

# The effects of abscisic acid administration in PCOS rat model and H2O2 induced human granulosa cell damage: A preliminary investigation

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## Research Article

**Keywords:** Abscisic acid, Polycystic ovary syndrome, Inflammatory factor, Oxidant stress, Cell apoptosis

**Posted Date:** April 13th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1516310/v1>

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# Abstract

**Background:** Polycystic ovary syndrome (PCOS) is commonly associated with metabolic disorders. Abscisic acid (ABA) is a phytohormone present in vegetables and fruits that can be naturally supplied by the dietary intake and has been previously studied for its benefits to human health, especially in diabetes people. It has been demonstrated that ABA plays a key role in glucose metabolism, inflammation, and tumor growth.

**Methods:** Wistar rats were implanted with letrozole-continuous-release pellets to induce a PCOS-like phenotype, subsequently treated with ABA or vehicle control. Bodyweight changes, Testosterone(T) level, fasting insulin measurements, and glucose tolerance tests have been investigated. A cell apoptosis model was established induced by H<sub>2</sub>O<sub>2</sub>. Cell viability was tested by cell counting Kit-8 (CCK-8). Cell apoptosis was tested by flow cytometry, and the oxidative stress state of cells was examined using ROS array.

**Results:** Body weights, glucose impairment, and T level were significantly improved in the ABA treated PCOS rat than in vehicle-treated control PCOS rats. ABA significantly reduced H<sub>2</sub>O<sub>2</sub>-induced KGNs cell apoptosis and ROS levels and increased cell viability.

**Conclusions:** The findings suggest that ABA could be a candidate adjunctive treatment for improving the insulin sensitivity and hyperandrogenemia of PCOS patients, and ameliorated H<sub>2</sub>O<sub>2</sub>-induced KGN cell apoptosis and oxidant stress.

## Background

Polycystic ovary syndrome (PCOS), a common endocrine disorder affecting reproductive women, is associated with reproductive and metabolic alterations, including hyperandrogenism, insulin resistance, polycystic ovaries, and infertility with 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 the mechanism of this pathology include hormonal imbalances, genetic abnormalities, and lifestyle and environmental 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) is a phytohormone widely found in fruits and vegetables in varying concentrations and has been demonstrated to behave as an endogenous hormone in mammals, which is produced and released from human pancreatic  $\beta$ -cells in response to high glucose concentration in healthy subjects (5). Mini-dose ABA is able to control the metabolic response to glucose availability through stimulating glucose uptake in skeletal muscle and adipose tissue with an insulin-independent mechanism, as well as increasing energy expenditure in the brown adipose tissue and white adipose tissues(6–8). ABA increases translocation of the glucose transporter GLUT4 to the plasma membrane and GLUT-4-dependent glucose uptake. The mechanism underlying ABA action 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 mice with ABA improved insulin sensitivity and suppresses obesity-related inflammation (10). In diabetic induced rats, ABA showed 60% of oxytocin stimulatory effects on myometrial contraction, and have a uterotonic effect, which indicated that ABA could be beneficial as a pre-labor prescription, especially in diabetic females (11). It is generally recognized that ABA is a safe ingredient. In sub-chronic toxicity studies following a 4-week and a 13-week dietary intervention with different concentrations of ABA in rats, no adverse toxicological effects were found for 90 d at intakes of up to 20,000 ppm (around 1,500 mg/kg body weight per day)(12). Moreover, in healthy subjects, the consumption of ABA from fig fruit extracts produced a significant and clinical reduction in postprandial glucose and insulin levels in response to a high-GI glucose drink(13). All in all, these studies indicated that ABA may be a possible biomedical application for PCOS.

Oxidative stress might support insulin resistance(IR) in PCOS and directly stimulate excess ovarian androgen production. Accumulating research has reported that oxidative stress induced by reactive oxygen species (ROS) may promote the development of IR and hyperandrogenism, and inflammatory cytokine might be associated with endothelial dysfunction, which are all key characteristics of PCOS(14, 15). ROS in the ovarian tissues was found significantly high expressed in the PCOS rat model induced by letrozole(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 when activated by physical or chemical stimuli, and ABA could stimulate functional activities of these cells, including migration, phagocytosis, and release of ROS and NO(17). Granulosa cells (GCs) are a type of ovarian cell, and metabolic activity serves a vital role in the regulation of oocyte quality. Oxidative stress damage in the follicular microenvironment contributes to the impairment of oocyte quality in PCOS. ROS is oxygen-derived molecules that include superoxide anions, H<sub>2</sub>O<sub>2</sub>, 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<sub>2</sub>O<sub>2</sub>-induced oxidative stress has not been reported yet.

Letrozole is believed to recapitulate both the endocrine and metabolic phenotypes of PCOS clinically and is widely used to induce PCOS experimentally in animal models(18). We have successfully established a letrozole-induced PCOS rat model with PCOS-like reproductive, endocrine, and metabolic phenotypes previously(19). An oxidative stress model of human ovarian granulosa cells (KGNs) induced by H<sub>2</sub>O<sub>2</sub> was established. In the present study, it 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.

## **Material And Methods**

### **Animals and experimental design**

Female Wistar rats were obtained from Chengdu Dashuo Experimental Animals Limited Company (Chengdu, Sichuan, China). All the animal procedures were performed in accordance with the ethical principle in animal research and approved by the Animal Ethics Committee of West China Second University Hospital of Sichuan University. Every effort was made to minimize the number of animals in each experimental group and to ensure minimal discomfort and pain. The rats were housed five per cage under standard conditions (12:12-h of light-dark cycle; at  $23 \pm 2^\circ\text{C}$ ; 55–65% humidity), with *ad libitum* access to food and tap water. At the age of 21 days, 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 letrozole-continuous-release pellets randomly (Innovative Research, USA.), containing 36 mg of letrozole (daily dose, 400 $\mu\text{g}$  daily). Control rats were implanted with a placebo which was lacking the bioactive molecule (Innovative Research, USA.), as we previously described(19). Following 7 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. PCOS group served as treatment control and received nothing but with same feed and rear environment, while the PCOS + ABA group received ABA orally at a dose of 0.125ug per kilogram of body weight as previously reported(12). All the rats were sacrificed after 2 weeks of ABA treatment.

## Tissue sampling and Histopathology

At 12 weeks of age, blood samples were obtained and stored at  $-20^\circ\text{C}$ . The rats were decapitated, and ovaries were excised, fixed in neutral buffered 4% paraformaldehyde for 24h, placed in 70% ethanol, dehydrated, and embedded in paraffin. After processing of formalin-fixed ovaries through paraffin embedding, samples were longitudinally and serially sectioned into 5- $\mu\text{m}$  thick slices and stained with hematoxylin and eosin (H&E). The ovarian tissues morphology was evaluated under a light microscope by two persons blinded to the origin of the sections.

## Measurement of Bodyweight, Testosterone level, Glucose tolerance, Fasting insulin

All rats were weighed weekly on electronic scales during the experiment. Testosterone(T) level was determined by GC-MS before and after ABA treatment. Fasting glucose (FPG) was measured by ACCU-CHEK (Roche, Switzerland) as previously mentioned after 8 hours of overnight fast. After that, rats were injected intraperitoneally with a bolus of 1g/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. Additionally, HOMA-IR (homeostasis model assessment of insulin resistance) was determined by fasting insulin and glucose values, and calculated as  $\text{Fasting insulin (mIU/L)} \times \text{Fasting glucose (mmol/L)} / 22.5$ . All kits contained standard samples for quality control and were used by manufacturer instructions.

## Cell Culture and ABA treatment

KGN cells were cultured in Dulbecco's modified eagle's medium/nutrient mixture F-12 (DMEM/F12), supplemented with 10% fetal bovine serum and 100IU/ml penicillin/streptomycin. The processing time and concentration of H<sub>2</sub>O<sub>2</sub> used in assays were chosen as previously described (20). Culture plates were inoculated with cells according to experimental requirements, and different concentrations (0, 5, 10, 50, 100, and 200μM) of H<sub>2</sub>O<sub>2</sub> culture solution were added when the cell fusion rate reached 85–90%. Following H<sub>2</sub>O<sub>2</sub> treatment at different time intervals (0.5h, 2h, 12h, and 24h), the time and concentration required for establishing the oxidative stress model using the CCK-8 kit (Meilun Biotechnology Co. Ltd., Dalian, China). Treatment with 100uM H<sub>2</sub>O<sub>2</sub> for 24h had been optimized for the following experiments. The treatment group was pretreated with ABA (0.001μM, 0.01μM, 0.1uM, and 1μM) followed by treatment of H<sub>2</sub>O<sub>2</sub>. ABA was dissolved in 0.1% Dimethyl sulfoxide(DMSO) and stored at -20°C. All solutions were freshly prepared from stock solutions prior to each experiment and the final concentration of DMSO was < 0.1%.

## **Cell viability by Cell Counting Kit-8 (CCK-8) assay**

KGN cells were incubated into a 96-well plate at a concentration of 5,000 cells/well. The original culture solution was drained out by flowing H<sub>2</sub>O<sub>2</sub> treatment. An aliquot of 100 μL CCK-8 solution was added to each well (CCK-8: culture solution = 1:9) of culture cells in a CO<sub>2</sub> incubator for another 1 h at 37°C. Absorbance was measured at 450 nm using a microplate reader. Cell viability was expressed as the percentage of the drug group to the control group (100%). Data represent the mean of three independent experiments.

## **Cell Apoptosis Assay by Flow Cytometry Analysis**

The apoptosis of KGNs was detected by Annexin V-FITC-PI double-staining assay (DOJINDO, Japan) according to the kit's instructions. Cultured KGNs were diluted at a concentration of 5×10<sup>5</sup> cells/ml and plated in 24-well plates. The culture solution was drained after KGN cells were cultured in various concentrations (0, 50, 100, 150, and 200 μM) of H<sub>2</sub>O<sub>2</sub> solution. The KGN cells were then digested using pancreatin without ethylene diamine tetraacetic acid (EDTA), and 1 ~ 5×10<sup>5</sup> cells/tube were collected. KGN cells were stained and tested using the Annexin V-FITC/PI apoptosis detection kit (Yisheng Biotechnology Co., Ltd., Shanghai, China). Cell apoptosis rate was analyzed by FlowJo 10.

## **ROS Detection by Flow Cytometry Analysis**

ROS level was detected by the Fluorometric Intracellular ROS Kit (MAK145) according to the manufacturer's instructions. The Fluorometric Intracellular ROS Assay Kit provides a sensitive, one-step fluorometric assay to detect intracellular ROS (especially superoxide and hydroxyl radicals) in live cells within 1 h of incubation. ROS reacts with a cell-permeable sensor, resulting in a fluorometric product (lex = 520/LEM = 605 nm) proportional to the amount of ROS present. ROS levels were analyzed by Flow Cytometry(FACS Calibur, American BD) according to the manufacturer's instructions. Briefly, add 100 μL/well (96-well plate) of Master Reaction Mix into the cell plate. Incubate the cells in a 5% CO<sub>2</sub>, 37°C

incubator for one hour. Treat cells with 20  $\mu\text{L}$ /well of test compounds in a suitable buffer. Measure the fluorescence intensity using a BD Accuri C6 flow cytometer. The data were analyzed using the FlowJo 10.

## Statistical analyses

Data were expressed as means  $\pm$  SD. Statistical analyses were performed with SPSS (version 19.0; SPSS, Chicago, IL) and Prism GraphPad (version 6.0, Graph-Pad Software, La Jolla, CA). 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.

## Results

### Establishment of PCOS rat model and effect of ABA therapy

The PCOS rat model was successfully induced by letrozole continuous-release pellets for 56 days and subsequently followed by ABA therapy for 21 days (Fig. 1A). Gained body weight, impaired insulin resistance, and elevated T level had been observed in PCOS rats, which were significantly reduced after ABA treatment (Fig. 1C-G). In addition, H&E staining of ovaries reveals 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).

### ABA can rescue cell viability and inhibit cell apoptosis in $\text{H}_2\text{O}_2$ -induced KGNs

The KGN cells were treated with 100 $\mu\text{M}$   $\text{H}_2\text{O}_2$ , followed by ABA exposure for 24h, to verify the antiapoptotic effects of ABA. Cell viability was tested using the CCK-8 method. The model cells were treated with 100 $\mu\text{M}$  ABA for 24h. As shown in Fig. 2, cell viability was significantly higher than in the negative control group. FACS analysis of KGN cell co-stained with FITC-conjugated annexin V and the nucleic acid stain SYTOX Red Dead cell stain revealed an increase in the apoptotic fraction for 24h compared to the serum-free medium control. The addition of ABA to serum-free medium reduced the L/A fraction after 24 h in serum-free medium. Therefore, the present results indicated that ABA exerted no toxic and antiapoptotic effect on KGNs.

### ABA lowered ROS levels induced by $\text{H}_2\text{O}_2$ in KGNs

We next investigated whether ABA functioned by decreasing ROS content in  $\text{H}_2\text{O}_2$  induced KGNs. As shown in Fig. 3, our results showed that ABA treatment inhibited the  $\text{H}_2\text{O}_2$ -induced increase of ROS levels in a dose-dependent manner, significantly at the concentration of 0.1 $\mu\text{M}$ . The present result indicated that ABA possesses antioxidant properties and increases the resistance of KGN cells to oxidation.

## Discussion

In the present study, we have made use of a well-established model of PCOS-implanted with letrozole pellets-to study its effects on insulin resistance, as we reported previously(19). We have evaluated that ABA treatment resulted in decreased testosterone concentration, body weight, and improvement of insulin resistance in PCOS rats induced by letrozole. Our further study revealed that ABA reversed H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis in KGNs, and also mitigated ROS activity in H<sub>2</sub>O<sub>2</sub>-induced KGNs, which may be closely associated with the alteration of ovary hormone release and IR. Therefore, the present results indicated that gained bodyweight, impaired insulin resistance, and higher T levels were significantly improved after ABA treatment in PCOS rats, suggesting that it may facilitate the treatment of PCOS. ABA significantly reduced cell apoptosis and ROS levels and increased cell viability. To our knowledge, these findings provided the first evidence dressing the role of ABA in maintaining KGNs survival and protecting against cell damage and oxidative stress in granulosa cells around oocytes.

In the present study, PCOS rats induced by letrozole in our study did not exhibit any significant changes in IPGTT, but induced insulin resistance with abnormal insulin levels and increased T levels, consistent with our previous study(19). These phenotypes are more similar to PCOS patients. ABA naturally originates from different dietary sources but also is endogenously produced by the carotenoid biosynthesis pathway. It has been demonstrated that chronic consumption of a supplement containing a low dose of ABA ameliorates the prediabetes markers. Moreover, it has also been proven that the improvement was greater in these subjects than in healthy ones, suggesting the beneficial influence of low-dose ABA supplementation in prediabetics(7). As PCOS is considered as a condition at a high risk of developing into prediabetes, or even diabetes, it reminds us that ABA may be a potential treatment for PCOS. In this paper, dietary ABA was displayed to possess the capacity to alleviate PCOS throughout a series of routine indicators, including reduced body weights, the rectification of abnormal T, and improved insulin resistance. Our data are consistent with the above results regarding promoting insulin sensitivity. Guri et al (10) showed that the ABA treatment in obese and prediabetic mice in regulating glucose metabolism is owing to its structural similarity to thiazolidinediones and its efficacy similar to that of these antidiabetic oral drugs. ABA may be a potent insulin-sensitizing compound with the ability to control systemic glycemic responses and skeletal muscle metabolism. The treatment of ABA-induced greater insulin sensitivity(12). Nevertheless, unlike thiazolidinediones, ABA exerts its hypoglycemic action, in mammals, by binding lanthionine synthetase C-like 2 (LANCL2) and acting on peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) (21). The data presented in our study and the published literature on ABA to date highlighted its ability to intersect the pathogenesis of PCOS and insulin resistance in multiple aspects.

Oxidative stress has been proved to be involved in the proliferation, differentiation, and maturation of follicles, which may closely relate to the number of meiotic I(MI) oocytes(14, 22). Oxidant stress has been implicated in many reproductive disorders including PCOS, Primary Ovarian Insufficiency (POI), endometriosis, infertility, and aging. ROS production plays a vital role in the induction of meiosis in the oocyte and higher ROS level has been seemed to impair oocyte maturation, and oxidative stress induces

granulosa cell discordant function and impacts oocyte quality(23, 24). Elevated ROS in the ovarian tissues had been found in letrozole-treated PCOS rats(16). In the meanwhile, the antioxidant protective effect of ABA has been extensively studied. Rafiepour et al(25) demonstrated that PPAR  $\gamma$  signaling cascade mediated by reducing ROS levels of ABA's antioxidant and antiapoptotic properties ABA was able to increase the antioxidant enzymes and peroxidase activities, as well as reduce MDA concentration,  $H_2O_2$  levels, and body weight in rats(26). As an antioxidant and antiapoptotic agent, ABA may provide a protective role by an endocrine or paracrine mechanism. Thus, the possible function of ABA from human KGNs might be able to provide nutrients and support to the oocytes. Therefore, it prompted us to inquire whether ABA contributes to granulosa cells' survival. There are many ways to detection of cell death. CCK-8 assay was employed to determine cell viability, annexin V/PI staining was used to measure the cell apoptosis rate. Here, we provided the first evidence demonstrating the role of ABA in inhibiting cell apoptosis, promoting cell viability, and also attenuating the increased expression of ROS levels in  $H_2O_2$  induced KGNs, suggesting that the level of ABA in follicular fluid might determine the developmental fate of ovarian follicles, and participating in the pathological process of PCOS.

Nevertheless, our study is a preliminary investigation of ABA in the PCOS rat model and ovarian function. 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. Further explorations could be clinically relevant for fertility in women, whether ABA could be used as an intervention to treat reproductive disorders.

## Conclusion

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

## Abbreviations

PCOS

Polycystic ovary syndrome

ABA

Abscisic acid

LANCL2

lanthionine synthetase C-like 2

IR

insulin resistance

ROS  
reactive oxygen species  
GC  
Granulosa cell  
KGN  
Granulosa tumor cell  
H<sub>2</sub>O<sub>2</sub>  
Hydrogen peroxide  
H&E  
hematoxylin and eosin  
HOMA-IR  
homeostasis model assessment of insulin resistance  
T  
Testosterone  
FPG  
Fasting glucose  
FINS  
Fasting insulin  
DMSO  
Dimethyl sulfoxide  
CCK8  
Cell Counting Kit-8  
EDTA  
ethylene diamine tetraacetic acid  
FACS  
Flow Cytometry

## **Declarations**

### **Ethics approval**

The experiments were approved by the Animal Ethics Committee of West China Second University Hospital of Sichuan University.

### **Consent for publication**

Not applicable

### **Availability of data and material**

Data are available upon request from the corresponding author

### **Competing interests**

The authors declare that they have no competing sssssssss.

## Funding

This work was supported by a grant from the National Natural Science Foundation of China (No.81671422) and a grant from the National Key Research and Development Program of China (No.2016YFC100206)

## Authors' contributions

All authors contributed to the study's conception and design. YDX, JLG 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 read and approved the final manuscript.

## Acknowledgments

None

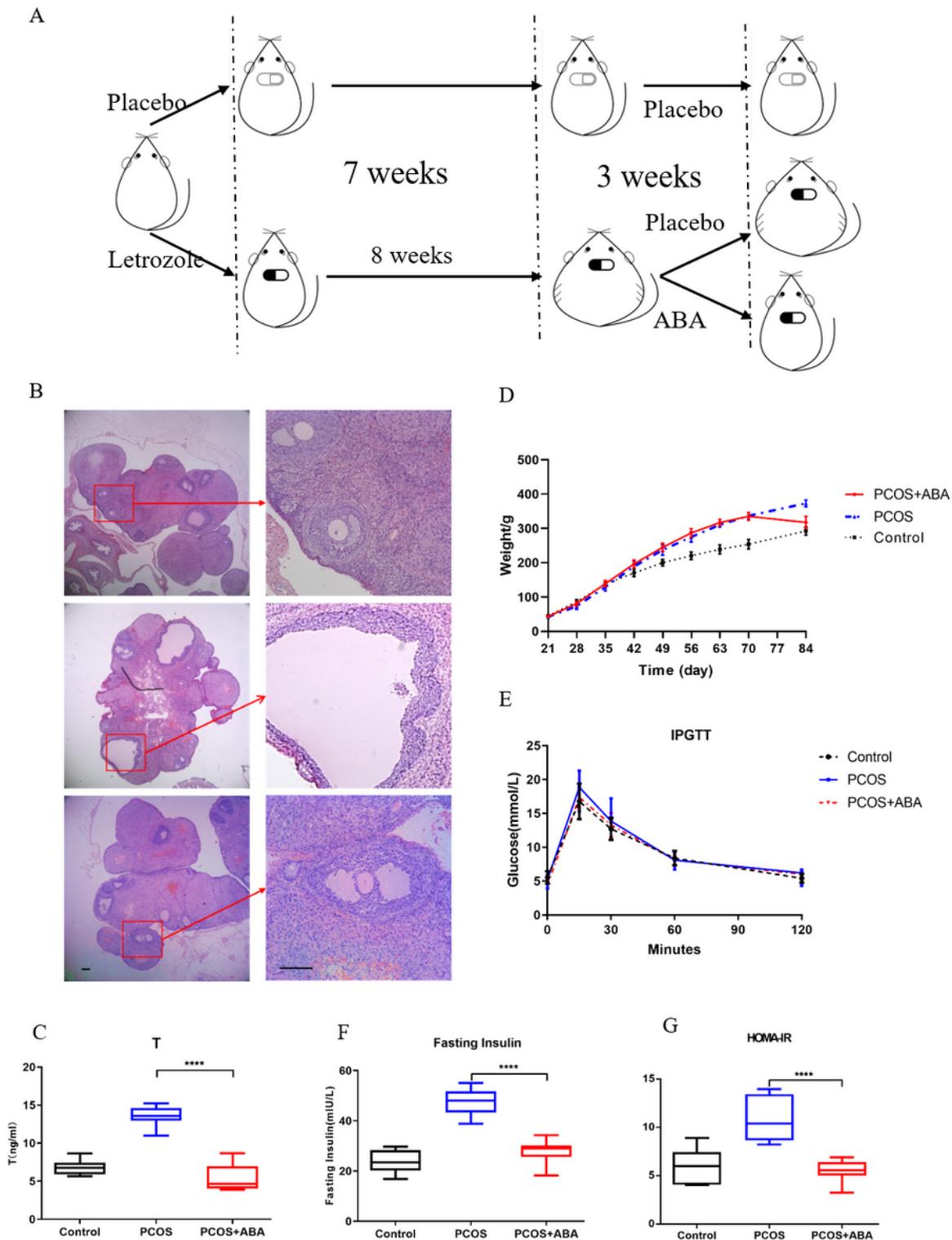
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## Figures

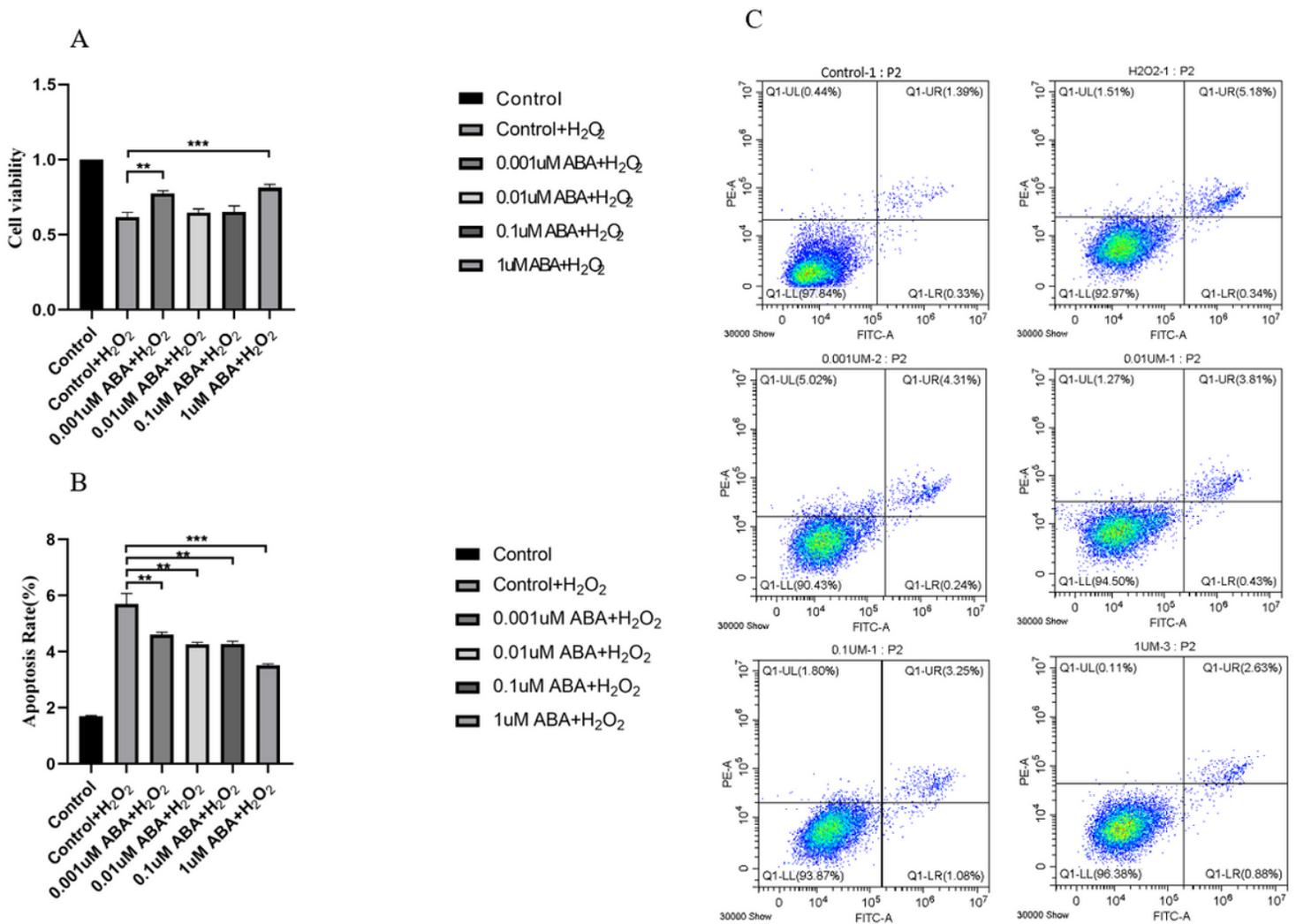


**Figure 1**

**Abscisic acid (ABA) treatment in polycystic ovary syndrome (PCOS) rat induced by letrozole.**

A. PCOS rat model was induced by subcutaneously implanted 90-day continuous released pellet of letrozole for 56 days, followed by 21 days of ABA treatment. B. ABA alleviated the abnormal morphology of the ovary of PCOS rats. (Scale bar=200 $\mu$ m) C. Serum level of testosterone decreased significantly after

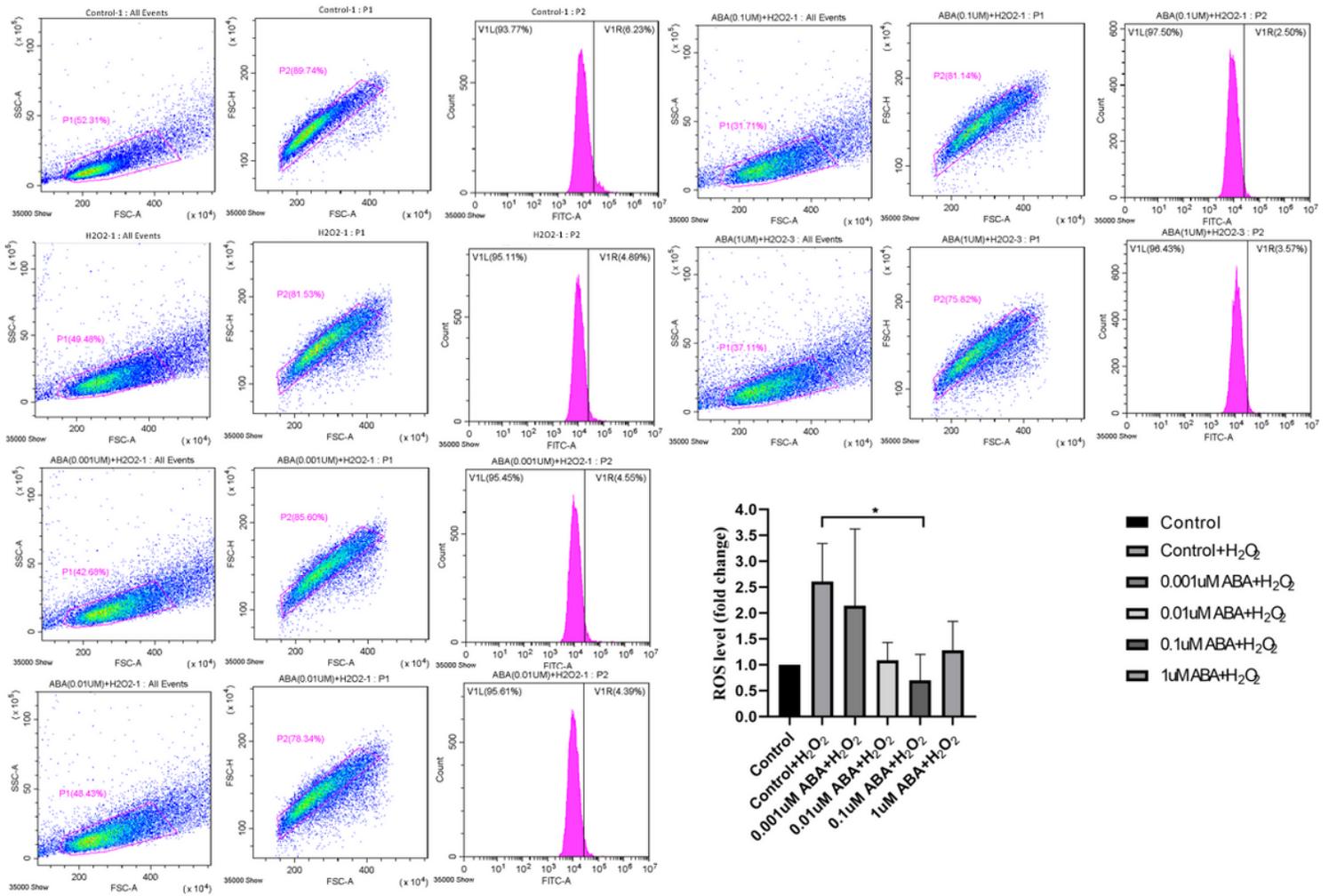
ABA treatment (PCOS+ABA versus PCOS, \*\*\*\*  $P < 0.0001$  by t-test). D. ABA significantly attenuated the increased body weight (PCOS versus Control, \*\*  $P < 0.01$  by repeated measure ANOVA; PCOS+ABA versus PCOS, \*\*  $P < 0.01$  by t-test). E. The increased glucose tolerance level changed significantly. E-F. ABA significantly reduced Fasting Insulin and HOMA-IR in PCOS rats (\*\*\*\*  $P < 0.0001$  by t-test).



**Figure 2**

### The effects of ABA on cell viability and apoptosis in H<sub>2</sub>O<sub>2</sub> induced KGNs

A. The cell viability of H<sub>2</sub>O<sub>2</sub> induced KGNs in the presence of different concentrations of ABA(A). B-C. Flow cytometry image and analysis of H<sub>2</sub>O<sub>2</sub> 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.



**Figure 3**

**The effects of ABA on reactive oxygen species (ROS) levels in H<sub>2</sub>O<sub>2</sub> induced KGNs**

Flow cytometry image and analysis of ROS levels of KGNs at different ABA concentrations in H<sub>2</sub>O<sub>2</sub> induced KGNs. \* P<0.05 was considered significant.