F12) (Gibco, New York, NY, USA), supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin. The processing time and concentration of H_2O_2 used in assays were chosen as previously described [21]. According to experimental requirements, cells were seeded on culture plates. When the cell fusion rate reached 85–90%, different concentrations (0, 5, 10, 50, 100, and 200 μM) of H_2O_2 were added to the culture medium. After H_2O_2 treatment at different time intervals (0.5 h, 2 h, 12 h, and 24 h), using the cell counting Kit-8 (CCK-8) (Meilun Biotechnology Co. Ltd., Dalian, Liaoning, China), the oxidative stress model was established with the required time and concentration. Treatment with 100 μM H_2O_2 for 24 h was optimized for the following experiments. The treatment group was pretreated with ABA (0.001 μM, 0.01 μM, 0.1 μM, and 1 μM) followed by treatment of H_2O_2. ABA was dissolved in 0.1% Dimethyl sulfoxide (DMSO) and stored at –20 °C. All solutions were freshly prepared from stock solutions before each experiment with a final concentration of DMSO $<$0.1%.

2.5 Cell Viability by CCK-8 Assay

KGN cells were incubated into a 96-well plate at a concentration of 5000 cells/well. The original culture solution was drained out by flowing H_2O_2 treatment. An aliquot of 100 μL CCK-8 solution was added to the cultured cells in each well (CCK-8: culture solution = 1:9), and cultured at 37 °C for an additional hour. Absorbance was measured at 450 nm using a microplate reader. Cell viability was expressed as the percentage of the drug group versus the control group (100%). Data represent the average of three independent experiments.

2.6 Cell Apoptosis Assay by Flow Cytometry Analysis

According to the instructions of the kit, apoptosis of KGNs was detected by Annexin V-FITC-PI double-staining assay (CK04, DOJINDO, Kumamoto, Japan). Cultured KGNs were diluted at 5×10^5 cells/mL concentration and cultured in 24-well plates. The culture solution was drained after KGN cells were cultured in various concentrations of H_2O_2 solution. The KGN cells were then digested using pancreatin without ethylenediaminetetraacetic acid, and 1~5 $\times$ 10^5 cells/tube were collected. Annexin V-FITC/PI apoptosis detection kit (40302-B, Yisheng Biotechnology Co., Ltd., Shanghai, China) was used to stain and test KGN cells. The data of cell apoptosis was analyzed using FlowJo 10.0 (TreeStar, Ashland, OR, USA).

2.7 ROS Detection by Flow Cytometry Analysis

According to the manufacturer's instructions, the ROS level was detected by the Fluorometric Intracellular ROS Kit (MAK145, Sigma-Aldrich, Darmstadt, Germany). The Fluorometric Intracellular ROS Assay Kit provides a sensitive, one-step fluorescence detection method that can detect intracellular ROS in live cells within 1 h of incubation (especially superoxide and hydroxyl radicals). ROS reacts with a cell-permeable sensor to produce a fluorometric measurement product (lex = 520/LEM = 605 nm) proportional to the amount of ROS present. ROS levels were analyzed by Flow Cytometry (FACS Calibur, American BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions: a primary reaction mixture of 100 µL/well (96-well plate) was added to the cell plate; cells were cultured in a 5% CO_2 incubator at 37 °C for one hour. Cells were treated with 20 µL/well of the test compound in the appropriate buffer; fluorescence intensity was measured with Flow Cytometry. FlowJo 10 was used to analyze the data.

2.8 Statistical Analyses

Data are expressed as means $\pm$ SD. Statistical analyses were performed with SPSS (version 19.0; SPSS Inc, Chicago, IL, USA) and Prism GraphPad (version 6.0, Graph-Pad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) with Bonferroni *post hoc* test was used to assess the significance of the differences between more than two groups, whereas two groups were compared by Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Establishment of PCOS Rat Model and Effect of ABA Therapy

The PCOS rat model was successfully induced by continuous-release letrozole pellets for 56 days and subsequently followed by ABA therapy for 14 days (Fig. 1A).

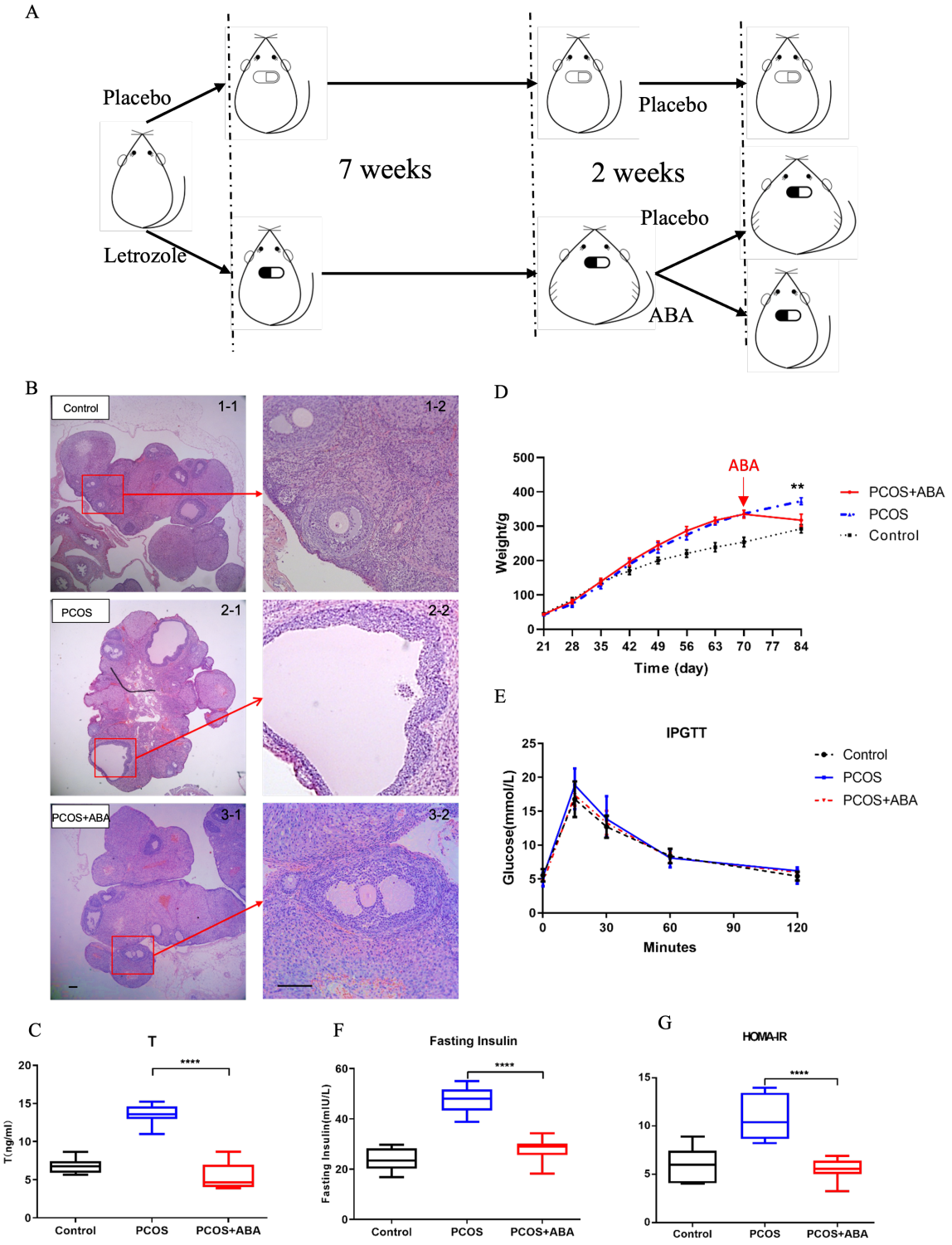

Fig. 1. Abscisic acid (ABA) treatment in Polycystic Ovary Syndrome (PCOS) rats induced by letrozole. (A) PCOS rats were induced for 49 days by subcutaneously implantation of 90-day continuously released letrozole pellet, followed by 14 days of ABA treatment. (B) 1-1 and 1-2 for Control, 2-1 and 2-2 for PCOS, 3-1 and 3-2 for PCOS + ABA, alleviated the abnormal ovarian morphology of the PCOS rats. (Scale bar = 200 μm). (C) Serum level of T decreased significantly after ABA treatment (PCOS + ABA versus PCOS, ****, $p <$ 0.0001 by *t*-test). (D) ABA significantly reduced weight gain (PCOS versus Control, **, $p < 0.01$ by repeated measure ANOVA; PCOS + ABA versus PCOS, **, $p < 0.01$ by *t*-test). (E) IPGTT for glucose tolerance showed no significant changes. (F,G) ABA significantly reduced fasting insulin and HOMA-IR in PCOS rats (****, $p < 0.0001$ by *t*-test).

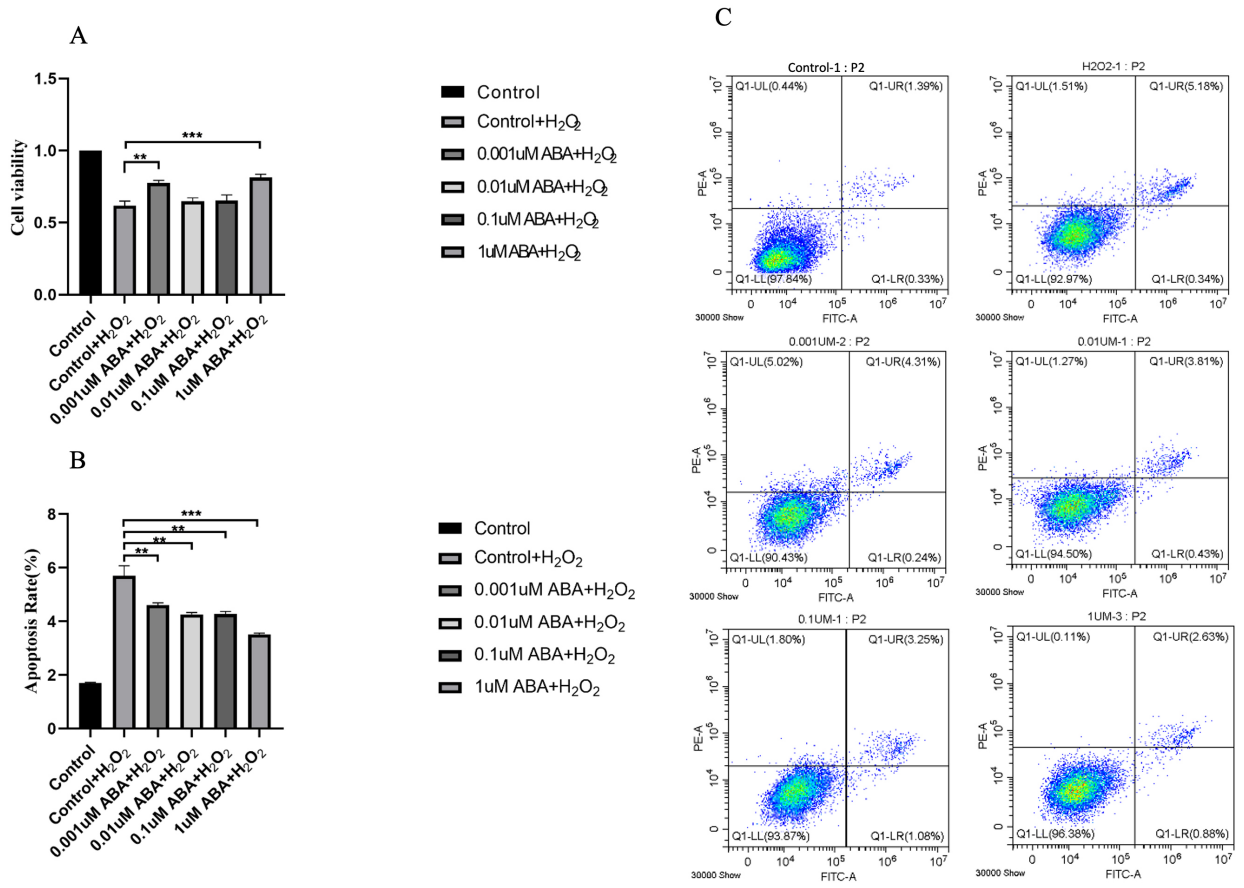

Fig. 2. The effects of ABA on cell viability and apoptosis in H_2O_2-induced KGNs. (A) The cell viability of H_2O_2-induced KGNs in the presence of different concentrations of ABA. (B,C) Flow cytometry image and analysis of H_2O_2-induced KGNs cell apoptosis treated with different ABA concentrations. Values represent the mean ± SD base on three independent experiments. **, $p < 0.01$ was considered significant; ***, $p < 0.001$ was considered significant.

Weight gain, impaired IR, and elevated T level were observed in PCOS rats, factors that were significantly reduced after ABA treatment (Fig. 1C,D,F,G). Intraperitoneal glucose tolerance test (IPGTT) for glucose tolerance showed no significant changes (Fig. 1E). In addition, H&E staining of ovaries revealed that a significant abnormal polycystic morphology and a greater number of cystic follicles were exhibited in the PCOS rat group, whereas, the number of cystic follicles was significantly smaller after ABA treatment (Fig. 1B).

3.2 ABA Can Rescue Cell Viability and Inhibit Cell Apoptosis in H_2O_2-Induced KGNs

The KGN cells were treated with 100 μM H_2O_2, followed by pretreated with ABA (0.001 μM, 0.01 μM, 0.1 μM, and 1 μM) exposure for 24 h, to verify the antiapoptotic effects of ABA. As shown in Fig. 2, cell viability was tested using the CCK-8 assay. After 24 hours of H_2O_2 treatment, cell density decreased, and cell shrinkage. However, pretreated with 0.001 μM and 1 μM ABA, cells were in better condition and had higher survival. Therefore, we speculate that ABA reduced the effects of H_2O_2 by regulating apoptosis. The FITC-Annexin V/PI apoptosis assay showed the apoptosis rate was increased by H_2O_2. Cell pretreated with increasing concentration of ABA showed a significantly decreased apoptosis rate compared to those in the H_2O_2 group. Therefore, the present results indicate that ABA exerted no toxic but antiapoptotic effect on KGNs.

3.3 ABA Lowered ROS Levels Induced by H_2O_2 in KGNs

We next investigated whether ABA acted by reducing ROS content in H_2O_2-induced KGNs. As shown in Fig. 3, our results demonstrate that ABA treatment inhibited the H_2O_2-induced increase of ROS levels in a dose-dependent manner, with significant effect at a concentration of 0.1 μM. The present result indicates that ABA possesses antioxidant activities and increases the resistance of KGN cells to oxidation.

4. Discussion

In the present study, we made use of a well-established model of PCOS implanted with letrozole pellets to study its effects on IR, as previously reported [20]. Our findings suggest that ABA treatment results in decreased T concen-

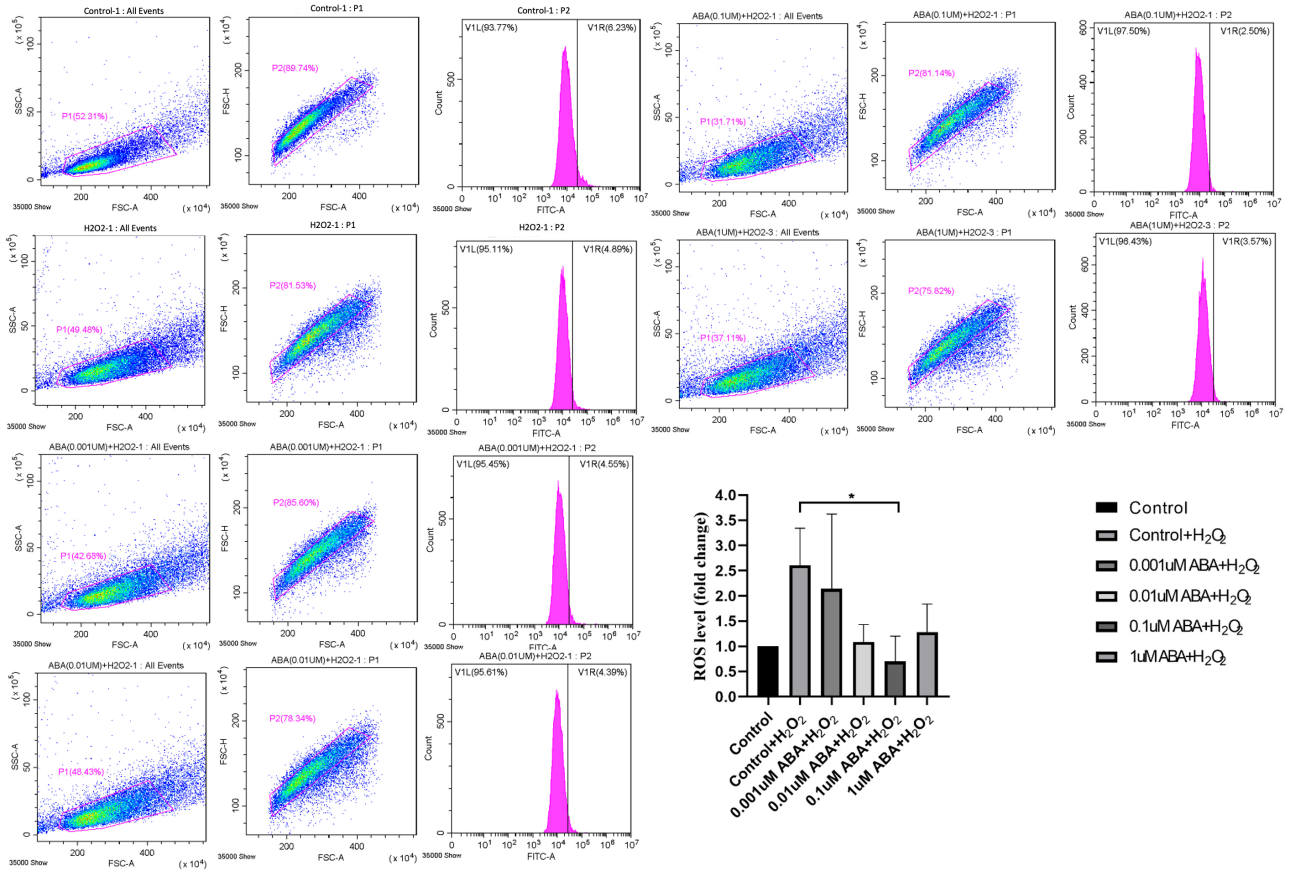

Fig. 3. The effects of ABA on reactive oxygen species (ROS) levels in H_2O_2-induced KGNs. Flow cytometry image and analysis of ROS levels of KGNs at different ABA concentrations in H_2O_2-induced KGNs. *, $p < 0.05$ was considered significant.

tration and body weight, as well as improvement of IR in PCOS rats induced by letrozole. Furthermore, ABA reversed H_2O_2-induced cell apoptosis in KGNs, and mitigated ROS activity in H_2O_2-induced KGNs, which may be closely associated with the alteration of ovary hormone release and IR. Therefore, the present results indicate that gained body weight, impaired IR, and higher T levels were significantly improved after ABA treatment in PCOS rats, suggesting that ABA may facilitate the treatment of PCOS. ABA significantly reduced cell apoptosis and ROS levels and increased cell viability. To our knowledge, these findings provide the first evidence of the role of ABA in maintaining the survival of KGNs and protection against cell damage and oxidative stress in GCs around oocytes.

In the present study, PCOS rats induced by letrozole did not exhibit any significant changes in IPGTT, but induced IR with abnormal insulin levels and increased T levels, consistent with our previous study [19]. These phenotypes are more similar to those of PCOS patients. Those PCOS rats also have been reported to exhibit drastic elevation of NF-kB levels, and the overexpression of NF-κB is strongly linked to impaired insulin secretion and excessive β-cell death [22]. ABA naturally originates from different dietary sources but also is endogenously produced by the carotenoid biosynthesis pathway. Magnone *et al.* [7] demonstrated that long-term consumption of supplements containing low doses of ABA ameliorate the prediabetes markers. Moreover, the authors proved that the extent of improvement in these subjects was greater than in healthy subjects, suggesting a beneficial influence of low doses of ABA supplements in prediabetics [7]. As PCOS is considered a condition at high risk of developing into prediabetes, or even diabetes, it underscores that ABA may be a potential treatment for PCOS.

Herein, dietary ABA was shown to alleviate PCOS throughout a range of routine indicators, including reduced body weight, the rectification of abnormal T, and improved IR. Our data are consistent with the above findings on promoting insulin sensitivity. Guri *et al.* [10] showed that ABA treatment of glucose metabolism regulation in obese and prediabetic mice is due to its structural similarity to thiazolidinediones, while its efficacy is similar to those of these anti-diabetic oral drugs. ABA may be an effective insulin-sensitizing compound, have the ability to control systemic glycemic responses and skeletal muscle metabolism. ABA treatment induced greater insulin sensitivity [12]. Nevertheless, unlike thiazolidinediones, ABA exerts its hypoglycemic effect in mammals by binding LANCL2 and acting on peroxisome proliferator-activated receptor gamma (PPARγ) [23]. ABA might diminish NF-

κB expression, eventually alleviating oxidative stress and inflammatory cytokines [18]. Our data and the published literature highlight the ability of ABA to intersect the pathogenesis of PCOS and IR in multiple aspects.

Oxidative stress has been proven to be involved in follicle differentiation, proliferation and maturation, which may be closely related to the number of meiotic I oocytes [14,24]. Oxidative stress has been associated with a number of reproductive disorders, including PCOS, Primary Ovarian Insufficiency, endometriosis, infertility, and aging. ROS production plays a vital role in inducing oocyte meiosis, higher ROS level appears to impair oocyte maturation, and oxidative stress can induce uncoordinated functions of granulosa cells, affecting oocyte quality [25,26]. Elevated ROS in the ovarian tissues had been found in letrozole-treated PCOS rats [16].

Meanwhile, the antioxidant protective effects of ABA have been widely studied. Rafiepour *et al.* [27] demonstrated that the PPARγ signaling cascade mediated by reducing ROS levels of ABA's antioxidant and antiapoptotic properties was able to increase the activity of the antioxidant enzymes and peroxidase, as well as decrease MDA concentrations, H_2O_2 levels, and body weights in rats [28]. As an antioxidant and antiapoptotic agent, ABA may play a protective role in an endocrine or paracrine mechanism. Thus, the possible function of ABA from human KGNs might be to provide nutrients and support the oocytes. Accordingly, we inquired whether ABA contributes to GCs' survival. There are many ways to detect cell death. CCK-8 assay was used to determine cell viability, while annexin V/PI staining was used to measure the rate of cell apoptosis. Here, we provide the first evidence demonstrating the role of ABA in inhibiting cell apoptosis, promoting cell viability, and attenuating the increased expression of ROS levels in H_2O_2-induced KGNs, indicating that the level of ABA in the follicular fluid may determine the growth of ovarian follicle fate, and participate in the pathological process of PCOS.

Our study is only a preliminary investigation of ABA in the context of the PCOS rat model and ovarian function. The possible mechanism of the effect of ABA on testosterone might be the potent phytoestrogenic property of ABA, as ABA treatment group showed remarkable improvement of ovulation and decreased abnormal cysts. Further research is needed to investigate the effects of ABA on ovary function using *in vivo* and *in vitro* studies, such as the effects on theca cell, angiogenesis, and folliculogenesis. Continued exploration could be clinically relevant for fertility in women, addressing whether ABA could be used as an intervention to treat reproductive disorders.

5. Conclusions

In conclusion, the current research uncovered a novel role of ABA in the regulation of the metabolic and endocrine imbalance of the PCOS model, demonstrating that ABA represses H_2O_2-induced cell apoptosis through scavenging cellular ROS. These findings not only provide potential avenues for ameliorating endocrine and metabolic alterations in PCOS patients by ABA, facilitating the treatment of PCOS, but also expand our understanding regarding the mechanism of follicular development under H_2O_2-induced dysfunction by supplementing ABA.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors contributed to the study's conception and design. YDX, JLG, YFZ, and LX performed the experiments and acquired the data. XHL and SWL participated in data analysis. YDX prepared the manuscript. LX revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All the animal procedures were performed by the ethical principle in animal research and approved by the Animal Ethics Committee of West China Second University Hospital of Sichuan University, No. 2018-013.

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Conflict of Interest

The authors declare no conflict of interest.

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