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Fenofibrate ameliorates letrozole-induced polycystic ovary in rats *via* modulation of PPARα and TNFα/CD95 pathway

M.A. MORSY[1,2], M. EL-HUSSIENY[3], N.M. ZENHOM[4], A.B. NAIR[1], K.N. VENUGOPALA[1,5], M.M.M. REFAIE[2]

[1]Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia
[2]Department of Pharmacology, [3]Department of Pathology, [4]Department of Biochemistry, Faculty of Medicine, Minia University, El-Minia, Egypt
[5]Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

Abstract. – OBJECTIVE: Polycystic ovary syndrome (PCOS) is a prevalent endocrine health problem during the childbearing period that seriously affects fertility in females. Fenofibrate, a peroxisome proliferator-activated receptor-alpha (PPARα) agonist, showed beneficial effects in models of endocrine disturbances. Thus, we evaluated the potential therapeutic effect of fenofibrate in experimental PCOS.

MATERIALS AND METHODS: Rats received oral fenofibrate (300 mg/kg/day) for three weeks following a three-week PCOS induction regimen using oral letrozole (1 mg/kg/day). We determined the changes in body weight, levels of serum testosterone, insulin, anti-Müllerian hormone (AMH), ovarian malondialdehyde (MDA), superoxide dismutase (SOD), and tissue tumor necrosis factor-alpha (TNFα) and CD95 protein expressions. The tissue expression of interleukin-10 (IL10) and PPARα genes was determined.

RESULTS: Letrozole-treated rats showed successful induction of PCOS, confirmed by histopathology and significantly increased body weight, testosterone, insulin, AMH, and MDA, and decreased SOD. Ovaries of untreated PCOS rats showed increased TNFα and CD95 and decreased PPARα and IL10 expression. Administration of fenofibrate ameliorated the letrozole-induced PCOS changes.

CONCLUSIONS: Fenofibrate-mediated amelioration of PCOS in rats is attributed partly to its antioxidant, anti-inflammatory, and anti-apoptotic properties and activation of PPARα.

Key Words:
Fenofibrate, Polycystic ovary syndrome, PPARα, TNFα, CD95, Anti-Müllerian hormone.

Introduction

Polycystic ovary syndrome (PCOS) is a common gynecological health problem that affects about 6-22% of females in the childbearing period. Patients usually present with hyperandrogenism, anovulatory cycles, and insulin resistance. Accumulating evidence implicates insulin resistance in the pathogenesis of PCOS by increasing ovarian androgen production in response to metabolic disturbances[1,2]. Other mechanisms include increased oxidative stress, which contributes to the initiation and progression of PCOS. Reactive oxygen or nitrogen species, when at normal levels, regulate several physiologic functions in female reproduction, such as oocyte maturation, folliculogenesis, steroidogenesis, corpus luteal function, and luteolysis. However, increased oxidative stress may contribute to follicular atresia and diminish the quantity and quality of oocytes[3,4]. Induction of oxidative stress stimulates the excessive release of inflammatory mediators, initiating tissue inflammation and disturbing the apoptotic pathway. Tumor necrosis factor-alpha (TNFα) is a multifunctional cytokine that influences the ovarian reproductive axis, inducing changes that closely resemble those found in PCOS[3,5].

Alterations in the female ovarian microenvironment could alter the normal physiological processes of programmed cell death and proliferation inside the ovaries. Regulation of apoptosis in follicular cells depends on the interplay between different autocrine and paracrine factors, which can be achieved via regulation of the CD95

Corresponding Author: Mohamed A. Morsy, Ph.D; e-mail: momorsy@kfu.edu.sa

(also known as Fas) system and the Bcl-2 family[6,7]. A delicate balance between the apoptotic and survival factors determines whether follicles continue developing or undergo atresia.

Peroxisome proliferator-activated receptors (PPARs) are a group of widely distributed nuclear receptors, including PPARα, PPARβ/δ, and PPARγ. These receptors regulate multiple cellular processes, such as lipoprotein metabolism, glucose homeostasis, immune response, cellular proliferation, and differentiation[8]. Furthermore, PPARα increases the gene expression of different antioxidant enzymes, improving the redox state and repressing inflammation and apoptosis[9]. Disordered fatty acid metabolism and insulin resistance are characteristics of defective PPARα signaling.

Several studies[10-16] showed the regulatory role of PPARs outside and inside the ovaries; they are crucial for maintaining normal metabolic functions and physiological processes and are promising targets in many pathological disorders. In contrast, PCOS increases the formation of free radicals and inflammatory mediators and stimulates the apoptotic cascade[4,5]. Fenofibrate, a specific ligand of PPARα, is commonly used to treat hyperlipidemia[10]. Moreover, fenofibrate shows pleiotropic effects, which are beneficial in many diseases[11-13]. Therefore, the current study aimed to evaluate the role of the PPARα agonist fenofibrate on letrozole-induced PCOS in female rats and to explore the different mechanisms involved in such effect, including the TNFα/CD95 pathway.

Materials and Methods

Drugs and Chemicals

Fenofibrate was from Minapharm Pharmaceuticals (Cairo, Egypt). Testosterone, insulin, and anti-Müllerian hormone (AMH) ELISA kits were from US Biological (Salem, MA, USA). Polyclonal TNFα and CD95 antibodies were from Thermo Fisher Scientific (Waltham, MA, USA).

Animals and Experimental Protocol

Six-week-old female Wistar albino rats from the National Research Center (Giza, Egypt), weighing 250-300 g, were kept in standard housing conditions and left to acclimatize for one week with free access to ordinary chow and water. This work was conducted in the Department of Pharmacology, Faculty of Medicine, Minia University, Egypt. The Faculty of Medicine board approved the experimental protocol (Approval No. 717:12/2020) following the EU directive 2010/63/EU.

The study comprised four randomly divided experimental groups (n = 7): Group I received oral 0.5% carboxymethylcellulose in water; Group II received oral fenofibrate (300 mg/kg/day)[12,17]; Group III received letrozole (1 mg/kg/day, orally)[18-20] for induction of PCOS, while in Group IV animals received combined oral treatment of letrozole (1 mg/kg/day) and fenofibrate (300 mg/kg/day). All treatment protocols continued for three weeks.

Sample Preparation

At the end of the three-week study protocol, animals were weighed and euthanized, then the ovaries of each rat were excised through an abdominal incision. Next, the ovaries were washed, weighed, and homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4). The homogenate was centrifuged at 4000 rpm for 15 min at 4°C, and the supernatant was kept at -80°C till used.

Determination of Serum Testosterone, Insulin, and AMH

BioAssay colorimetric ELISA kits were used to determine serum testosterone, insulin, and AMH, following the instructions by the manufacturers.

Determination of Malondialdehyde (MDA)

The oxidative stress marker MDA, the main decomposition product of lipid peroxidation, reacts with thiobarbituric acid forming a pink chromogen, which is measured spectrophotometrically at 535 nm and expressed as nmol/g tissue[21].

Determination of Superoxide Dismutase (SOD)

The activity of ovarian SOD was determined based on its ability to inhibit the oxidation of pyrogallol. The SOD unit is the amount of enzyme that inhibits 50% of pyrogallol oxidation. The absorbance of the sample was measured at 420 nm over 3 min, and SOD activity was expressed as unit/g tissue[22].

Histopathological Evaluation

Immediately after euthanization, the ovaries were dissected, weighed, fixed in 10% formalin for 24 h, embedded in paraffin wax, and sectioned

at 5 μm. Sections were stained with hematoxylin and eosin and blindly examined under a light microscope (Olympus CX23LEDRFS1, Olympus, Tokyo, Japan). The number of cystic follicles and corpora lutea of rat ovaries of various groups was determined for semiquantitative analysis.

Immunohistochemical Examination

Sections were placed on positively charged slides, deparaffinized in xylene, and rehydrated through descending graded alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigens were retrieved using sodium citrate buffer (pH 6.0) and boiled in a microwave. Primary antibodies against TNFα or CD95 (dilution 1:100 and 1:50, respectively) were applied and incubated overnight in a humidity chamber. Tissue sections were washed, treated with the secondary antibody for 30 min, and then 3,3′-diaminobenzidine was added to develop a brown color reaction. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted.

Examination of sections was done under light microscope magnification ×200. The cytoplasmic TNFα reactivity was evaluated by considering the percentage of positive cells as follows: 0, no stained cells; 1, ≤25% stained cells; 2, >25% and ≤50% stained cells; 3, >50% and ≤75% stained cells; and 4, >75% stained cells[23]. The expression of CD95 was graded as follow: 0, no positive cells; 1, = ≤10% positive cells; 2, >10 to 40% positive cells; 3, >40 to 70% positive cells; 4, >70 positive cells[24].

Real-Time Polymerase Chain Reaction (PCR)

We used the RiboZol RNA extraction reagent (AMRESCO, Solon, OH, USA) to extract the total RNA from the homogenized ovarian specimens following the manufacturer's instructions. The Revert Aid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was used to synthesize cDNAs. The ovarian expression of interleukin-10 (IL10) and PPARα was determined by real-time PCR (Real-Time PCR Detection System, Kapa Biosystems, Wilmington, MA, USA) using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific). For IL10, forward primer: 5′-AAAGCAAGGCAGTGGAGCAG-3′, and the reverse primer: 5′-TCAAACTCATTCATGGCCTTGT-3′[25]; for PPARα, the forward primer: 5′-ACGATGCTGTCCTCCTTGATG-3′, and the reverse primer: 5′-GCGTCTGACTCGGTCTTCTTG-3′[26]; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the forward primer: 5′-GTCGGTGTGAACGGATTTG-3′ and reverse primer: 5′-CTTGCCGTGGGTAGAGTCAT-3′[27] were added to the reaction mixture. The relative expression of IL10 or PPARα was calculated using the comparative cycle threshold method[28]. The results for all experimental samples were graphed as the relative expression compared with the control gene (GAPDH).

Statistical Analysis

The results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Dunnett multiple comparison test. GraphPad Prism software (version 5; La Jolla, CA, USA) was used for statistical analysis. The results were significant when the calculated *p*-value was less than 0.05.

Results

Effect of Fenofibrate on Rat Bodyweight Changes and Serum Testosterone, Insulin, and AMH

The PCOS group showed significant increases in rat body weight and the serum PCOS biomarkers: testosterone, insulin, and AMH compared with the vehicle-treated negative control group. Administration of fenofibrate significantly decreased all the measured parameters compared with the untreated PCOS group (Table I).

Histopathological Findings

Neither the control nor the fenofibrate group showed any histopathological changes in the ovaries. The ovaries of these groups showed normal follicular growth distribution with incidental cystic follicles, and visible corpora lutea, indicating ovulation. The newly formed corpora lutea were superficially located in the ovary and composed of large, polygonal, and finely vacuolated luteal cells.

In the untreated letrozole-induced PCOS group, all ovaries were acyclic, showing multiple large cystic follicles lined by one to multiple layers (1-4 cell layers) of cuboidal or flattened granulosa cells resting on a thin wall and encircled by theca cells. At the same time, no newly formed corpora lutea were observed in the ovaries, indicating ovulation arrest. While in the fenofibrate-treated PCOS group, unremarkable numbers of cystic follicles were observed, but there were corpora

Table I. Effect of fenofibrate (FN) on bodyweight changes and serum testosterone, insulin, and anti-Müllerian hormone (AMH) levels in letrozole-induced polycystic ovary syndrome (PCOS).

Group	Body weight changes (g)	Testosterone (ng/ml)	Insulin (ng/ml)	AMH (ng/ml)
Control	54 ± 2.6	0.16 ± 0.01	0.3 ± 0.02	1.8 ± 0.04
FN	50 ± 2.4	0.17 ± 0.01	0.3 ± 0.03	1.9 ± 0.03
PCOS	92 ± 4.0[a]	2.30 ± 0.09[a]	1.8 ± 0.05[a]	6.2 ± 0.4[a]
PCOS+FN	78 ± 4.5[a,b]	0.39 ± 0.05[a,b]	1.2 ± 0.07[a,b]	3.0 ± 0.3[b]

Values are representations of 7 observations in each group as mean ± SEM. Results are considered significantly different when $p < 0.05$. [a]Significant difference compared to the control group and [b]significant difference compared to the untreated PCOS group.

lutea, indicating the occurrence of ovulation. Besides, a significant increase in the number of corpora lutea and a significant reduction in the number of cystic follicles were observed in this group compared with the untreated PCOS group (Figure 1 and Table II).

Effect of Fenofibrate on Ovarian MDA and SOD

In the PCOS group, the oxidative stress marker MDA level significantly increased while the antioxidant enzyme SOD activity significantly decreased compared with the control group. On the

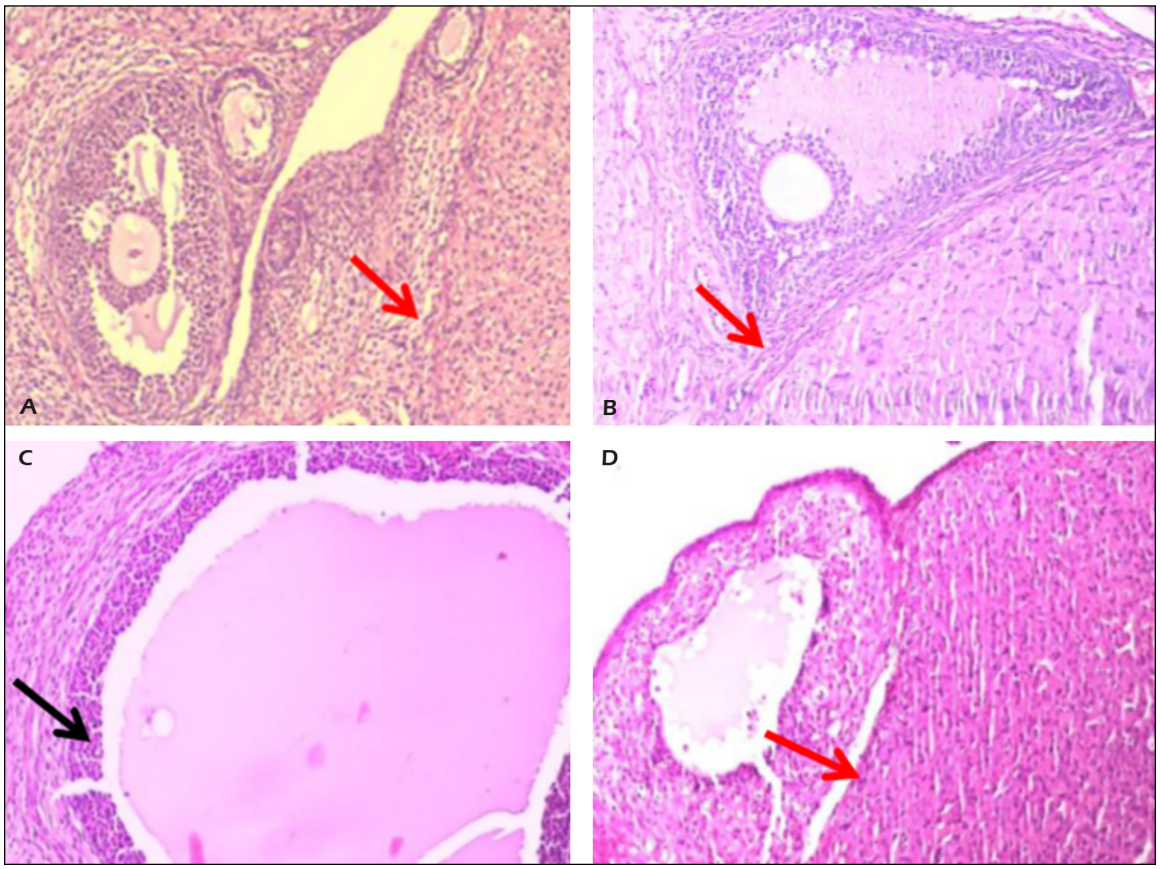

Figure 1. Histopathological evaluation of rat ovaries (hematoxylin and eosin, ×200). **A-B,** Control and fenofibrate group featuring normal ovarian architecture. There was normal follicular growth distribution. Corpora lutea were seen, indicating ovulation. Newly formed corpora lutea are superficially located in the ovary and composed of large polygonal finely vacuolated luteal cells (*red arrow*). **C**, The polycystic ovary syndrome (PCOS) group with dilated cystic follicles lined by a few layers of cells (*black arrow*). **D**, The PCOS+fenofibrate group with corpora lutea indicated ovulation (*red arrow*).

Table II. The number of cystic follicles and corpora lutea of rat ovaries.

Group	Cystic follicles	Corpora lutea
Control	0.3 ± 0.2	6 ± 0.4
FN	0.4 ± 0.2	6 ± 0.4
PCOS	8.1 ± 0.5[a]	1 ± 0.2[a]
PCOS+FN	2 ± 0.2[a,b]	5 ± 0.4[b]

Values are representations of 7 observations in each group as mean ± SEM. Results are considered significantly different when $p < 0.05$. [a]Significant difference compared to the control group and [b]significant difference compared to the polycystic ovary syndrome (PCOS) group. FN: fenofibrate.

other hand, the PCOS+fenofibrate group showed a significant decrease in MDA level and a significant increase in SOD activity compared with the PCOS group (Table III).

Immunohistochemical Evaluation of TNFα Expression

In the control and fenofibrate groups, almost negative TNFα expression was observed in granulosa cells of ovarian follicles and corpora lutea. However, the expression of this cytokine was strong in the cells of cystic follicles and ovarian follicles of the PCOS group. Instead, slightly positive TNFα expression was detected within the granulosa cells of ovarian follicles and corpora lutea in the PCOS+fenofibrate group (Figure 2). Semiquantitative analysis of ovarian TNFα expression showed a significant increase in the PCOS group compared with the control group, while the PCOS+fenofibrate group showed a significant decrease in its expression (Figure 2).

Table III. Effect of fenofibrate (FN) on malondialdehyde (MDA) and superoxide dismutase (SOD) in letrozole-induced polycystic ovary syndrome (PCOS).

Group	MDA (nmol/g tissue)	SOD (unit/g tissue)
Control	27 ± 1.6	9213 ± 204
FN	25 ± 1.5	8557 ± 380
PCOS	72 ± 5.8[a]	3999 ± 368[a]
PCOS+FN	44 ± 2.7[a,b]	7229 ± 370[a,b]

Values are representations of 7 observations in each group as mean ± SEM. Results are considered significantly different when $p < 0.05$. [a]Significant difference compared to the control group and [b]significant difference compared to the polycystic ovary syndrome (PCOS) group. FN: fenofibrate.

Immunohistochemical Evaluation of CD95 Expression

Ovaries of the control and fenofibrate groups were almost negative for CD95 expression. The untreated PCOS ovaries showed positive expression of CD95 within the granulosa cells lining the cystic follicles and trivial positivity within the surrounding follicles, which was significantly attenuated in the fenofibrate-treated PCOS group (Figure 3). Furthermore, semiquantitative analysis of ovarian CD95 expression showed a significant increase in CD95 in the PCOS group compared with the control and the PCOS+fenofibrate groups (Figure 3).

Real-Time PCR of IL10 and PPARα Gene Expression

The fold changes of IL10 and PPARα gene expression in different groups were calculated relative to GAPDH as a reference gene to standardize mRNA expression (Figure 4). The results of this experiment revealed significant upregulation of IL10 and PPARα genes in the PCOS+fenofibrate group compared with the PCOS group, which showed significantly downregulated IL10 gene expression compared with the control group. The expression of PPARα was undetectable in the PCOS group.

Discussion

PCOS, a common reproductive hormonal disturbance with systemic-metabolic disorders, affects about 6-22% of reproductive-age women and markedly impairs their fertility. Women with PCOS usually present with several metabolic abnormalities, including obesity, insulin resistance, hyperinsulinemia, diabetes, dyslipidemia, hypertension, and impairment of the normal metabolic processes[1,2]. The mechanisms involved in the pathogenesis of PCOS are far from being well-understood. Besides, finding new therapies for early curative intervention in PCOS patients is necessary. The current study evaluated the role of PPARα agonist fenofibrate in controlling letrozole-induced PCOS and explored the different mechanisms involved in mediating its curative effect. The results showed significant increases in body weight, serum testosterone, insulin and AMH, and ovarian MDA, TNFα, and CD95 and significant decreases in SOD, PPARα, and IL10 in rats with PCOS, with typical histopathological ovarian features

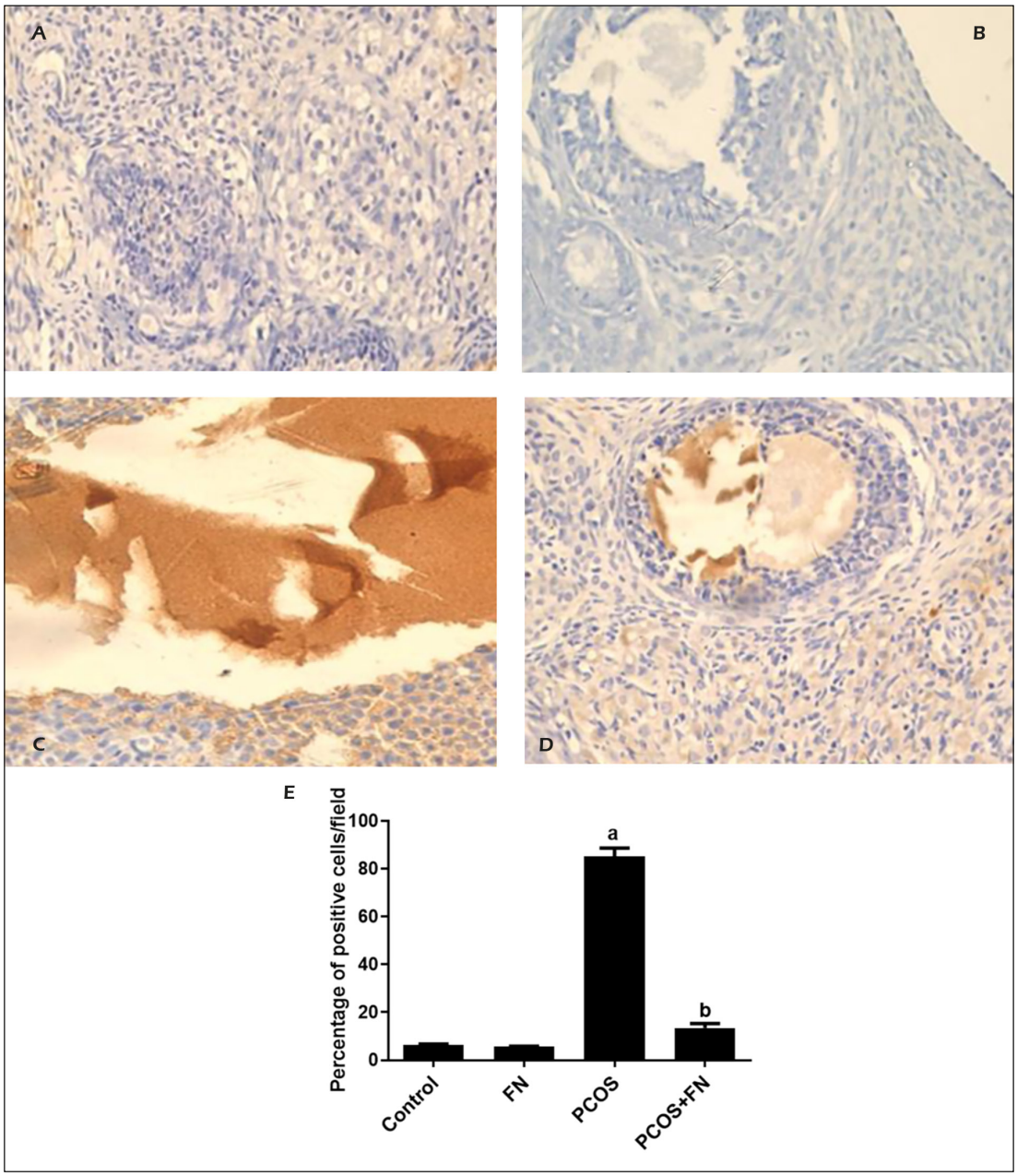

Figure 2. Effect of fenofibrate (FN) on ovarian tumor necrosis factor-alpha (TNFα) immunohistochemical staining in letrozole-induced polycystic ovary syndrome (PCOS) in rats (×200). TNFα expression of rat ovary of (**A**) control, (**B**) FN-treated, (**C**) PCOS, and (**D**) PCOS+FN-treated groups, and (**E**) semiquantitative analysis of TNFα expression. Results are mean ± SEM ($n = 7$) of the percentage of TNFα positive cells/field. [a,b]Significantly different ($p < 0.05$) from control and PCOS groups, respectively.

of PCOS. Importantly, our results showed for the first time that fenofibrate can prevent the pathological changes leading to the development of experimental PCOS.

Insulin resistance is one of the most important contributing factors in the pathogenesis of PCOS. Insulin resistance in PCOS is characterized by obesity, hyperandrogenism, and increased insulin secretion in response to metabolic abnormalities. Hyperinsulinemia, in turn, stimulates fat storage and disturbs cholesterol and lipoprotein metabolism. Furthermore, insulin directly stimulates the steroidogenic enzyme cytochrome P450c17 and promotes the conversion of cholesterol to progesterone and subsequently into androgen. In addition, insulin directly promotes the pituitary

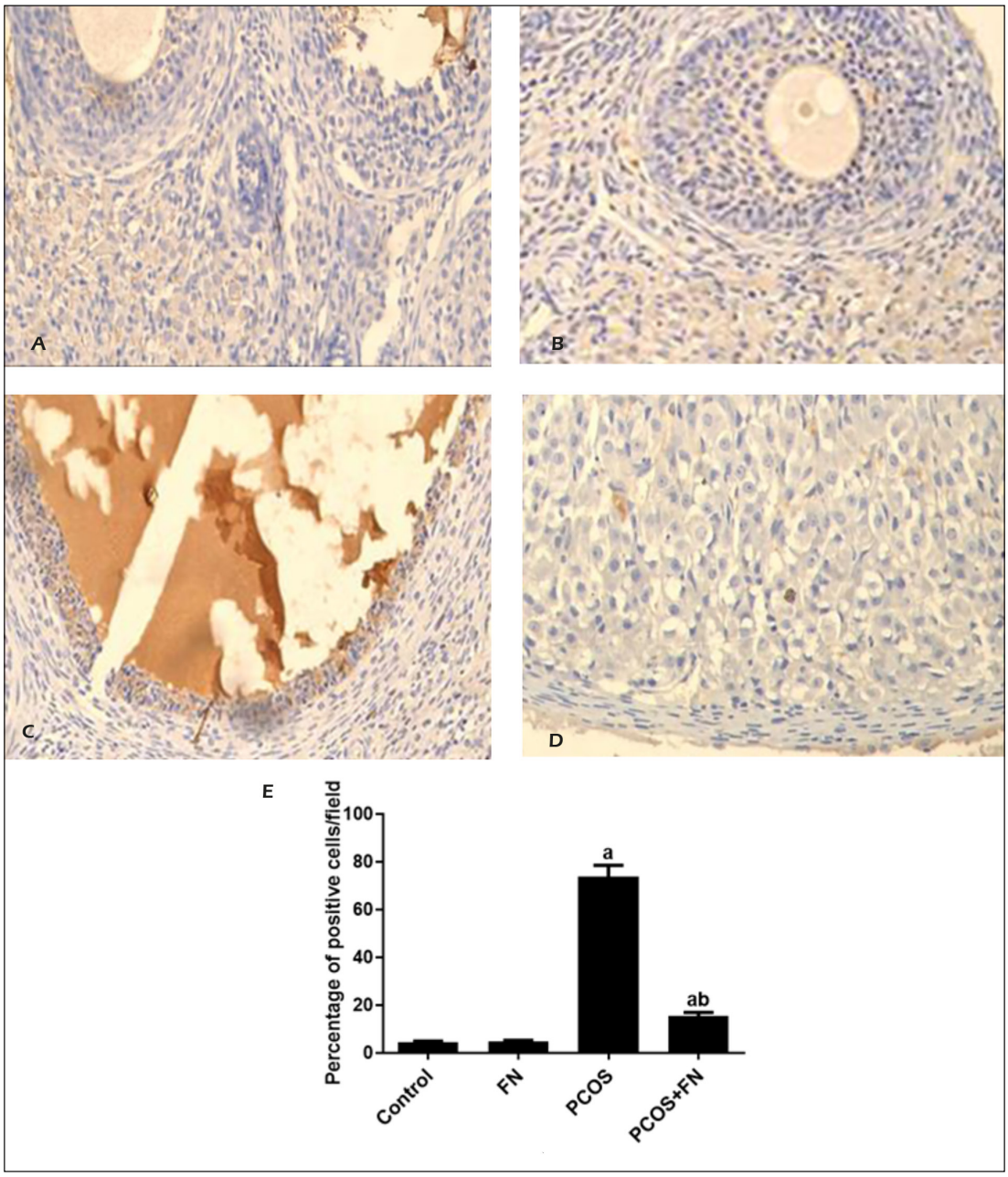

Figure 3. Effect of fenofibrate (FN) on ovarian CD95 immunohistochemical staining in letrozole-induced polycystic ovary syndrome (PCOS) in rats (×200). CD95 expression of rat ovary of (**A**) control, (**B**) FN-treated, (**C**) PCOS, and (**D**) PCOS+FN-treated groups, and (**E**) semiquantitative analysis of CD95 expression. Results are mean ± SEM (n = 7) of the percentage of CD95 positive cells/field. [a,b]Significantly different ($p < 0.05$) from control and PCOS groups, respectively.

secretion of luteinizing hormone, which activates its receptors on theca cells to increase androgen production. On the other hand, abdominal obesity associated with elevated androgen leads to metabolic disorders, promoting more insulin production[1,29,30]. Alternatively, serum AMH, an important marker in PCOS[31], is increased due to the androgen-induced excess of small antral follicles[18]. The present study showed significant increases in the body weight, serum testosterone, insulin, and AMH levels of the letrozole-induced PCOS rats, which is in keeping with the previous findings by others[19,20,32,33]. In agreement with the current results, fenofibrate treatment decreased the body weight and insulin levels in previous studies[34-36]. The ability of fenofibrate to decrease

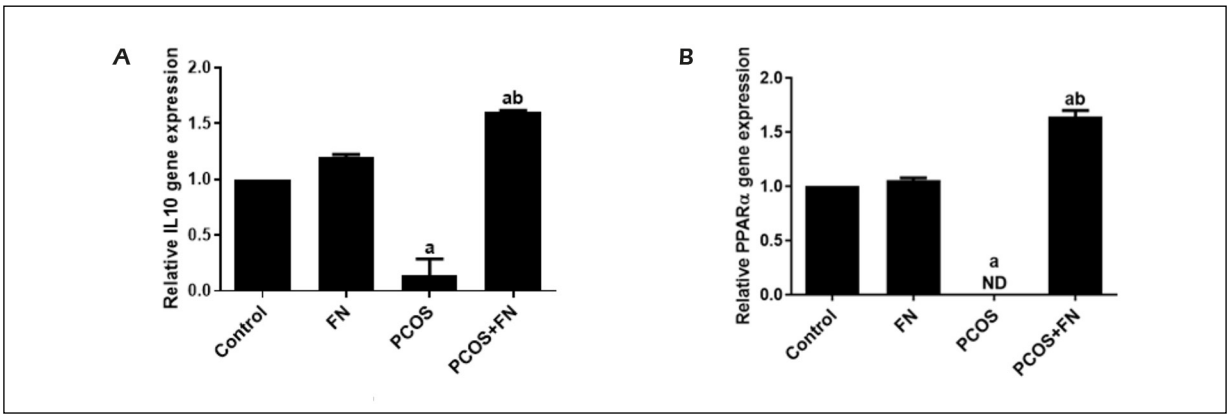

Figure 4. Effect of fenofibrate (FN) on relative gene expression of ovarian (**A**) interleukin-10 (IL10) and (**B**) peroxisome proliferator-activated receptor-alpha (PPARα) in letrozole-induced polycystic ovary syndrome (PCOS) in rats using real-time polymerase chain reaction. Results are mean ± SEM ($n = 7$). [a,b]Significantly different ($p < 0.05$) from control and PCOS groups, respectively. ND: non-detected.

testosterone and AMH seems to be secondary to its ameliorative effect against PCOS.

The increased formation of free radicals, and subsequent disturbance of the oxidant/antioxidant balance, are highly involved in the pathogenesis of PCOS. The limited capacity of the ovaries to resist oxidative stress and the deleterious effects of reactive oxygen species-activated signaling[3-5] are responsible for PCOS-associated follicular atresia, diminished number and quality of oocytes, insulin resistance, obesity, and hyperandrogenism[37-40]. Previous studies of letrozole-induced PCOS[41] showed increased MDA and decreased SOD activities, supporting the current findings. Moreover, in harmony with our results, fenofibrate can modulate these parameters in premature ovarian failure[17].

Induction of inflammation with improper release of several pro-inflammatory cytokines and diminished anti-inflammatory mechanisms is another critical factor in the pathogenesis of PCOS. IL10, an anti-inflammatory and immune-regulating cytokine, can inhibit the synthesis of pro-inflammatory cytokines, including TNFα, and decrease insulin resistance[42]. Moreover, a decrease or polymorphism in IL10 is associated with PCOS; meanwhile, IL10 can reduce PCOS-induced inflammation[43,44]. On the contrary, elevated levels of TNFα were evident in PCOS patients[45,46]. Besides, TNFα stimulates apoptosis in cultured ovarian follicles[47] and promotes insulin resistance and hyperandrogenemia[48], which can contribute to PCOS pathogenesis. The previous reports of increased TNFα in letrozole-induced PCOS[49], and its modulation by fenofibrate in premature ovarian failure[17] support the results of the current study.

The interaction between pro-apoptotic and pro-survival signals in the ovarian tissue dictates cell life or death. Several molecular pathways, including the CD95 system, which involves the interaction of CD95, an apoptosis-inducing receptor, and its ligand CD95L, contribute to this cell death-promoting side of this balance. In healthy women, only one follicle develops into a mature one, followed by ovulation in each menstrual cycle; the remnant follicle undergoes atresia in varying developmental stages. However, in women with PCOS, there are numerous small antral follicles in bilateral ovaries with an inability to produce mature follicles; thus, follicular development arrests, and no appearance of a dominant follicle, with persistent anovulation[50,51]. Estrogen is considered a survival factor for the maintenance of granulosa cells; consequently, inhibition of aromatase increases apoptosis of granulosa cells by reducing the conversion of androgen to estrogen[52]. Similarly, AMH causes follicular atresia in the PCOS, probably by indirectly inhibiting estrogen production[53]. In the current study, decreasing the number of secondary follicles in the fenofibrate-treated PCOS rats may be attributed to lowering AMH levels, which leads to activation of aromatase expression and eventually support folliculogenesis[51,54].

The PPARs are nuclear hormone receptors involved in different cellular functions, including ovarian physiology, as they can bind to estrogen response elements, regulate the expression of

aromatase, and inhibit progesterone production. In addition, these receptors can modulate the expression and activity of proteolytic enzymes that affect tissue remodeling and angiogenesis during follicular development, maturation, ovulation, and luteal formation. PPARs are regulated by different factors such as fibrates, insulin, arachidonic acid, eicosanoid metabolites, thiazolidinediones, and some non-steroidal anti-inflammatory drugs[55,56]. Fenofibrate is a PPARα agonist with antidyslipidemic, anti-inflammatory, antioxidant, and anti-apoptotic effects[9,12,17,57]. These established properties of fenofibrate explain the curative effect of fenofibrate against letrozole-induced PCOS observed in the current study.

Diagnosis of PCOS requires two out of the three Rotterdam criteria: oligo/anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovaries on ultrasound[58]. On the other hand, fenofibrate could ameliorate the PCOS-associated conditions, such as weight gain[35], dyslipidemiam[59], insulin resistance[60], endometrial hyperplasia[61], high blood pressure[62], depression[63], heart problems[64], stroke[65], sleep apnea[66], and non-alcoholic fatty liver disease[67].

Conclusions

Concomitant treatment of female rats with fenofibrate protected the ovary against letrozole-induced PCOS. The current study showed that fenofibrate confers ovarian antioxidant (via decreasing MDA and increasing SOD), anti-inflammatory (via decreasing TNFα and increasing IL10), and anti-apoptotic effects (via decreasing CD95) and an increase in PPARα. Thus, the current results warrant further investigation of the possible curative effects of fenofibrate and its target receptor PPARα in PCOS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Data Availability

Data are contained within the article or available upon reasonable request from the corresponding author.

Ethical Approval

The experimental protocol was approved by the Institutional Research Ethics Committee of Faculty of Medicine, Minia University (Approval No. 717:12/2020).

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Authors' Contribution

Conceptualization, M.A.M. and M.M.M.R.; methodology, M.A.M., M.E., N.M.Z., A.B.N., K.N.V., and M.M.M.R.; formal analysis, M.A.M., M.E., N.M.Z., A.B.N., K.N.V. and M.M.M.R.; writing, M.A.M., M.E., N.M.Z. and M.M.M.R.; funding acquisition, M.A.M. All authors have read and agreed to the published version of the manuscript.

ORCID ID

M.A. Morsy: https://orcid.org/0000-0002-6752-9094;
M. El-Hussieny: https://orcid.org/0000-0002-1756-940X;
N.M. Zenhom: https://orcid.org/0000-0003-3970-784X;
A.B. Nair: https://orcid.org/0000-0003-2850-8669;
K.N. Venugopala: https://orcid.org/0000-0003-0680-1549;
M.M.M. Refaie: https://orcid.org/0000-0001-6211-9558.

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