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An Experimental Study on The Oxidative Status and Inflammatory Levels of A Rat Model of Polycystic Ovary Syndrome Induced by Letrozole and A New High-Fat Diet

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Abstract

Background: Although there are numerous animal models of polycystic ovary syndrome (PCOS), they often fail to accurately replicate the reproductive and metabolic phenotypes associated with PCOS. The objective of this study is to assess oxidative status and inflammatory levels in a rat model of PCOS subjected to a new high-fat diet (HFD) in combination with letrozole.

Materials and Methods: In this experimental study, mature, six-week-old female Sprague-Dawley rats (n=20) were divided into four groups: control (standard diet); letrozole (letrozole plus a standard diet); HFD; and letrozole+HFD. After 16 weeks, the rats underwent vaginal smear analysis, measurement of hormonal and lipid profiles, and an oral glucose tolerance test (OGTT). Ovarian tissue morphology, oxidative parameters, and inflammatory status were evaluated.

Results: The experimental groups exhibited anoestrus profiles in the vaginal smears and abnormal ovarian morphology, which was not observed in the control group. Steroid hormone levels were significantly higher in the letrozole+HFD group compared to the other groups (P=0.00). The experimental groups also showed abnormal glucose levels and lipid metabolism. The relative expression levels of inflammatory genes were significantly elevated in the experimental groups compared to the control group (P=0.00), and the letrozole+HFD group exhibited the highest expression level (P=0.00). The HFD, letrozole, and letrozole+HFD groups demonstrated significantly increased levels of malondialdehyde (MDA) and reactive oxygen species (ROS), while the levels of enzymatic antioxidants were significantly reduced compared to the control group (P=0.00).

Conclusion: The combination of a new HFD and letrozole treatment induces inflammation and oxidative stress (OS) in a rat model of PCOS. This model accurately exhibits abnormal metabolic phenotypes and disruptions in hormonal profiles associated with PCOS.

Keywords: High-Fat Diet, Inflammation, Oxidative Stress, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects various stages of a woman's reproductive life (1, 2). The clinical manifestations of women with PCOS are highly variable and often include menstrual dysfunction, hyperandrogenism, and metabolic disorders such as obesity, insulin resistance, type 2 diabetes, hypertension, and dyslipidaemia, with an increased risk of cardiovascular disease (3-6). Additionally, oxidative stress (OS) and low-grade inflammation are factors in the pathogenesis of this syndrome. OS in women with PCOS is associated with metabolic disorders, and an association exists between markers of OS and inflammation with high androgen levels. However, as our understanding of the pathological symptoms of PCOS grows, it is important to examine this disorder from an inflammatory and oxidative perspective. This may lead to a better understanding of the underlying mechanisms of PCOS, which is essential for transitioning from a symptomatic treatment approach to a definitive treatment approach. Recent studies have established the presence of low-grade inflammation and

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OS in women with PCOS. OS is defined as an "oxidant/antioxidant imbalance in favour of the former, which leads to cell damage," while low-grade inflammation is a pathological condition characterised by elevated inflammatory markers (1).

Despite the high prevalence of PCOS, its etiopathogenesis remains unclear (6). Ethical limitations for human studies have led to the development of animal models to further understand PCOS pathogenesis. Among the various animal models that exhibit a wide range of features related to human PCOS, rodent models provide a versatile tool for determining the exact biological mechanisms involved in the development of PCOS (7). PCOS models in rodents have been induced through various methods, including prenatal and postnatal treatment with androgens, oestrogens, aromatase inhibitors, anti-progesterone factors, high-fat diets (HFD), permanent light exposure, and genetic manipulations (8, 9).

PCOS induced by letrozole and HFD are two widely used models that have received special attention regarding morphological, endocrine, and metabolic characteristics; however, investigations of OS and inflammation are insufficient. Letrozole, a cytochrome P450 aromatase inhibitor, blocks the conversion of testosterone to oestradiol (8, 10), leading to hyperandrogenism (7, 8). Letrozole also causes similar changes to those seen in women with PCOS, such as weight gain, increased ovarian size, thickening of the theca inner cell layer, and anovulation (7, 11). However, in the letrozole model, no metabolic abnormalities such as insulin resistance, adiposity, and dyslipidaemia have been observed. On the other hand, HFD-induced PCOS is associated with metabolic disorders similar to those observed in some women with PCOS (12). Previous HFD models did not adequately simulate metabolic factors. Therefore, in this study, we aim to establish a rat model of PCOS using letrozole in combination with a new HFD to investigate hormonal, metabolic, oxidative, and inflammatory status.

Materials and Methods

Animals

In this experimental study, 20 female Sprague-Dawley rats, aged 6 weeks and weighed 180-200 g, were obtained from the Pasteur Institute of Iran. The rats were housed in the animal facility under controlled conditions of a 12/12-hour light/dark cycle, temperature range of 22-24°C, and humidity of 45 ± 2%.

Experimental design

The rats were randomly divided into four groups with five rats per group. i. The control group consumed a standard laboratory diet that consisted of 3.14 kcal/g with an energy supply ratio of 21.5% protein, 65% carbohydrate, and 4% fat. Additionally, they received 5 ml of 0.5% carboxymethyl cellulose (CMC) as a vehicle once per day for four weeks. ii. Letrozole group rats received daily gavages of letrozole (1 mg/kg body weight) dissolved in 0.5 ml of CMC for four weeks. They were also fed the standard laboratory diet. iii. HFD group rats consumed an HFD composed of 5.3 kcal/g, with an energy supply ratio of 20% protein, 36% carbohydrate, and 40% fat. The HFD also contained 1.25% cholesterol, 23.1 g/L d-fructose, and 18.9 g/L d-glucose. Similar to the control group, they received the vehicle (CMC) daily for 16 weeks. iv. The letrozole+HFD group received letrozole dissolved in 0.5 ml of 0.5% CMC daily for four weeks through gavage. They were fed the HFD for 16 weeks.

The rats were measured weekly to assess changes in weight. Oestrous cycle changes were determined by evaluating the cellular composition of vaginal smears ten weeks after initiation of gavage, as described previously (13). At the end of the experiment, all rats were euthanized by decapitation under deep anaesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg).

Oral glucose tolerance test

During the last week of the experiment, an oral glucose tolerance test (OGTT) was performed. The rats fasted for 15 hours, then blood samples were collected from the tail veins (time 0). The rats subsequently received a glucose solution (2 g/kg body weight) through gavage. Additional blood samples were collected from the tail veins at 30, 60, and 120 minutes after glucose administration to measure glucose levels with the Decont Personal Accu-check system. Additionally, the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated 48 hours after OGTT using the following formula: fasting plasma insulin (mU/l)×fasting plasma glucose (mmol/l)/22.5. Insulin and glucose concentrations were measured by enzyme-linked immunosorbent assay (ELISA; Merck/Merck Millipore, Hungary).

Hormone assay

Serum testosterone, progesterone, and oestradiol levels were measured using an ELISA kit (Demeditec, Germany) following the manufacturer's instructions. In order to accurately assess hormonal changes, blood samples were taken from all of the rats at similar stages of the oestrous cycle.

Lipid profile assay

Serum levels of total cholesterol (TC, mmol/L), triglycerides (TG, mmol/L), low-density lipoprotein (LDL, mmol/L), and high-density lipoprotein (HDL, mmol/L) were assessed using standard colorimetric methods.

C-reactive protein assay

Serum C-reactive protein (CRP) contents were analysed by serological analysis. An ELISA kit (Millipore's MILLIPLEX® MAP Rat/Mouse CRP Single Plex, USA) was used for this purpose.

Histological studies

Ovarian samples were taken from mice at similar phases of the oestrous cycle. The ovaries were serially sectioned at 5 µm from the centre and stained with haematoxylin and eosin (H&E). Ovarian follicles in different growth phases, including preantral, antral, and atretic follicles, as well as the corpus luteum, were evaluated in every fifth section of the largest cross-sectional area. Follicles were classified as previously described (14). Additionally, the thickness of the largest follicular wall, including the theca and granulosa layers, was measured.

Gene expression analysis

Total RNA was extracted from ovaries using TRIzol (Qiagen, USA) following the manufacturer's instructions. The RNA was treated with DNase I (Cinnagen, Iran). cDNA synthesis was performed using a RevertAid kit (Fermentas, MD, USA) according to the manufacturer's protocol. Gene-specific primer sets were designed using AlleleID software version 7.5 (DBA Oligo, Inc., USA). The primer sequences are listed below:

Tnf-α-F: 5′-TCAGCCTCTTCTCATTCC-3′
R: 5′-ACTTCTCCTCCTTGTTGG-3′
Mcp-1-F: 5′-TGGTGGTCTGTGGTGCTAAG-3′
R: 5′-AACTGGAGGCTTGGTAGAATGAG-3′
Il-1β-F: 5′-CACTATTCCTAATGCCTTCC-3′
R: 5′-TCTGAGAGACCTGACTTG-3′
Ef1-F: 5′-AGTCGCCTTGGACGTTCTT-3′
R: 5′-CCGATTACGACGATGTTGATGTG-3′

Real-time polymerase chain reaction (PCR) was performed using an ABI StepOne machine (Applied Biosystems, ABI, USA) and RealQ Plus SYBR Green (Ampliqon, Denmark). The qPCR protocol included one cycle at 95ºC for 15 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 45 seconds, following the MIQE guidelines (15). The Ef1 gene was used as the reference gene. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. A no-template control tube for each gene was included in all experiments.

Evaluation of oxidative status

Tissue supernatant was prepared from ovarian tissue to evaluate total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) activities, and malondialdehyde (MDA) levels as previously described (16, 17). The production of reactive oxygen species (ROS) in ovarian tissue was measured using the 2′,7′- dichlorodihydrofluorescein (DCHF) probe, following a method described previously (17). TAC levels were measured using the ferric reducing/ antioxidant power (FRAP) method as previously described (17) and presented as mol/L. MDA levels were assessed as an index of lipid peroxidation, following a previously described method (16), and presented as nmol/mg protein. GPX, SOD, and CAT activities were measured according to previously described methods (16). GPX activity was defined as the conversion of NADPH to NADP and measured by monitoring absorption changes at 340 nm. One unit of SOD activity was measured by calculating the 50% inhibition of nitro blue tetrazolium reduction. CAT activity was calculated based on absorbance changes in one minute and presented as µMol/min/mg protein. The total protein concentration in the tissue supernatant was measured using the Lowry assay method (18).

Statistical analysis

The data were analysed using SPSS version 16 software package for Windows (SPSS Inc., Chicago, IL, USA). The statistical analysis involved conducting one-way ANOVA followed by Bonferroni post hoc analysis to assess group differences. The Bonferroni adjustment was applied to control for multiple comparisons and maintain the overall significance level. This adjustment helps reduce the likelihood of obtaining false positive results when conducting multiple pairwise comparisons and ensures that the observed differences between groups are not simply due to random chance. A $P<0.05$ was considered statistically significant and indicated meaningful differences between the groups. The results are presented as mean ± SD, providing an indication of the central tendency and variability of the data.

Ethical consideration

All procedures were approved by the Laboratory Animal Ethics Committee of Damghan University, Damghan, Iran (IR.BSDU.REC.1399.14).

Results

Weight changes

There were no significant differences in body weights among the different groups at the start of the experiment. However, after the first four weeks of treatment, the weight of the rats in the HFD-treated and letrozole+HFD-treated groups significantly increased compared to the control and letrozole groups ($P=0.002$, Table 1). Conversely, there was no significant difference in weight between the letrozole and control groups. Interestingly, the letrozole+HFD group showed the highest weight gain compared to the other groups ($P=0.004$, Table 1).

Vaginal smears

The rats treated with letrozole and letrozole+HFD exhibited complete acyclicity. The vaginal smears of the letrozole-treated and letrozole+HFD-treated rats showed a higher presence of leukocytes, which indicated pseudo dioestrus. In contrast, the vaginal smears of the control and HFD rats displayed a regular oestrous cycle (Fig.1).

Table 1: Changes in body weights of rats under different treatment conditions

Weeks/Groups	Control	Letrozole	HFD	Letrozole+HFD
6	199 ± 7.7	202.5 ± 7.0	195.2 ± 6.6	202.5 ± 10.1
7	222.5 ± 7.3	224.7 ± 7.5	219.0 ± 9.2	228.3 ± 11.3
8	236.5 ± 7.3	240.3 ± 12.5	236.5 ± 16.4	240.8 ± 10.1
9	261.5 ± 6.1	264.3 ± 7.0	258.5 ± 9.8	264.5 ± 9.8
10	291.5 ± 5.1[a]	296.2 ± 5.7[a]	301.5 ± 5.1[b]	321.2 ± 5.7[c]
11	315.5 ± 5.2[a]	320.8 ± 5.5[a]	325.5 ± 5.1[b]	345.8 ± 5.5[c]
12	337.5 ± 4.1[a]	346.3 ± 5.0[a]	352.5 ± 5.1[b]	386.3 ± 5.0[c]
13	338.5 ± 5.5[a]	354.8 ± 13.2[a]	353.5 ± 5.1[b]	394.8 ± 13.2[c]
14	363.5 ± 7.1[a]	370.7 ± 7.3[a]	388.5 ± 5.1[b]	425.7 ± 7.3[c]
15	392.5 ± 5.3[a]	399.2 ± 6.4[a]	417.5 ± 5.1[b]	454.2 ± 6.4[c]
16	410.5 ± 5.1[a]	418.3 ± 7.3[a]	448.5 ± 5.1[b]	486.3 ± 7.3[c]
17	435.5 ± 6.4[a]	445.2 ± 17.2[a]	473.5 ± 6.4[b]	513.2 ± 17.2[c]
18	475.8 ± 11.7[a]	485.2 ± 11.9[a]	509.5 ± 4.4[b]	565.2 ± 11.9[c]
19	496.0 ± 13.3[a]	507.5 ± 14.7[a]	549.7 ± 4.3[b]	587.5 ± 14.7[c]
20	515.8 ± 11.7[a]	520.0 ± 11.0[a]	559.5 ± 10.3[b]	610.0 ± 11.0[c]
21	533.0 ± 12.0[a]	547.5 ± 15.6[a]	581.7 ± 4.4[b]	637.5 ± 15.6[c]

Data are presented as mean ± SD. The body weights of the rats were recorded on a weekly basis. Different letters indicate a significant difference among the groups. The statistical analysis involved conducting one-way ANOVA followed by Bonferroni post hoc analysis. HFD; High-fat diet.

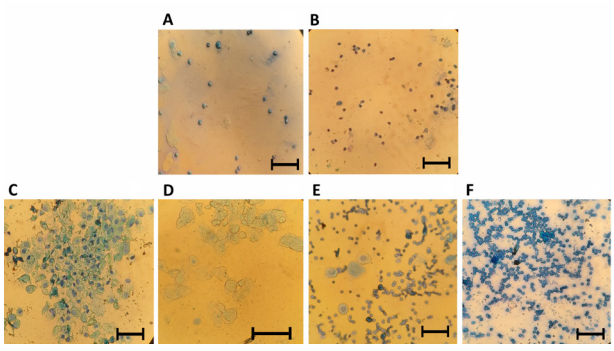

Fig.1: Cellular types in vaginal smears of the experimental groups. **A.** Vaginal smear from the letrozole group. **B.** Vaginal smear from the letrozole+HFD group. Vaginal smears from the HFD and control groups with regular oestrous cycles. **C.** Proestrus. **D.** Estrus. **E.** Metestrus. **F.** Dioestrus (scale bar: 100 µm). HFD; High-fat diet.

Oral glucose tolerance test

There were significant differences in blood glucose levels (BGL) among all the experimental groups at 0, 60, and 120 minutes. The letrozole+HFD group showed significantly higher BGL at 0 minutes, 60 minutes, and 120 minutes compared to the other groups (P=0.00). Additionally, the letrozole and HFD groups exhibited significantly increased BGL at 0, 60, and 120 minutes compared to the control group (P=0.03). BGL of the HFD group at 0 minutes, 60 minutes, and 120 minutes were significantly higher than those of the letrozole group (P=0.00, Table 2).

Homeostasis model assessment of insulin resistance

Homeostasis model assessment of insulin resistance (HOMA-IR) significantly increased in the letrozole+HFD group compared to the other groups (P=0.004). Furthermore, HOMA-IR significantly increased in the letrozole and HFD groups compared to the control group (P=0.03). The HFD group had significantly higher HOMA-IR than the letrozole group (P=0.01, Table 2).

Ovarian morphology

Ovarian sections from the control and HFD groups exhibited several corpus luteous (CL) and follicles at different growth stages, while no CL was observed in the letrozole and letrozole+HFD groups (Fig.2). The ovary weights in both the control and HFD groups were significantly higher than in the letrozole and letrozole+HFD groups (P=0.00). The number of preantral follicles was significantly higher in the letrozole and letrozole+HFD groups compared to the control and HFD groups (P=0.00, Table 2).

There was no significant difference between the control and HFD groups, while the number of preantral follicles in the letrozole+HFD group was significantly higher than in the letrozole group (P=0.00).

No significant difference was observed in the number of antral follicles between the control and HFD groups. However, the number of antral follicles in the letrozole+HFD and letrozole groups was significantly higher than in the control and HFD groups (P=0.009). The CL count in both the control and HFD groups was significantly higher than in the letrozole and letrozole+HFD groups (P=0.00). Additionally, there were significantly less atretic follicles in the control group compared to the other groups (P=0.00). The letrozole+HFD group

exhibited the highest number of atretic follicles (P=0.001), and the number of atretic follicles in the letrozole+HFD group was significantly higher than in the letrozole group (P=0.01).

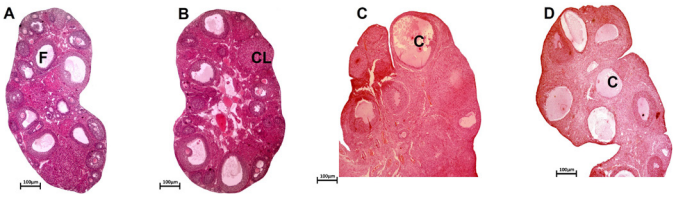

Fig.2: Ovarian histology. Micrographs correspond to the largest section of the haematoxylin-eosin (H&E) stained ovary. **A.** Ovary from the control group. **B.** Ovary from the HFD group. **C.** Ovary from the control letrozole group. **D.** Ovary from the control letrozole+HFD group (scale bar: 100 µm). HFD; High-fat diet, F; Follicle, CL; Corpus Loteum, and C; Cyst.

The mean number of total cystic follicles showed a significant difference among all groups. The control group had a significantly lower number of cystic follicles compared to the other groups. In the letrozole+HFD group, the mean number of total cystic follicles was significantly higher compared to the HFD and letrozole groups (P=0.002). Additionally, the thickness of the follicle wall in the letrozole and letrozole+HFD groups was significantly increased compared to the control and HFD groups. However, the thickness of the follicle wall in the letrozole+HFD group showed a significant decrease compared to the letrozole group (P=0.013).

Hormone assay

Oestrogen levels were significantly lower in the letrozole and letrozole+HFD groups compared to the control and HFD groups (P=0.004). There was no significant difference between the letrozole and letrozole+HFD groups (P=0.001). Additionally, there was no significant difference in oestrogen levels between the control and HFD groups. Progesterone levels were significantly decreased in the letrozole and letrozole+HFD groups compared to the control and HFD groups (P=0.009). No significant difference was observed in progesterone levels between the control and HFD groups. The same pattern was observed for the letrozole and letrozole+HFD groups. Serum testosterone was significantly higher in the letrozole+HFD group compared to the other groups (P=0.001). Testosterone levels did not significantly change in the HFD group compared to the control group. However, testosterone levels were significantly increased in the letrozole group compared to the control group (P=0.03, Table 2).

Lipid profile assay

TC and LDL levels in the letrozole+HFD group were significantly higher than in the other groups (P=0.001). There was no significant difference between the letrozole and HFD groups in terms of TC and LDL levels. The TC and LDL levels in the control group were significantly lower than in the other groups (P=0.006). Additionally, there was no significant difference among HDL levels (P=0.004, Table 2).

C-reactive protein assay

CRP levels were significantly increased in the letrozole+HFD group compared to the other groups (P=0.014). Furthermore, CRP levels were significantly higher in the letrozole and HFD groups compared to the control group (P=0.001). However, CRP levels in the HFD group were significantly lower than in the letrozole group (P=0.006, Table 2).

Table 2: Ovarian morphological parameters, lipid and hormonal profile, and CRP of the experimental groups

Groups	Control	Letrozole	HFD	Letrozole+HFD
Ovary weight (Kg)	0.15 ± 0.02^{a}	0.27 ± 0.05^{b}	0.16 ± 0.04^{a}	0.25 ± 0.02^{b}
Preantral follicle	4.50 ± 1.05^{a}	9.50 ± 1.05^{b}	5.17 ± 0.98^{a}	14.00 ± 2.00^{b}
Antral follicle	3.00 ± 0.63^{a}	0.67 ± 0.52^{b}	3.00 ± 1.10^{a}	0.50 ± 0.55^{b}
Corpus luteum	4.17 ± 1.17^{a}	0.00 ± 0.00^{b}	1.33 ± 0.52^{c}	0.00 ± 0.00^{b}
Atretic follicles	0.83 ± 0.75^{a}	6.83 ± 1.17^{b}	2.17 ± 0.75^{c}	11.67 ± 1.63^{d}
Cystic follicles	0.50 ± 0.55^{a}	5.67 ± 1.033^{b}	3.33 ± 1.03^{c}	11.83 ± 1.06^{d}
Thickness of follicle wall (µm)	61.5 ± 7.31^{a}	121.83 ± 7.65^{b}	68.00 ± 3.63^{a}	135.83 ± 14.80^{b}
Oestrogens (pg/ml)	13.5 ± 3.08^{a}	4.67 ± 1.86^{b}	15.17 ± 4.58^{a}	5.50 ± 2.07^{b}
Testosterone (pg/ml)	0.19 ± 0.04^{a}	0.33 ± 0.05^{b}	0.19 ± 0.04^{a}	0.46 ± 0.05^{c}
Progesterone (ng/ml)	53.33 ±7.20^{a}	23.50 ± 6.57^{b}	54.33 ± 8.94^{a}	22.33 ± 5.47^{b}
TC (mmol/ml)	2.28 ± 0.35^{a}	2.95 ± 0.32^{b}	3.27 ± 0.27^{b}	3.89 ± 0.25^{c}
HDL (mmol/ml)	1.18 ± 0.19	1.03 ± 0.35	1.10 ± 0.34	1.05 ± 0.50
LDL (mmol/ml)	0.30 ± 0.04^{a}	0.43 ± 0.030^{b}	0.44 ± 0.03^{b}	0.50 ± 0.03^{c}
CRP (pg/ml)	14.02 ± 1.28^{a}	21.68 ± 1.03^{b}	17.35 ± 0.82^{c}	30.36 ± 1.44^{d}
HOMA-IR	5.09 ± 0.17	7.85 ± 0.40	12.48 ± 1.28	14.64 ± 1.17
BGL at 0 minute	5.93 ± 0.26	6.77 ± 0.25	7.43 ± 0.17	7.82 ± 0.12
BGL at 60 minutes	6.93 ± 0.25	8.22 ± 0.24	9.37 ± 0.25	10.92 ± 0.21
BGL at 120 minutes	6.13 ± 0.26	7.27 ± 0.25	8.23 ± 0.2	8.82 ± 0.12

Values are presented as mean ± SD. One-way ANOVA was performed for data analysis. A P<0.05 indicates statistical significance. Different letters are used to indicate significant differences within the same row. HFD; High-fat diet, HOMA-IR; Homeostasis model assessment of insulin resistance, TC; Total cholesterol, LDL; Low-density lipoprotein, HDL; High-density lipoprotein, BGL; Blood glucose levels, and CRP; C-reactive protein.

Gene expression analysis

We evaluated the mRNA transcripts of *Tnf-α*, *Wnt4*, *Il-1β*, and *Mcp-1* as they are involved in ovarian tissue inflammation (Fig.3). The relative mRNA levels of *Tnf-α* showed significant differences among the experimental groups (P=0.003). *Tnf-α* mRNA levels significantly increased in the letrozole+HFD group compared to the other groups (P=0.012), while there was no significant difference between the letrozole and HFD groups. Additionally, the expression level of *Tnf-α* mRNA showed a significant increase in the experimental groups compared to the control group (P=0.009). Relative expression levels of *Il-1β* increased significantly in the letrozole+HFD group compared to the other groups (P=0.009). However, there was no significant difference between the letrozole and HFD groups. Furthermore, the expression levels of *Mcp-1* mRNA in the letrozole+HFD group significantly increased compared to the other groups (P=0.012), while there was no significant difference between the letrozole and HFD groups. The expression level of *Mcp-1* mRNA in the control group was significantly lower compared to the other groups (P=0.001).

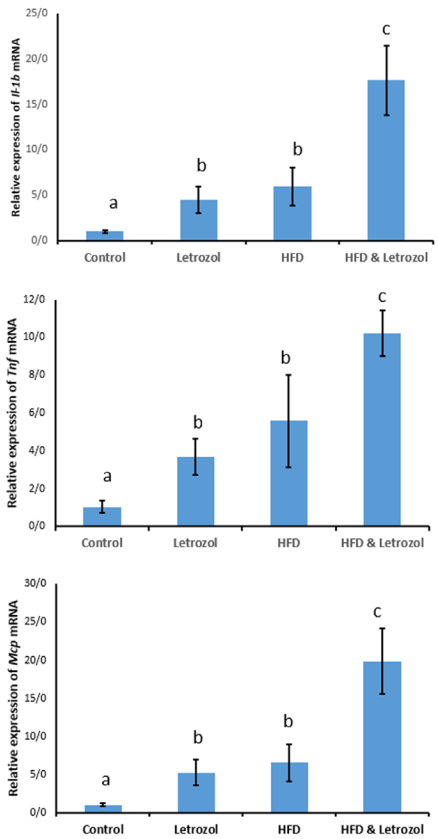

Fig.3: Relative mRNA expression levels of *Il-1β*, tumour necrosis factor-alpha (*Tnf-α*), and *Mcp-1*. The data are presented as mean ± SD, obtained from three independent experiments. P<0.05 indicate statistical significance. Different letters indicate significant differences among the groups. HFD; High-fat diet.

Evaluation of oxidative status

Figure 4 shows the OS parameters in the experimental groups. ROS significantly increased in the letrozole+HFD group compared to the other groups (P=0.002). In the letrozole group, there was a significant increase in ROS levels compared to the HFD group (P=0.006), while both groups had significantly higher ROS levels than the control group (P=0.003). TAC levels significantly decreased in the experimental groups compared to the control group (P=0.009). Furthermore, TAC levels were significantly lower in the letrozole+HFD group compared to the HFD and letrozole groups (P=0.002). Additionally, TAC levels were significantly lower in the letrozole groups compared to the HFD group (P=0.001).

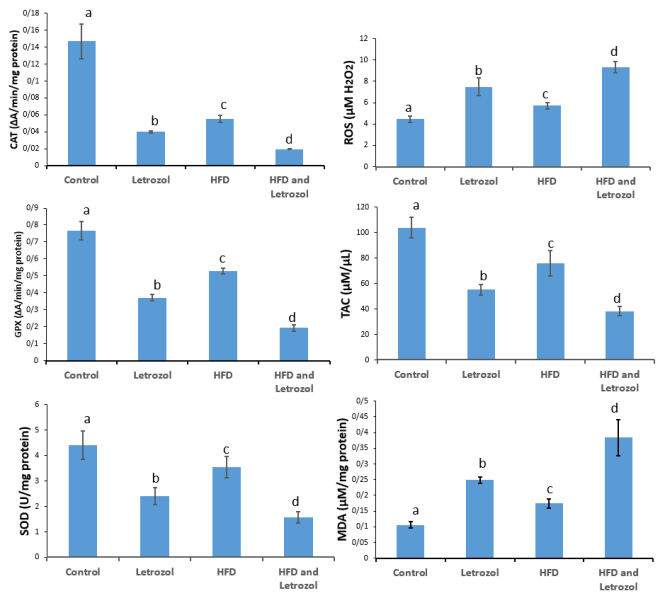

Fig.4: Oxidative stress (OS) parameters in ovarian tissue of the experimental groups. The results are presented as mean ± SD. Different letters indicate significant differences between groups (P<0.05). CAT; Catalase, ROS; Reactive oxygen species, GPX; Glutathione peroxidase, TAC; Total antioxidant capacity, SOD; Superoxide dismutase, and MDA; Malondialdehyde.

The concentrations of MDA in the letrozole+HFD group were significantly higher than in the other groups (P=0.006). Similarly, the MDA concentration was significantly lower in the HFD group compared to the letrozole groups, while both groups had significantly higher MDA levels than the control group (P=0.003).

CAT, GPX, and SOD were significantly decreased in the letrozole+HFD group compared to the other groups (P=0.003). Conversely, the activities of CAT, GPX, and SOD were significantly higher in the control group compared to the other groups (P=0.005). CAT, GPX, and SOD in the letrozole and HFD groups were significantly lower compared to the control group (P=0.006), while the activities of CAT, GPX, and SOD were significantly higher in the HFD group compared to the letrozole group (P=0.001).

Discussion

The results of the present study align with previous studies conducted in rats regarding the animal model characteristics and ovarian and endocrine changes associated with PCOS (8, 19). However, the novelty of

this study lies in the utilisation of a new diet regimen combined with letrozole in the rat model, as well as the investigation of inflammatory and oxidative profiles. This study successfully established a rat PCOS model that used a new HFD in conjunction with letrozole, which resulted in the manifestation of PCOS characteristics observed in humans.

Several studies have examined the reproductive and metabolic characteristics of rodent models of PCOS. HFD induce various metabolic changes in animals. The combination of androgens with HFD can induce a PCOS model that exhibits both ovarian and metabolic characteristics of PCOS (9). Vaginal smear analysis is widely accepted as a key indicator of ovarian physiological function (13). In the PCOS rat model, acyclic vaginal smears were observed, which indicated disrupted oestrous cycles in the letrozole+HFD group. Hormonal profiles were also disrupted and led to morphological changes in the ovarian tissues. Histopathological assessment revealed a high number of cystic follicles with thickened walls, attributed to the thickened theca cell layer, as well as a decrease in the corpus luteum, which was consistent with findings from other studies (9).

The PCOS rats exhibited a significant increase in body weight. In recent years, the role of nutrition in PCOS has been recognized in reproductive research, particularly the strong association between obesity and PCOS. Obese individuals are more susceptible to menstrual irregularities, hyperandrogenism, and hirsutism (12). Therefore, diet plays a crucial role in the incidence and severity of PCOS (20). HFD can exacerbate the effects of androgens, which leads to glucose intolerance and insulin resistance (8). The results of the present study support previous findings where HFD and letrozole significantly contributed to weight gain (19). Overweight individuals are widely known to experience hyperandrogenism, anovulation, and are at increased risk for metabolic syndrome (8, 9), which aligns with the results of the present study. Hyperandrogenism is a major factor responsible for abnormal ovarian physiology and a prominent characteristic of PCOS, often leading to irregular reproductive cycles. In this study, letrozole+HFD-treated rats exhibited anovulation and hyperandrogenism. Therefore, the new HFD diet combined with letrozole shows promise in inducing a PCOS model by impairing glucose intolerance, insulin resistance, and hormonal profiles.

Letrozole inhibits the conversion of androgens to oestrogens in the ovary (8, 10, 19), which results in decreased oestrogen and progesterone levels and increased testosterone levels (7). The hormonal changes observed in the letrozole and letrozole+HFD-treated rats in this study support these findings. The hormonal alterations in HFD and letrozole+HFD-treated rats could be attributed to insulin resistance and hyperinsulinemia. Insulin activates the inositol phosphoglycan pathway in theca cells by reducing hepatic sex hormone-binding globulin production, enhancing the effect of LH on theca cells, and ultimately causing hyperandrogenism (19). Moreover, hyperinsulinemia disrupts gonadotropin secretion and increases LH receptor expression, which stimulates the ovaries to produce androgens, and leads to impaired follicular growth and anovulation. The findings of our study are in line with previous research that support the role of insulin resistance, hyperandrogenism, and abnormal gonadotropin secretion as key factors in the pathophysiology of PCOS. However, there is an ongoing debate of whether insulin resistance precedes the other criteria. The insulin-gonadotropin-like activity affects ovarian steroidogenesis, disrupts insulin signalling in the brain, and ultimately impacts ovulation and body weight. Therefore, an assessment of glucose intolerance using the OGTT method is considered more useful than measuring fasting glucose levels alone when evaluating PCOS patients (21). In our study, the results indicate that the OGTT test can effectively reflect insulin resistance, which is consistent with previous findings (10). Feeding adult female rats with an HFD for 120 days can induce insulin resistance and infertility. Our results suggest that letrozole+HFD treatment may have a more direct effect on insulin signalling pathways compared to letrozole alone. However, describing the underlying mechanisms will be a challenge for future studies.

Another aspect examined in our study was the lipid profile. Both HFD and letrozole exhibited abnormal lipoprotein profiles. It is estimated that 70% of PCOS patients have abnormal serum lipid levels. Insulin resistance is believed to be responsible for reducing the activity of lipoprotein lipase and consequently causing dyslipidaemia (19). In our study, both HFD and letrozole, either alone or in combination, increased LDL and TG levels. Elevated LDL and TG levels have also been reported in HFD-treated C57BL/6 mice (22). In letrozole-treated rats, higher TG and LDL levels were observed, and this supported the results of a study where letrozole increased both TG and TC levels (10). In contrast, another study reported no effect on the lipid profile (7, 11).

Objectives of the present study included the evaluation of OS and inflammatory markers in a rat model of PCOS and an investigation of the role of nutrition and obesity in the pathogenesis of PCOS. Growing evidence suggests that inflammation plays a significant role in the development of PCOS. Persistent moderately elevated levels of CRP are characteristic of low-grade chronic inflammation, which is a systemic and chronic condition. PCOS is considered to be part of this inflammatory group. Based on this, our study aimed to examine whether gene expression of inflammatory markers and CRP levels increased in the PCOS rat model. Low-grade chronic inflammation has been associated with insulin resistance syndrome. Our study showed a significant increase in CRP levels in the HFD, letrozole, and letrozole+HFD groups compared to the control group that had normal oestrous cycles. This finding supported with previous data that demonstrated a correlation between CRP levels, obesity, and insulin sensitivity (23). Adipose tissue-

derived cytokine expression is believed to play a key role in low-grade chronic inflammation (1), which aligns with our results of increased gene expressions of inflammatory markers in the HFD, letrozole, and letrozole+HFD groups compared to the control group. In other words, visceral adipose tissue may contribute to the features of low-grade chronic inflammation (1). These observations also support the correlation between insulin sensitivity and CRP reported by Festa et al. (23).

The involvement of OS in PCOS is well-established. Studies show that diet-induced OS, documented by increased ROS production and NFkB activation, leads to an inflammatory response (24). Glucose absorption can affect the production and secretion of Tnf-α and IL-6 from circulating monocytes in PCOS. These findings are consistent with the results of our study. Furthermore, markers of OS are associated with the insulin resistance index (2, 24), which aligns with our observation of changes in the oxidative profile and insulin resistance index in the PCOS rat model. Additionally, hyperandrogenaemia during PCOS can trigger an inflammatory response induced by diet. Administration of oral androgens has been shown to activate mononuclear cells, leading to ROS production, NF/kB activation, and increased Tnf-α mRNA. Therefore, OS may contribute to the induction and/or exacerbation of PCOS (6).

Conclusion

The present study results demonstrate that the combination of a new HFD and letrozole is suitable for studying both the ovarian and metabolic features of PCOS and may be useful for evaluating new treatments. In addition to ovarian changes, this regimen closely mimics clinical PCOS by inducing OS, inflammation, and metabolic disorders in rats.

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Authors' Contributions

S.F.M.; Performed the experiments, meticulously executing the planned procedures and ensuring the accurate collection of data. S.Z.; Played in the inception and design of the experiments, demonstrating a profound understanding of techniques, drafted the paper, crafting a comprehensive and insightful manuscript that presented the research findings and their implications coherently. M.N.; Diligently analyzed the data, employing sophisticated statistical methods to draw meaningful conclusions from the experimental results. Their expertise in data analysis provided valuable insights into the observed trends and correlations. H.H.-M.; Contributed by providing essential reagents, materials, and analysis tools that were crucial to the successful execution of the experiments. Their expertise and support significantly enhanced the experimental process. All authors read and approved the final manuscript.

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