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# Letrozole Rat Model Mimics Human Polycystic Ovarian Syndrome and Changes in Insulin Signal Pathways

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**Background:** The aim of this study was to explore whether letrozole and high-fat diets (HFD) can induce obese insulin-resistant polycystic ovary syndrome (PCOS) with all reproductive and metabolic phenotypes in a rat model.





**Material/Methods:** Twenty-four 3-week-old female Sprague-Dawley rats were randomized into 4 groups: control, Letrozole, HFD, and Letrozol+HFD. The PCOS model was induced by 12 weeks of Letrozole treatment (1 mg/kg p.o. dissolved in 0.5% CMC solutions once daily) and HFD. Ovarian morphology, estrous cyclicity, hormonal status, body weight, glucose and insulin tolerance, lipid profile, and insulin signaling pathway were investigated.

**Results:** The rat model manifests anovulatory cycles and PCO morphology, increased body weight, elevated testosterone levels, abnormal glucose and lipid metabolism, and insulin resistance. The rat model also expresses significantly decreased phosphorylation of 6 essential signaling proteins – INSR, IRS, PI3K, AKT, ERK1, ERK2 – in the PI3K/AKT and MAPK/ERK pathways in the classic insulin-sensitive tissues (e.g., quadriceps femoris muscle, omentum majus, and liver), as well as non-classic target ovary tissues. Disrupted insulin signaling contributes to the decrease in insulin sensitivity and compensatory hyperinsulinemia in PCOS rats.

**Conclusions:** Continuous administration of letrozole and high-fat diets can induce PCOS, metabolic phenotypes, and disrupted activation of the insulin signaling pathway.

**MeSH Keywords:** **Insulin Resistance • Models, Animal • Obesity • Polycystic Ovary Syndrome**

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## Background

Polycystic ovary syndrome (PCOS) is the most common gynecological endocrine disease, with an incidence ranging from ~6% to ~20% [1]. PCOS increases the risk of long-term metabolic complications such as type 2 diabetes mellitus (T2DM) and atherosclerosis [2]. Overweight and obesity exacerbate the reproductive and metabolic disorders of PCOS [3,4]. Studies in the United States and Australia reported the highest prevalence of obesity among PCOS women, at 61% and 76%, respectively [5,6]. In women with PCOS, obesity is mainly characterized by abdominal accumulation of body fat [7]. Hyperinsulinemia exists independently of obesity and may, therefore, be an independent factor in PCOS [7]. Specific metabolic changes, such as hyperinsulinemia, insulin resistance, and metabolic syndrome, may already be present before the onset of PCOS and can mediate the onset of PCOS [8]. Consequently, it is critical to explore the pathogenesis of obesity and insulin resistance in PCOS.

However, the etiology of the PCOS is still obscure. One hypothesis is that PCOS is an inherited ovarian disease in which excess androgens in early life lead to PCOS in adulthood [9]. Prenatal testosterone-treated sheep and nonhuman primates show strikingly similar features to human PCOS [10,11]. However, these animal models are prohibitively costly, hard to house, and not subject to gene editing [12]. Various strategies with androgen modeling such as dihydrotestosterone, dehydroepiandrosterone, and testosterone propionate have also been used to induce PCOS in rodents [2]. However, in these rats, unlike in women with PCOS, ovarian weight is reduced, and typical gonadotropin levels, lipid profiles, and other metabolic characteristics are lacking [13]. Therefore, there is no criterion standard PCOS model of rodents. It is necessary to develop an animal model to comprehensively evaluate all reproductive, endocrine, and metabolic phenotypes [14].

Letrozole is an aromatase inhibitor that prevents testosterone from converting to estradiol, thereby increasing blood and ovarian androgen levels [13,15]. Letrozole induces similar PCO changes to that of PCOS women, such as increased ovarian weight and size, a thickened theca interna cell layer, and anovulation [9,16,17]. However, in this model, no metabolic aberrations were observed, inclusive of adiposity, insulin sensitivity, and dyslipidemia [2]. Adipose tissue inflammation is central to the pathogenesis of obesity-associated insulin resistance, T2DM, and hepatic steatosis [18,19]. High-fat diets (HFD)-induced adipose tissue expansion is accompanied by hyperinsulinemia, LH hypersecretion, female infertility, and hypertestosteronemia, much like in some women with PCOS [20]. What is the origin of PCOS with obesity and insulin resistance? Whether it is hyperandrogenism causes hyperinsulinemia, or vice versa, hyperinsulinemia causes hyperandrogenism, remains to be determined [21]. In this study, we

attempted to answer these questions and determine whether continuous administration of letrozole and high-fat diets, from pre-adolescence to adulthood, induces PCOS and metabolic phenotypes similar to that of human obese insulin-resistant PCOS. Ovarian morphology, estrous cyclicity, hormonal status, body weight, glucose and insulin tolerance, lipid profile, and insulin signaling pathway were investigated in all rats.

## Material and Methods

### Animals

Twenty-four adolescent, 3-week-old female Sprague-Dawley rats weighing 68–90 g were purchased from Shanghai Slac Laboratory Animal Co. (Shanghai, China). All rats were housed ( $n=6$  per cage) under controlled conditions (21–22°C, 55–65% humidity, reversed 12-h light-dark cycle) with free access to food and water [9]. The Laboratory Animal Ethics Committee of Fujian University of Traditional Chinese Medicine approved the research. Ethical standards of animal care were observed.

### Experimental PCOS model and treatment

Twenty-four rats were randomly divided into 4 groups: control, letrozole, HFD, and Letrozol+HFD.

Control group ( $n=6$ ): The rats were fed with a standard laboratory diet (3.85 kcal/g, energy supply ratio: protein 20%, carbohydrate 70%, fat 10%) and received vehicle only [0.5% of carboxymethyl cellulose (CMC) 2 ml/kg body weight] once daily p.o.

Letrozole group ( $n=6$ ): The rats were administered letrozole (Jiangsu Hengrui Medicine Co., China) at a concentration of 1 mg/kg p.o. dissolved in 0.5% CMC solutions once daily and fed with the same standard laboratory diets as the control group [22].

HFD group ( $n=6$ ): The rats were fed with a high-fat diet (5.24 kcal/g, energy supply ratio: protein 20%, carbohydrate 20%, fat 60%) and received the same vehicles as the control group.

Letrozol+HFD group ( $n=6$ ): The rats were administered similar letrozole as in the letrozole group and fed with the same high-fat diet as the HFD group.

All the treatment periods were 12 weeks. At the end of the experiment, all rats were sacrificed by decapitation for evaluation.

### Vaginal smears

After 8 weeks of gavage, vaginal smears were obtained from all rats, fixed with 10% formalin, and stained with hematoxylin and eosin. The changes in the estrus cycle were observed under a light microscope for 10 consecutive days.

### Body weights

All rats were weighed to observe the changes in weight and to calculate drug doses every week.

### Glucose and insulin tolerance tests

Glucose tolerance testing was performed after fasting for 15 h, and the insulin tolerance test was performed after fasting for 2 h. Glucose (2 g kg<sup>-1</sup> body weight) and insulin (Actrapid, Novo Nordisk; 0.75 U kg<sup>-1</sup>) were administered intraperitoneally (i.p.). Blood glucose levels were measured at 0, 15, 30, 45, 60, and 120 min with a Bayer Contour glucometer using tail vein blood [19].

### Tissue sample collection

Twenty-four hours after the last letrozole gavage, all rats were anesthetized (2% pentobarbital sodium, i.p.). Trunk blood was obtained, and sera were stored in a freezer at -20°C for the subsequent use. The next step was to quickly remove and clean the quadriceps femoris muscle, omentum majus, liver, and bilateral ovaries. Left ovaries were immobilized in 4% paraformaldehyde solution, successively sectioned at 4 µm, and stained with hematoxylin and eosin. Quadriceps femoris muscle, omentum majus, liver, and right ovaries were rapidly frozen in liquid nitrogen for molecular biological analyses [12,23].

### Hormone assays

Serum testosterone, progesterone, estradiol, and insulin levels were detected by RIA using the kit provided by Tianjin Union Pharmaceutical Technology Co. (Tianjin, China) according to the manufacturer's use instructions. The homeostasis model assessment of insulin resistance (HOMA-IR) was conducted using the homeostasis model assessment, and the calculation formula was:  $HOMA-IR = FINS \times FPG / 22.5$  [2].

### Lipid measurements

Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were determined by enzymatic colorimetric test technique. Fasting blood glucose (FPG) was determined by an automated glucose oxidation method in a Beckman autoanalyzer (Beckman Instruments, Palo Alto, CA).

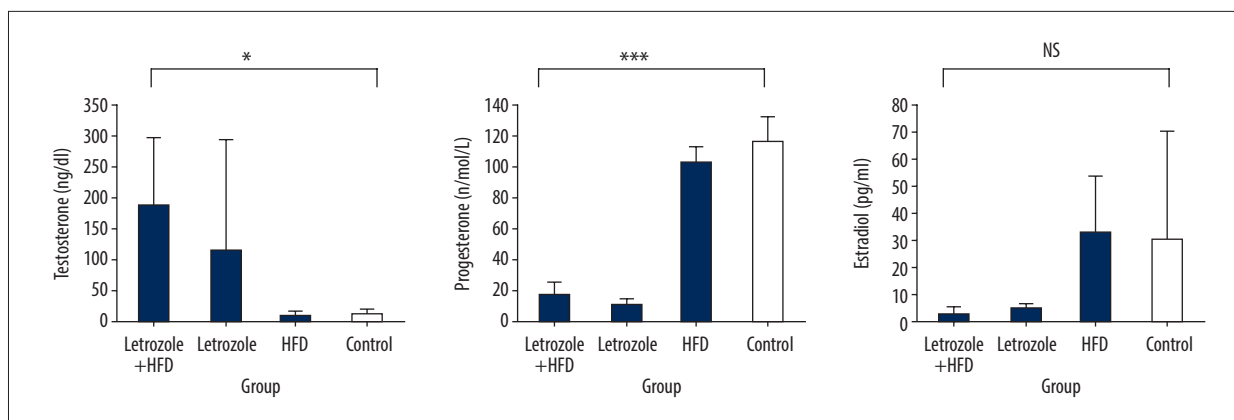
### Ovarian morphology

The ovaries were fixed with 4% paraformaldehyde, conventionally dehydrated, paraffin-embedded, sectioned with 4-µm thickness, routinely dewaxed and hydrated, stained with hematoxylin for 15 min, differentiated with 1% hydrochloric acid and alcohol, stained with 1% eosin solution, dehydrated and sealed with neutral gum, and analyzed under a conventional birefringence microscope by 2 individuals who did not know the source of the sections [24].

Follicles were counted based on the following definitions. Primordial follicle: surrounded by a monolayer of follicular epithelial cells [25]. Primary follicle: the monolayer or multilayer follicular epithelial cells surrounding the oocyte are equally prismatic or highly prismatic [25]. Secondary follicle: follicle in which oocyte was covered with more than 2 layers of granulosa cells and in which antrum formation commenced [25]. Graafian (tertiary) follicle: follicle that possesses a single and large antrum filled with follicular fluid, in which granulosa cells surround the antrum, and the oocyte is surrounded by granulosa cells (cumulus cells) [25,26]. Atretic follicle: degenerated zona pellucida or oocyte, along with pyknosis in granulosa cells, with granulosa cells and cell debris within the antral cavity [25,27].

### Western blot analysis

Fresh-frozen quadriceps femoris muscle, omentum majus, liver, and ovary tissues collected from all rats were lysed in RIPA lysis buffer (radioimmunoprecipitation assay buffer) containing protease and phosphatase inhibitors (ThermoFisher Scientific, USA). Tissue lysates were centrifuged for 20 min at 12,000 rpm at 4°C in a microcentrifuge, and the supernatants were aspirated for the following analysis. Equal amounts of protein (30–50 µg) were resolved on SDS-PAGE gel and transferred onto a PVDF membrane. Membranes were blocked in blocking buffer [5% nonfat milk diluted in TBST (0.05% Tween-20 in 10 mM Tris; 0.15M NaCl, pH7.4 (Tris-buffered saline))] for 1 h at room temperature, followed by incubation overnight (4°C) with phospho-specific antibodies for insulin receptor (INSR, Y1361), insulin receptor substrate-1 (IRS-1, Y-612), phosphoinositide 3-kinase (PI3K, Y607), extracellular signal-regulated kinases 1 and 2 (ERK1, Y204; ERK2, Y187), provided by Abcam (Cambridge, United Kingdom), and protein kinase B (AKT, Thr308), provided by CST (Danvers, USA), which are essential signaling proteins activated by insulin. β-actin (Santa Cruz Biotechnology, CA, USA) was used as the loading control. Protein bands were acquired using darkroom development techniques for chemiluminescence, and band density was measured using ImageJ software (National Institutes of Health). Because there were too many samples that could not be exposed at the same time and because different lengths of exposure could affect



**Figure 1.** Sex steroid concentrations in Letrozole+HFD, Letrozole, HFD, and control groups of rats. Serum testosterone, progesterone, and estradiol levels were determined by RIA. Data are presented as mean±SEM of 6 rats and analyzed by one-way ANOVA with Dunnett's post hoc test. \* P 0.05, \*\* P 0.01, \*\*\* P 0.001 compared with control group. NS – Not significant.

band density, protein levels were determined as fold changes to each control group after normalization of each protein (band density) with its corresponding loading control ( $\beta$ -actin band density) and calculated by the following formula:  $T_{\text{protein level}} = (E_{\text{band density}} / E_{\text{corresponding } \beta\text{-actin band density}}) / (C_{\text{band density}} / C_{\text{corresponding } \beta\text{-actin band density}})$ . T indicates 6 target phospho-proteins being measured; E stands for experimental groups, C represents control group [28].

### Statistical analyses

SPSS software (version 17.0; SPSS, Inc., Chicago, IL) was used for statistical evaluations. We used one-way ANOVA with Dunnett's post hoc test to compare the effects on body weight, area under the curve (AUC) of glucose and insulin tolerance, lipid profile, sex steroid concentrations, and protein levels of insulin signaling pathway among groups [9]. Values are expressed as mean±SEM.  $P < 0.05$  was considered statistically significant.

## Results

### Letrozole+HFD model rats had endocrine phenotypes similar to human obese PCOS

#### Sex steroid concentrations

In general, the main principle to induce polycystic ovary models is exposure to excess androgen, the biochemical hallmark of PCOS. One of the critical unanswered questions regarding the etiology of PCOS is from where, in the human female, does the excess androgen come? One hypothesis is that the ovary is genetically predisposed to hypersecrete androgens [11]. The letrozole model relies on inhibiting aromatase from increasing endogenous androgens instead of treating animals with exogenous androgens, which is consistent with the hypothesis of hypersecretion of

ovarian androgens [29]. As Figure 1 depicts, compared with control rats, the serum testosterone concentrations of letrozole+HFD rats were significantly increased ( $P < 0.05$ ), whereas HFD rats had normal testosterone levels ( $P > 0.05$ ). Letrozole+HFD rats had hyperandrogenism, while HFD rats did not, suggesting that obesity and hyperinsulinemia alone cannot cause hyperandrogenism. The progesterone concentrations were significantly lower ( $P < 0.001$ ) and the estradiol concentrations were also lower in the letrozole+HFD rats, indicative of anovulation.

#### Vaginal smears

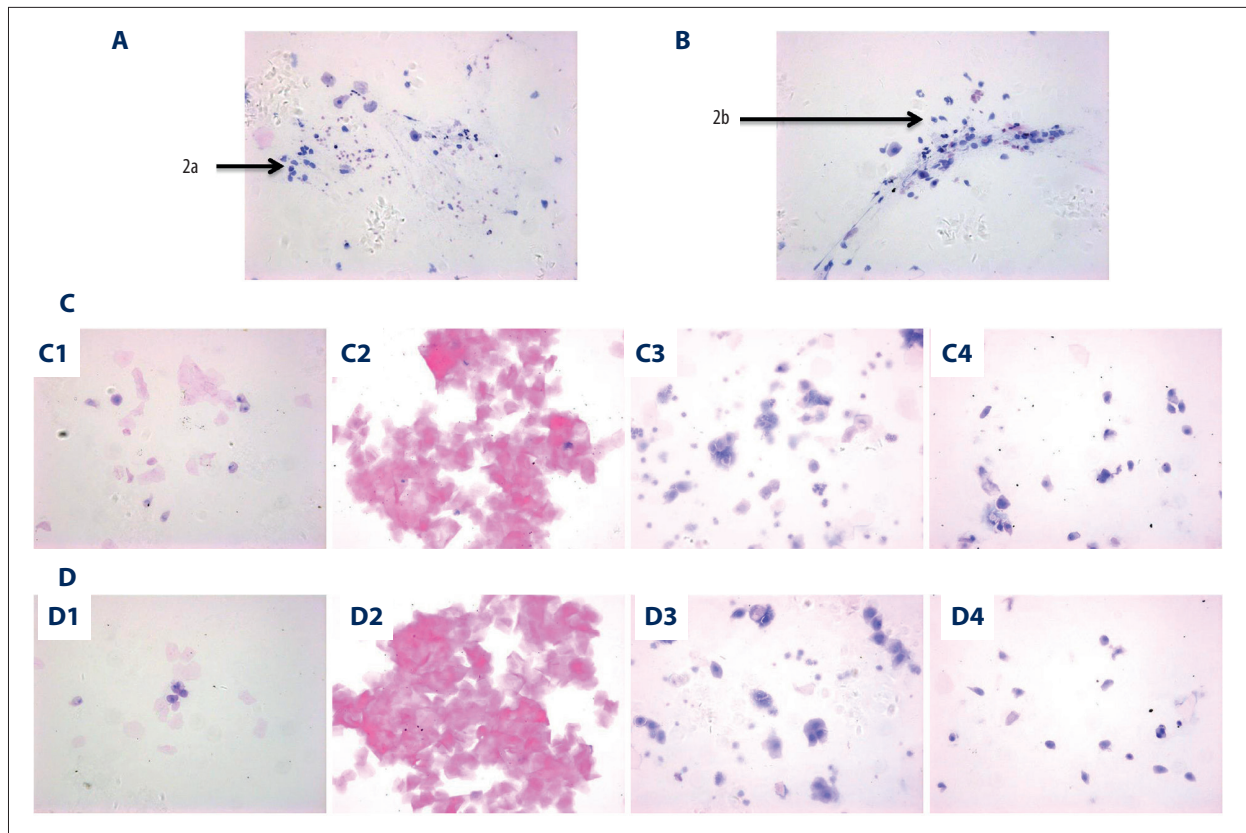
Vaginal smears staining results of the letrozole+HFD group and letrozole group showed many leukocytes and few keratinocytes (Figure 2A, 2B). These results denote that the rats of both groups were in the diestrus phase and had no ovulation. In contrast, rats of the HFD group and control group still maintained a regular estrus cycle (Figure 2C, 2D).

#### Ovarian morphology

Multiple luteal follicles and follicles at different development stages with normal multilayered granulosa cells were observed in the pathological sections of ovaries of the control group and HFD group (Figure 3C, 3D), indicative of ovulatory cycles. Compared with the control group, rats of the letrozole+HFD group and letrozole group had more and larger diameter atretic follicles, with more thickened theca interna cell layers, presenting typical polycystic ovary changes (Figure 3A, 3B) and anovulation.

The above observations strongly suggest that letrozole and high-fat diets, administered in rats from 3-week-old to 15-week old, can induce the PCOS model, recapitulating similar ovarian and endocrine features to those in human obese insulin-resistant PCOS.





**Figure 2.** Vaginal smears in Letrozole+HFD, Letrozole, HFD, and control groups of rats. (A) Vaginal smear from a Letrozole+HFD rat. (B) Vaginal smear from a Letrozole rat. 2a & 2b – leukocytes. (C) Vaginal smears from an HFD rat, showing a regular estrus cycle. (D) Vaginal smears from a control rat, showing a regular estrus cycle. C1, D1 – proestrus; C2, D2 – estrus; C3, D3 – metestrus; C4, D4 – diestrus.

### Letrozole+HFD model rats have metabolic phenotypes similar to those of human obese PCOS

#### Weight

At the start of the experiment, the 4 groups of rats had no significant difference in body weight. The weight of the rats in the letrozole+HFD group increased significantly from 8 weeks to 15 weeks after high-fat diet feeding, compared with that of the control rats and letrozole rats ( $P < 0.001$ , Figure 4), showing “metabolic obesity”.

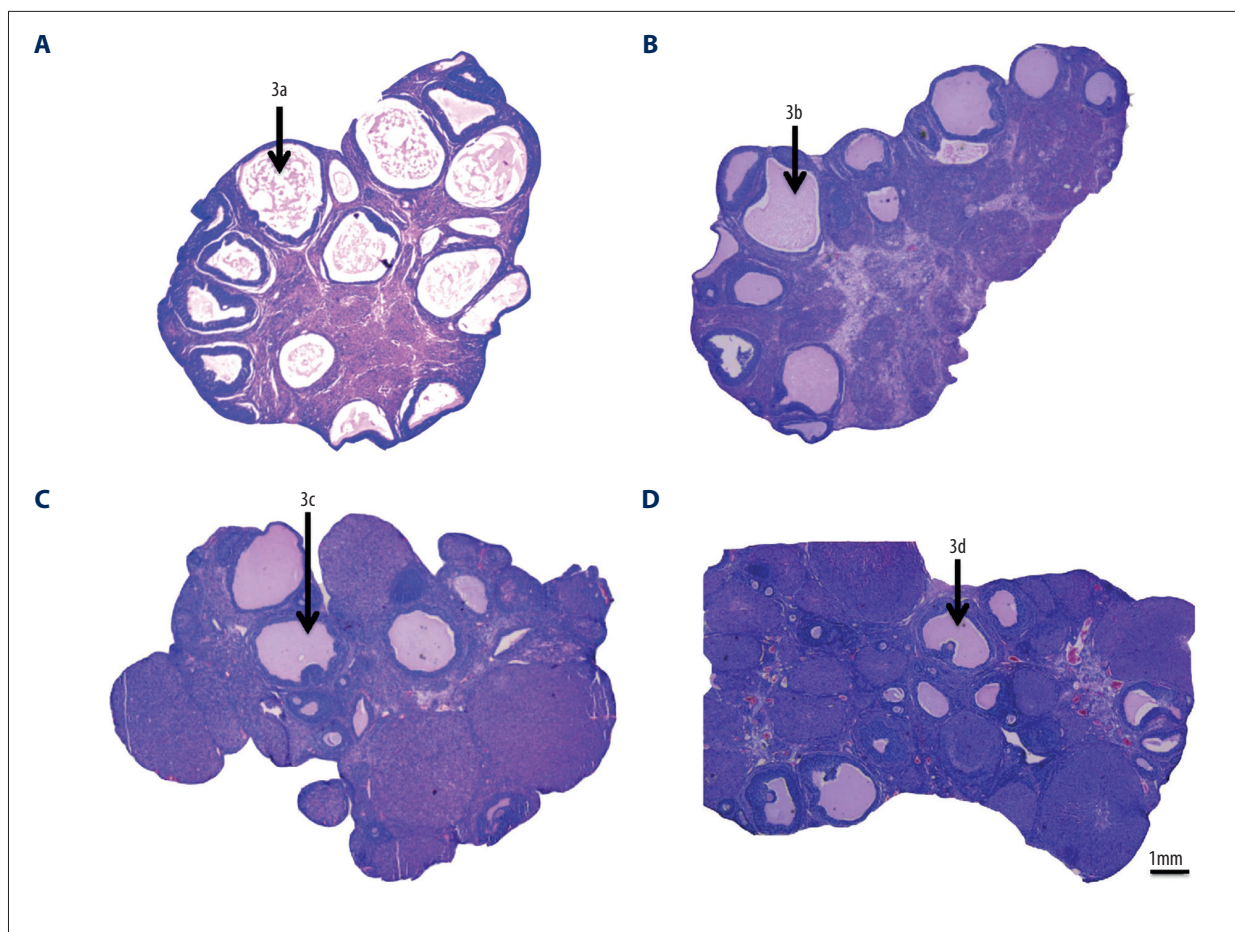
#### Glucose and insulin tolerance tests

PCOS women have a significantly higher risk of impaired glucose tolerance (IGT) and T2DM when they are younger [30]. The fasting glucose levels in PCOS women are not reliable predictors of the glucose levels at 2 h after an oral glucose challenge, particularly in IGT patients who have the highest risk for subsequent development of T2DM [31]. In the glucose tolerance experiment tests, blood glucose levels at 15 min, 60 min, and 120 min were significantly higher in the letrozole+HFD

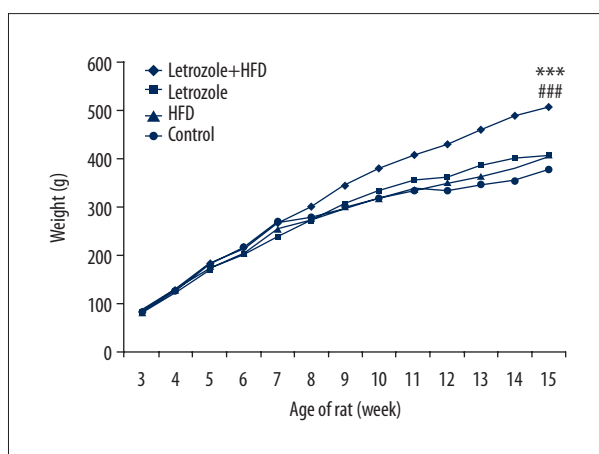
group ( $P < 0.05$ , Figure 5A), and the area under the curve (AUC) of glucose tolerance tests of letrozole+HFD rats was more significant than that of control rats and letrozole rats ( $P < 0.05$ , Figure 5B). Rats in the letrozole+HFD group had higher blood glucose levels at 0 min, 15 min, and 120 min and larger AUC than the control and letrozole rats in insulin tolerance tests ( $P < 0.05$ , Figure 5C, 5D). These results show that letrozole+HFD rats have impaired glucose concentration after tolerance and normal fasting blood glucose (FPG), which closely mimic the clinical features and glucose metabolism of PCOS women.

#### Index of insulin resistance

Obese PCOS women have deteriorated metabolic manifestations, and lean PCOS women manifest metabolic differences, including hyperinsulinemia and insulin resistance, compared with non-PCOS females with similar BMI [32,33]. Excess androgen in women predisposes them to pancreatic  $\beta$ -cell dysfunction [34,35], and prenatal androgen excess is critical in programming the metabolic milieu in the developing fetus in animal models with increased fasting glucose, IGT, and hyperinsulinemia [36–39]. As Figure 6 shows, letrozole+HFD rats



**Figure 3.** Ovarian morphology in Letrozole+HFD, Letrozole, HFD and control groups of rats. (A) Ovary from a Letrozole+HFD rat. (B) Ovary from a Letrozole rat. (C) Ovary from an HFD rat. (D) Ovary from a control rat. 3a & 3b – atretic follicles; 3c & 3d – graafian (tertiary) follicles.



**Figure 4.** Growth curves of Letrozole+HFD, Letrozole, HFD, and control groups of rats from 3 weeks of age to 15 weeks of age. Data are presented as mean±SEM of 6 rats and analyzed by one-way ANOVA with Dunnett's post hoc test. \*\*\*  $P < 0.001$  compared with the control group at 15 w. ###  $P < 0.001$  compared with Letrozole group at 15 w.

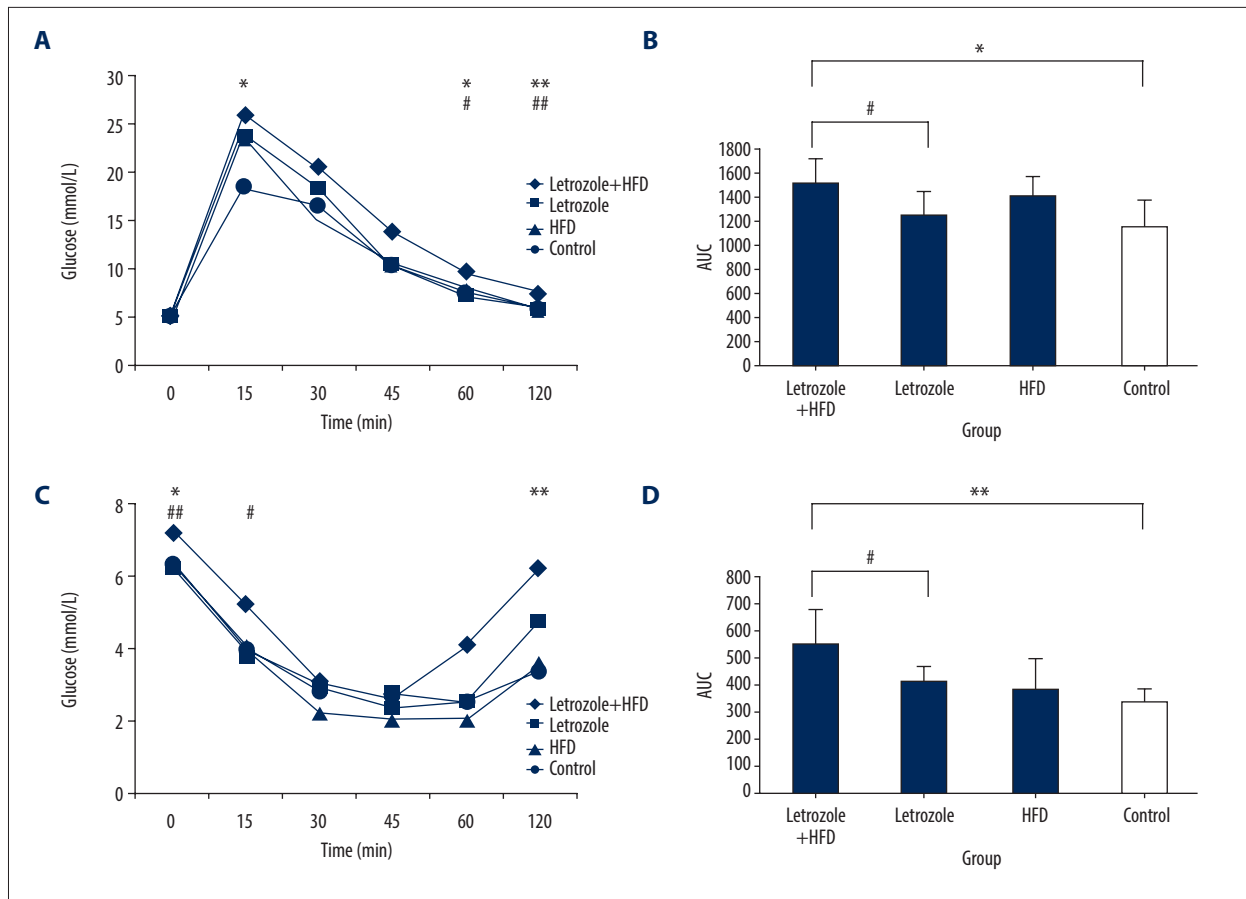
also had abnormal insulin resistance indices such as higher fasting insulin (FINS) levels and HOMA-IR compared with the controls ( $P < 0.05$ ).

#### Lipid profiles

It has been estimated that 70% of PCOS women have dyslipidemia [40,41], and the TC/HDL-C ratio is positively associated with cardiovascular disease events [12,42]. The rats induced by letrozole and high-fat diets in this study presented lipid abnormalities similar to PCOS women, including higher levels of TC ( $P < 0.05$ , Figure 7A), LDL-C ( $P < 0.001$ , Figure 7C), and TC/HDL ratio ( $P < 0.01$ , Figure 7D), but no significant difference was found in the levels of HDL-C ( $P > 0.05$ , Figure 7B).

#### Letrozole+HFD model rats had disrupted insulin signaling pathway activity

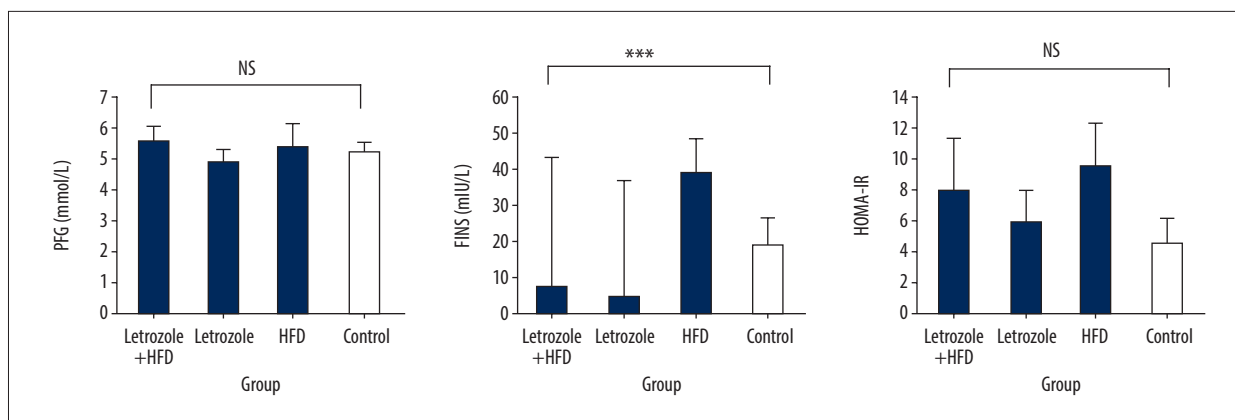
It is estimated that approximately 50–90% of women with PCOS have varying degrees of insulin resistance [43,44]. Insulin



**Figure 5.** Blood glucose levels and AUC of glucose and insulin tolerance tests in Letrozole+HFD, Letrozole, HFD, and control groups of rats were measured with a Bayer Contour Glucometer. **(A)** Blood glucose during glucose tolerance tests. **(B)** AUC of glucose tolerance tests. **(C)** Blood glucose during insulin tolerance tests. **(D)** AUC of insulin tolerance tests. Data are presented as mean±SEM of 6 rats and analyzed by one-way ANOVA with Dunnett's post hoc test. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared with control group. #  $P < 0.05$ , ##  $P < 0.01$  compared with Letrozole group.

resistance and associated compensatory hyperinsulinemia are critical in the pathogenesis of PCOS. Increasing insulin sensitivity by weight loss or drug therapy improves ovulatory function, menstrual cyclicity, fertility, and hyperandrogenic and metabolic features [45–48]. Insulin signaling transduction involves a series of reactions triggered by the binding of insulin to the INSR ligand, followed by autophosphorylation of the receptor and activation of the receptor tyrosine kinase, leading to tyrosine phosphorylation of IRS1 [49]. Binding of IRS1 to the regulatory subunit p85 of PI3K through Src homology 2 (SH2) domains results in the activation of PI3K, which produces phosphatidylinositol-4,5-triphosphate (PIP2) and phosphatidylinositol-3,4,5-triphosphate (PIP3) in the membrane [50]. PIP3 is an allosteric activator of 3-phosphoinositide-dependent protein kinases (PDK) and is involved in the activation of downstream target protein AKT [50,51] and mediation of metabolic effects, including glucose disposal into skeletal muscle [52]. Another characterized insulin signaling pathway is the mitogen-activated protein kinase (MAPK)/ERK pathway, which mediates

cell growth and steroidogenic effects [52]. The primary signal transduction step of the MAPK/ERK signaling pathway is the tertiary cascade enzyme reaction. Ras/Raf/MEK/ERK is its main pathway. Once activated, all members of the Raf family can initiate the phosphorylation cascade, whereby Raf activates MEK, and MEK in turn activates ERK [53]. However, contrary to the complexity observed in Raf activation, MEK and ERK are fully activated simply by phosphorylation of activated fragments in their respective kinase domains [53]. The activation/phosphorylation of 6 essential signaling proteins in the PI3K/AKT and MAPK/ERK pathways activated by insulin was investigated to access the activity of insulin signaling. The phosphorylation levels of INSR, IRS, AKT, and ERK1 were significantly lower in the classic insulin-sensitive tissues of letrozole+HFD rats, such as quadriceps femoris muscle, omentum majus, and liver, as well as ovary tissues ( $P < 0.01$ , Figure 8A, 8B, 8D, 8E). The letrozole+HFD rats also had lower levels of phosphorylated PI3K in the liver and ovary ( $P < 0.001$ , Figure 8C) and phosphorylated ERK2 in the quadriceps femoris muscle, liver, and



**Figure 6.** Index of insulin resistance in Letrozole+HFD, Letrozole, HFD, and control groups of rats. Fasting blood glucose (FPG) was measured by an automated glucose oxidation method. Fasting insulin (FINS) levels were determined by RIA, and the homeostasis model assessment of insulin resistance (HOMA-IR) was evaluated by the homeostasis model assessment and calculated by the following formula:  $HOMA-IR = FINS \times FPG / 22.5$ . Data are presented as mean  $\pm$  SEM of 6 rats and analyzed by one-way ANOVA with Dunnett's post hoc test. \* P 0.05 compared with the control group. NS – not significant.

ovary ( $P < 0.001$ , Figure 8F) compared with the control and letrozole rats. These changes contributed to the disruption of insulin sensitivity and compensatory hyperinsulinemia in PCOS model rats.

This study shows that the ovarian and metabolic abnormalities in rats induced by letrozole and high-fat diets closely resembled those in human obese insulin-resistant PCOS; they manifested anovulatory cycles and PCO morphology, increased body weight, elevated testosterone levels, abnormal glucose and lipid metabolism, and insulin resistance.

## Discussion

Our findings of animal model features such as polycystic ovary and endocrine changes were consistent with those from previously published rat studies using letrozole [54,55]. However, the disrupted activities of the insulin signaling pathway in letrozole+HFD rats were the most important findings.

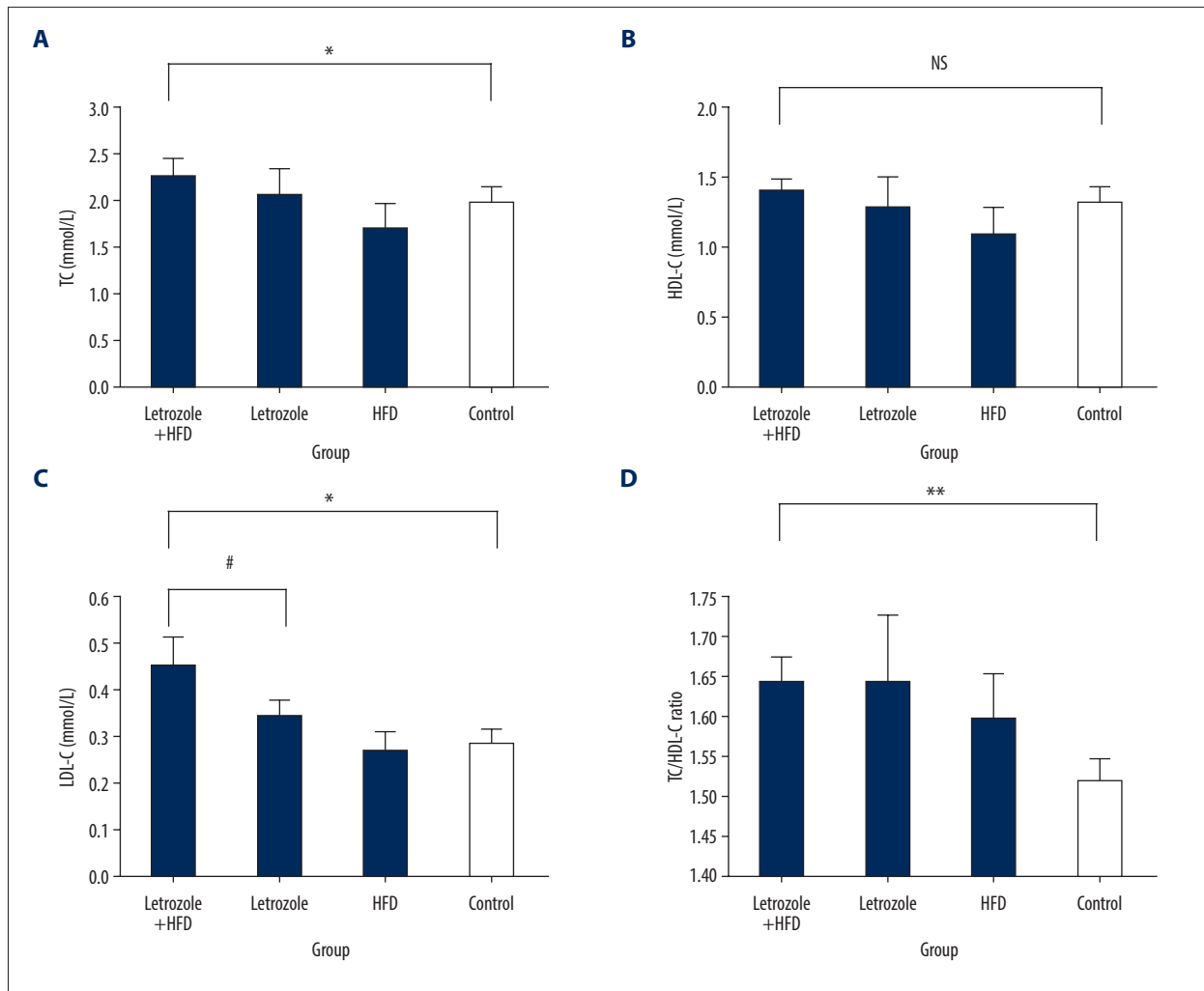
Deficiency in activity of aromatase is a hormone production disorder in the ovaries, which is considered to be the cause of PCOS [9,54]. Since it catalyzes the rate-limiting step of the synthesis of estrogens from androgens, decreased enzyme activity can lead to an increase in ovarian androgens and the development of PCOS [22]. The letrozole model is based on aromatase deficiency-induced classic PCOS and may be an effective co-treatment with other treatments that induce cardiometabolic aberrations to study these factors in PCOS [2]. In this study, the letrozole+HFD group and letrozole group showed polycystic ovary changes, including increased numbers and larger diameters of atretic follicles, decreased numbers of granulosa cell

layers, and thickened white membranes (Figures 1, 2), indicating that rats in both groups were not ovulating.

In letrozole-induced rat models, the most commonly used procedure is implanting a subcutaneous pellet in a 21-day-old rat, with letrozole doses varying from 100 to 400  $\mu\text{g}/\text{d}/100$  g body weight for a 90-day continuous release [14,56,57]. Figure 4 shows that in the letrozole+HFD rats, weight increased significantly from 8 weeks to 15 weeks, and the letrozole doses should be adjusted accordingly, which cannot not be accomplished by use of a constant-release pellet. For this reason, we improved the procedure by using oral administration and adjusting dosage every week.

In addition to the genetic causes of predisposition to androgens hypersecretion, environmental factors, particularly the effect of diet, clearly play an essential part in the presentation and severity of PCOS, if not in the origin of the disorder itself [58]. High-fat diets can exacerbate the effect of androgens on adiposity, glucose intolerance, pronounced liver steatosis, insulin resistance, and depressive behaviors [59,60]. There is robust evidence that increased body weight exacerbates hyperandrogenism, anovulation, and metabolic risk in PCOS [61,62]. It is generally accepted that high-fat diets can be used to generate a valid rodent model of metabolic syndrome with insulin resistance and compromised  $\beta$  cell function [63–65]. Therefore, we used letrozole to develop endogenous hyperandrogenism and high-fat diets to induce obesity and hyperinsulinemia, which both contribute to the formation of the obese insulin-resistant PCOS model. As Figures 4 and 5 show, after high-fat diet feeding, the letrozole+HFD group showed metabolic obesity and impaired glucose intolerance compared with the control group and letrozole group.



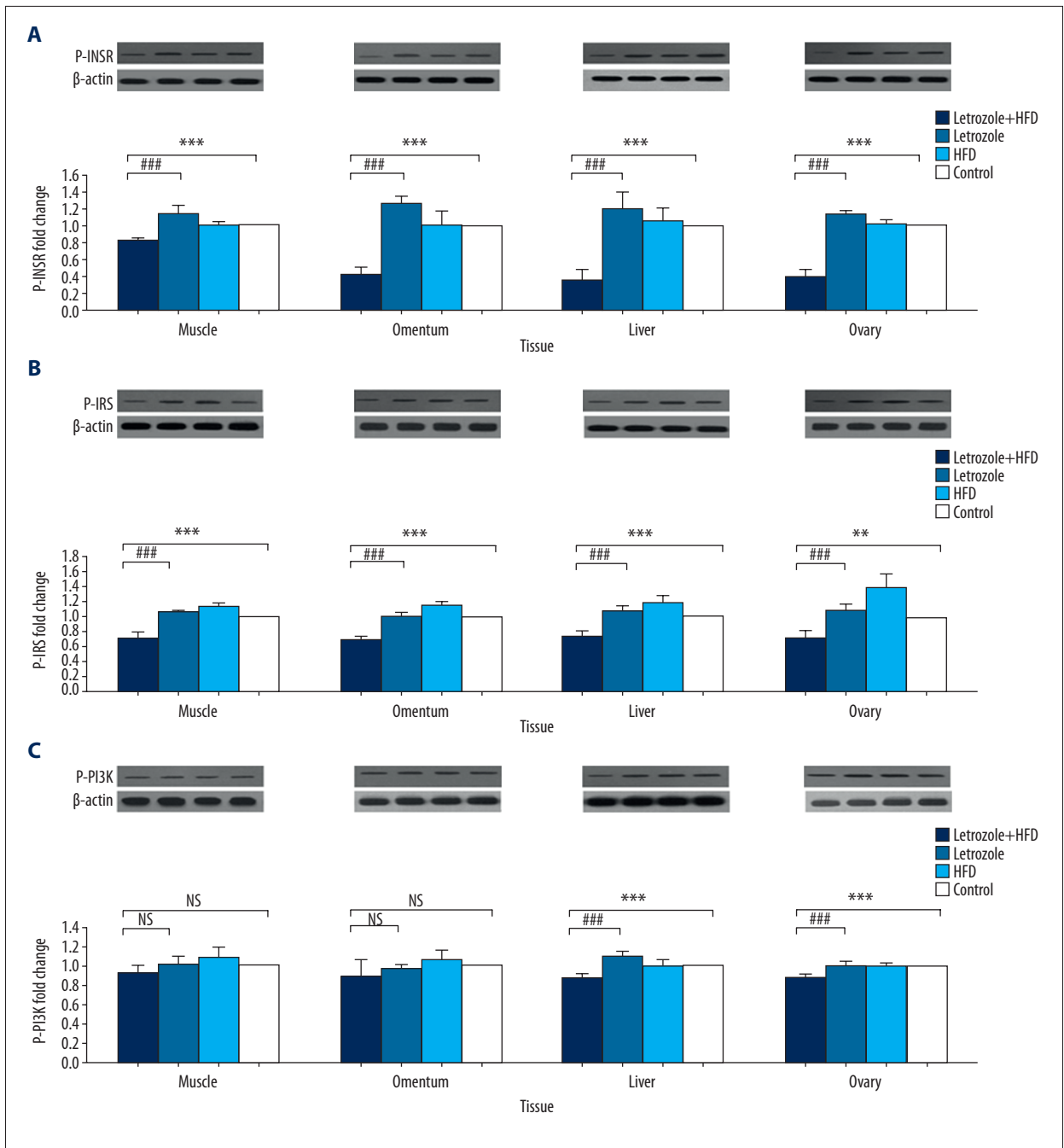


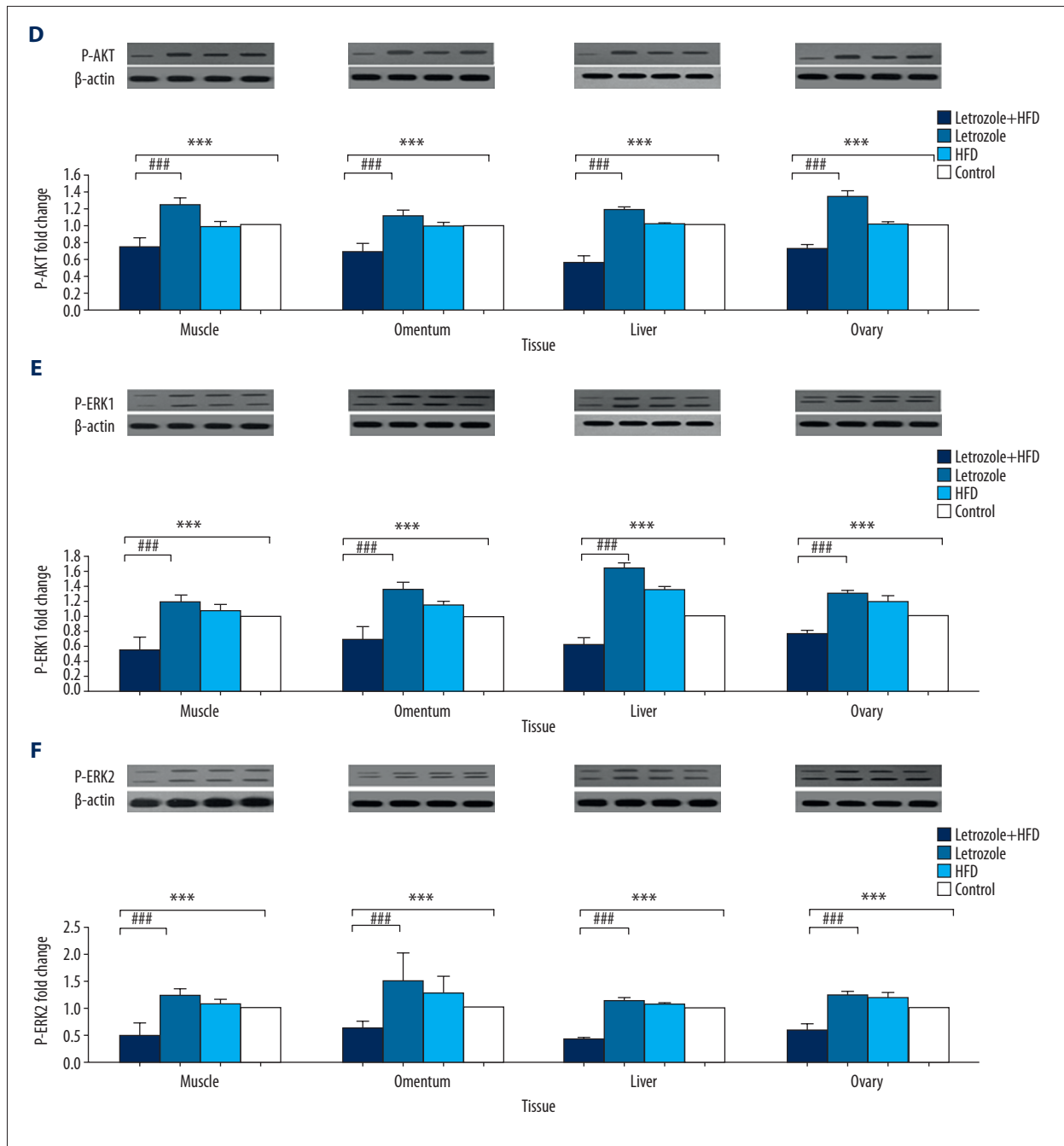
**Figure 7.** Lipid profiles in Letrozole+HFD, Letrozole, HFD, and control groups of rats. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were measured by enzymatic colorimetric test technique. **(A)** Total cholesterol. **(B)** High-density lipoprotein cholesterol. **(C)** Low-density lipoprotein cholesterol. **(D)** TC/HDL-C ratio. Data are presented as mean±SEM of 6 rats and analyzed by one-way ANOVA with Dunnett's post hoc test. \* P 0.05, \*\* P 0.01, \*\*\* P 0.001 compared with control group. # P 0.05, ## P 0.01 compared with Letrozole group. NS – not significant.

The striking results of the study were that the activation/phosphorylation of 6 essential signaling proteins – INSR, IRS, PI3K, AKT, ERK1, and ERK2 – in the PI3K/AKT and MAPK/ERK pathways activated by insulin were significantly lower in the letrozole+HFD group classic insulin-sensitive tissues such as quadriceps femoris muscle, omentum majus, and liver, as well as ovary tissues (Figure 8). Several experimental studies reported that the mRNA and protein levels of IRS-2 and PI3K in the ovaries of PCOS rats were significantly decreased [66,67]. Our findings of disrupted activities of the insulin signaling pathway in the ovaries of letrozole+HFD group rats were consistent with previous research. Furthermore, we found that disrupted activation of insulin signal transduction also existed in the classic insulin-sensitive tissues of rats, such as quadriceps femoris muscle, omentum majus, and liver, which contributes

to the impairment of insulin sensitivity and compensatory hyperinsulinemia in PCOS rats.

This study has several limitations. First, the expression levels of INSR, IRS, PI3K, AKT, ERK1, and ERK2 mRNA were not determined. Second, the model of obesity and insulin resistance induced by high-fat diets took 12 weeks to establish and was very time-consuming. Third, changes in protein expression in the insulin signaling pathway were not verified by antagonists or agonists. In the next step, metformin or other insulin sensitizers will be used to treat PCOS rats to verify the changes of the insulin signaling pathway or to assess new therapeutic methods in this model.





**Figure 8. (A–F)** The activity of insulin signaling in Letrozole+HFD, Letrozole, HFD, and control groups of rats. Protein contents in quadriceps femoris muscle (Muscle), omentum majus (Omentum), liver and ovary tissues were examined by Western blot with phospho-specific antibodies for insulin receptor (INSR, Y1361), insulin receptor substrate-1 (IRS-1, Y-612), phosphoinositide 3-kinase (PI3K, Y607), protein kinase B (AKT, Thr308), and extracellular signal-regulated kinases 1 and 2 (ERK1, Y204; ERK2, Y187). Band density was determined using ImageJ software (National Institutes of Health) and expressed as fold changes to each control group after normalization of each protein (band density) with its corresponding loading control (β-actin band density). Data are presented as mean±SEM of 6 rats and were analyzed by one-way ANOVA with Dunnett's post hoc test. \* P 0.05, \*\* P 0.01, \*\*\* P 0.001 compared with control group. NS – not significant.

## Conclusions

Continuous administration of letrozole and high-fat diets can induce PCOS, metabolic phenotypes, and disrupted activation of insulin signal transduction in the rat model. Further studies using metformin or other insulin sensitizers in PCOS rats are required to verify the changes we found in the insulin signaling pathway.

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