Promising Drug Candidates for the Treatment of Polycystic Ovary Syndrome (PCOS) as Alternatives to the Classical Medication Metformin

Hanan S. Anbar[a]*, Nisha Yarifbhai Vahora[b] , Hajra laghman Shah[b], Mariyam Mohammed Azam[b],Tamanna Islam[b], Fatima Hersi[c], Hany A. Omar[c,d,e], Wolfgang Dohle[f], Barry V. L. Potter[f], and Mohammed I. El-Gamal[c,g,h]

[a] Department of Clinical Pharmacy and Pharmacotherapeutics, Dubai Pharmacy College for Girls, Dubai 19099, United Arab Emirates

[b] Dubai Pharmacy College for Girls, Dubai 19099, United Arab Emirates

[c] Research Institute for Medical and Health Sciences, University of Sharjah, Sharjah 27272, United Arab Emirates

[d] Department of Pharmacy Practice and Pharmacotherapeutics, College of Pharmacy, University of Sharjah, Sharjah 27272, United Arab Emirates

[e] Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt

[f] Medicinal Chemistry & Drug Discovery, Department of Pharmacology, University of Oxford, Oxford OX1 3QT, United Kingdom

[g] Department of Medicinal Chemistry, College of Pharmacy, University of Sharjah, Sharjah 27272, United Arab Emirates

[h] Department of Medicinal Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

* Corresponding author

E-mail address: dr.hanan@dpc.edu

Abstract

Polycystic ovary syndrome (PCOS) is a prevalent hormonal disorder that affects women of reproductive age. It is characterized by abnormal production of androgens, typically present in small quantities in females. This study aimed to investigate the therapeutic potential of Irosustat (STX64), STX140, and compound **1G** as new drug candidates for treatment of letrozole-induced PCOS in female Wistar rats. 36 rats were divided into six groups of equal size. PCOS was induced in all groups, except the normal control group, by administering letrozole orally (1 mg/kg/day for 35 days). The onset of abnormal estrous cycle was confirmed by examining daily vaginal smears under a microscope. Subsequently, each rat group was assigned to a different treatment regimen, including one control group, one letrozole group, one metformin group (500 mg/kg/day) as a reference drug, and the other groups received a different drug candidate orally for 30 days. After treatment, blood collection was performed for biochemical measurements and determination of oxidative stress markers. The rats were dissected to separate ovaries and uterus for morphological, histological, and western blotting studies. Treatment with the drug candidates improved the ovaries and uterus weight measurements compared to the untreated PCOS group. The three tested drug candidates demonstrated promising improvements on lipid profile, blood glucose level, testosterone, progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol levels. In addition, Western blotting confirmed their promising effects on Akt, mTOR, and AMPK-α pathways. This study led to discovery of three promising drug candidates for management of PCOS as alternatives to metformin.

Keywords: Irosustat; Letrozole; Metformin; PCOS; STX64; STX140.

1. Introduction

Polycystic Ovary Syndrome (PCOS) is a prevalent endocrine disorder that primarily affects women in their reproductive age. Research indicates that 5-10% of females aged between 18 to 44 years are afflicted with this condition. According to the National Institute of Health Office of Disease Prevention, this translates to approximately 5 million women in the U.S.(Ndefo *et al.*, 2013). The underlying pathophysiological mechanism responsible for PCOS remains unclear. However, significant features of the disorder include hyperandrogenism and/or hyperandrogenemia, oligo-ovulation, and polycystic ovarian morphology (Guzick, 2004; Goodarzi *et al.*, 2011). Abnormal folliculogenesis and gonadotropin production, particularly luteinizing hormone hypersecretion, also contribute to the development of this condition. A common feature of PCOS is insulin resistance, partly caused by adipose tissue dysfunction. It results in compensatory hyperinsulinemia, which contributes to hyperandrogenism by stimulating ovarian androgen secretion and inhibiting hepatic sex hormone-binding globulin production (Goodarzi *et al.*, 2011). The woman may also suffer from lipid imbalance and oxidative stress (Atef *et al.*, 2019). Although not a defining characteristic, obesity frequently complicates PCOS (Guzick, 2004). PCOS is diagnosed by excluding other conditions that could cause the development of similar features.

Women with the polycystic ovarian syndrome usually have several health complications, such as menstrual dysfunction, infertility, hirsutism, acne, obesity, and metabolic syndrome (Norman *et al.*, 2007). PCOS increases the risk of development of type 2 diabetes mellitus, gestational diabetes and/or other pregnancy-related complications, venous thromboembolism, cerebrovascular and cardiovascular events as well as endometrial cancer (Azziz *et al.*, 2016).

Treatment of PCOS should be individualized according to the patient's needs; goals of therapy may be to reduce hyperandrogenic symptoms, induce ovulation, regulate menstruation and prevent cardio-metabolic complications (Goodarzi *et al.*, 2011). Medications traditionally used to manage symptoms of PCOS include metformin, combined oral contraceptive pills, spironolactone, and local treatments for hirsutism and acne (Witchel *et al.*, 2019).

Metformin, an insulin sensitizer, increases the insulin sensitivity of cells and counteracts hyperinsulinemia. Consequently, androgen levels decrease and there is improvement in menstrual irregularity and ovulatory function (Costello *et al.*, 2007; Yang and Choi, 2015). Oral Contraceptive

Pills (OCPs) decrease androgen levels and increase sex hormone-binding globulins, which seem to improve the management of symptoms such as hirsutism, acne, and hair loss (Soares *et al.*, 2009). However, various studies conducted on the effectiveness of metformin and OCPs have revealed that metformin may not be effective for all women with PCOS. Specifically, some women did not experience any improvement in insulin sensitivity until they combined metformin therapy with efforts to reduce their body weight. Therefore, lifestyle modifications are essential in conjunction with metformin therapy. Regarding treatment with OCPs, some studies have shown that insulin levels increased while others reported negative effects on insulin levels. Although metformin has been shown to improve body mass index (BMI), OCPs did not produce any significant changes or have an adverse effect on BMI levels. Furthermore, studies indicate that metformin and OCPs do not consistently improve lipid levels (Yang and Choi, 2015).

Metformin typically exhibits a favorable safety profile, although it may produce gastrointestinal complications, including nausea and diarrhea. However, it poses a severe risk of lactic acidosis (Stang *et al.*, 1999). On the other hand, OCPs may lead to undesirable outcomes such as weight gain and cardiovascular and thromboembolic events (Yang and Choi, 2015). Consequently, exploring alternative PCOS treatment medications could yield more effective therapeutic options.

Irosustat (STX64, Figure 1) is a steroid sulfatase enzyme (STS) inhibitor studied clinically up to Phase II to date for women with advanced hormone-dependent breast or endometrial cancer and for prostate cancer in men. According to Reed *et al*., STS is distributed throughout the body and may contribute to the regulation of androgens production (Reed *et al.*, 2005). STS has a role in normal male and female reproduction. Purohit *et al*. concluded the effectiveness of STS inhibitors in conditions such as endometriosis (Purohit *et al.*, 2008).

STX140 (Figure 1) is a steroidal bis-sulfamate drug candidate whose *in vitro* antiproliferative activity against breast carcinoma cells and *in vivo* in breast cancer models *inter alia* have been reported and also including activity against an *in vivo* model of multiple sclerosis. It acts through irreversible inhibition of the STS enzyme and disruption of microtubulin poymerization. Inhibition of STS leads to reduced conversion of inactive sulfated metabolites of steroidal hormones such as estradiol and estrone to the active forms of the hormones. Therefore, it was decided to test the impact of these

effects of the STS inhibitors, STX140 and Irosustat, a pure STS inhibitor, on a PCOS model (Newman *et al.*, 2008; Anbar *et al.*, 2021).

Compound **1G** (Figure 1) is a coumarin sulfonate derivative that has been reported as an inhibitor of lipopolysaccharide-induced nitric oxide (NO) and prostaglandin E_2 production in RAW 264.7 macrophages. It produces these activities by inhibiting cyclooxygenase-2 (COX-2) enzymatic activity and inducible nitric oxide synthase (iNOS) protein expressions (Jang *et al.*, 2014). These *in vitro* anti-inflammatory activities inspired us to test its activity against a PCOS model.

The main objective of this study was to investigate the efficacy of these three drug candidates, Irosustat (STX64), STX140, and **1G**, as potential PCOS medications in the letrozole-induced PCOS model in rats. Results were compared with those of metformin as a reference standard. Various morphological, biochemical, and histopathological assays were conducted and results are reported herein.

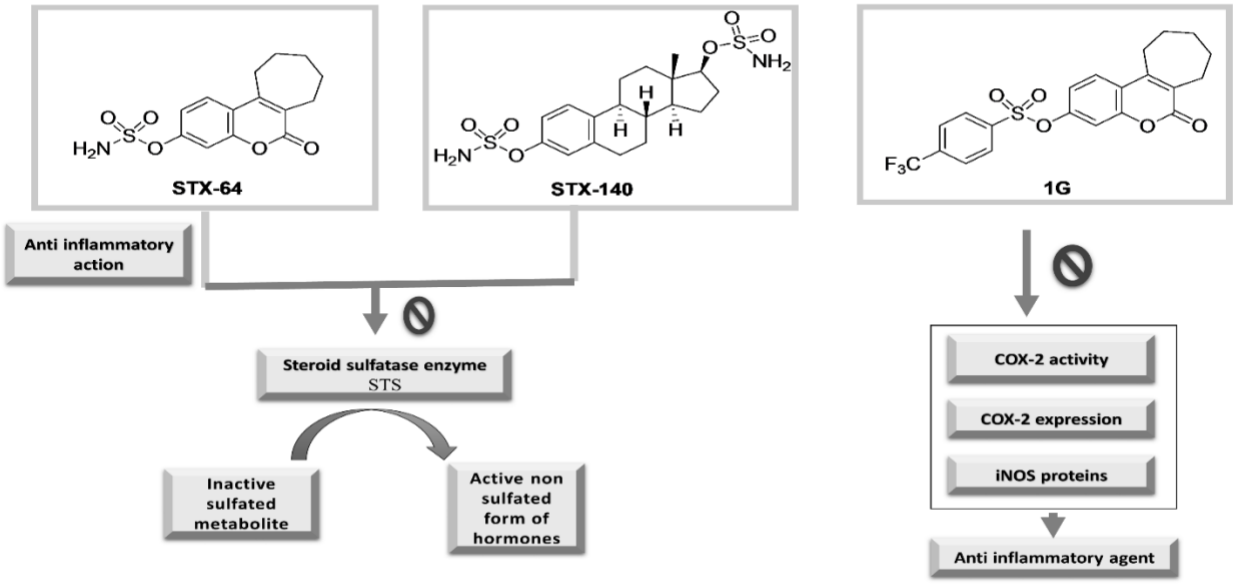

Figure 1. Structures and mechanisms of action of STX64 (Irosustat), STX140, and compound **1G**.

2. Materials and Methods

2.1. Animals

Thirty-six female, non-pregnant Wistar rats between 4-5 months old were isolated from males for three weeks before being transferred to the experimental area. The experimental area was a well-ventilated space where the temperature ranged from 23 ± 2 °C with a 12-hour light/dark cycle. Their body weight was between 170-230 grams (Mesbah *et al.*, 2015). Standard rat feed and water were provided throughout the experiment. Acclimatization was ensured by keeping the rats in the experimental area for two weeks before beginning the drug administration. The experiment was approved by the Ethical Research Committee of Dubai Pharmacy College for Girls, Dubai, United Arab Emirates (REC/UG/2021/02). All the procedures were carried out according to the ethical standards of lab experimental animals.

2.2. Chemicals and Reagents

Carboxymethylcellulose (CMC) was purchased from Sigma Aldrich (Missouri, USA). Letrozole (Femara ®, Cairo, Egypt) was obtained as Femara®, Novartis Pharmaceuticals. Metformin was purchased from Merck Pharmaceuticals. STX140 and Irosostat (STX64) and compound 1G were synthesized as previously described.

2.3. Animal Model of PCOS

The animals were randomly assigned into six groups of six rats each. The first group was reserved as normal control and administered with 1g/100 mL CMC only. To the rest of the groups were administrated letrozole 1 mg/kg/day dissolved in 1g/100 mL CMC by oral gavage for 35 consecutive days for induction of PCOS (Oner *et al.*, 2010). During the last week, vaginal smears were taken from one rat of each group by washing out the vagina with a small amount of normal saline using a dropper, then placing the fluid on a slide and allowing it to dry. 1% crystal violet was used to stain the dry slide. The rat's estrus cycle was observed in the smear under a light microscope using 10x and 40x lenses. An irregular estrus cycle - which confirms induction of PCOS - does not follow the usual sequence of proestrus, estrus, metestrus and diestrus, and there will appear to be the prolongation of the diestrus phase for up to 4 or 5 days (Marcondes *et al.*, 2002) as in Figure 2A and 2B.

2.4. Treatment

Once PCOS was induced, the six groups were designated with one treatment each: Control (1g/100 mL CMC only), Letrozole (1 mg/kg/day), Metformin (500 mg/kg/day) and the rest were new drug

treatment groups: STX140 (15 mg/kg/day), STX64 (20 mg/kg/day), **1G** (20 mg/kg/day). The dose of metformin is the same as that used in the PCOS model in rats in other reported studies (Zhang *et al.*, 2017; Karateke *et al.*, 2018; Xu *et al.*, 2022). The doses of the new treatments were determined by first performing an LD_{50} study and the doses that did not kill the animals due to overt toxicity were used in this study. The drug candidates were dissolved in 1% CMC and orally administered to the rats for 30 consecutive days [23]. During the last week of treatment, vaginal smears were again taken from each group to confirm the improvement in PCOS by observing the estrous cycle under a light microscope using 10x and 40x lenses. The detection of the proestrus phase indicates a regular estrous cycle for 12 hours, then the estrus phase for another 12 hours, metestrus for the next 21 hours and finally, the diestrus phase, which lasts for 57 hours. Therefore, the cycle is regular if the progression of proestrus, estrus, metestrus, and diestrus over the correct period is observed (Ajayi and Akhigbe, 2020).

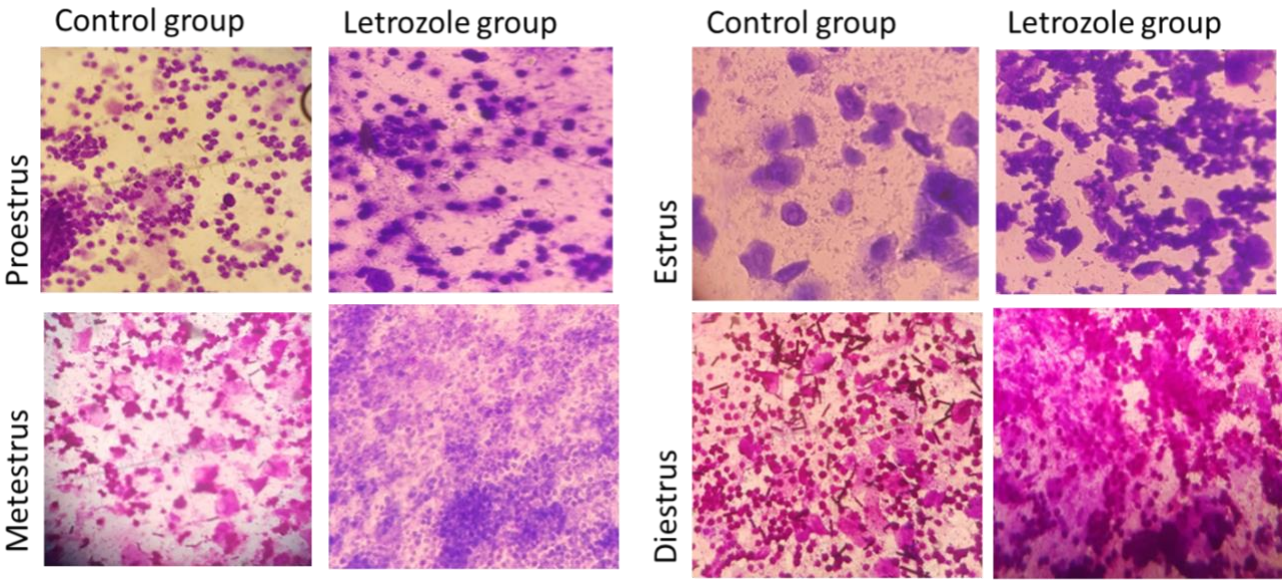

Figure 2A. Vaginal Cytology comparison between normal control group and letrozole- groups. Proestrus (nucleated epithelial cells mainly), estrus (anucleated cornified cells), metestrus (leukocytes, cornified, and nucleated epithelial cells all with an equal number) and diestrus (leukocytes mainly).

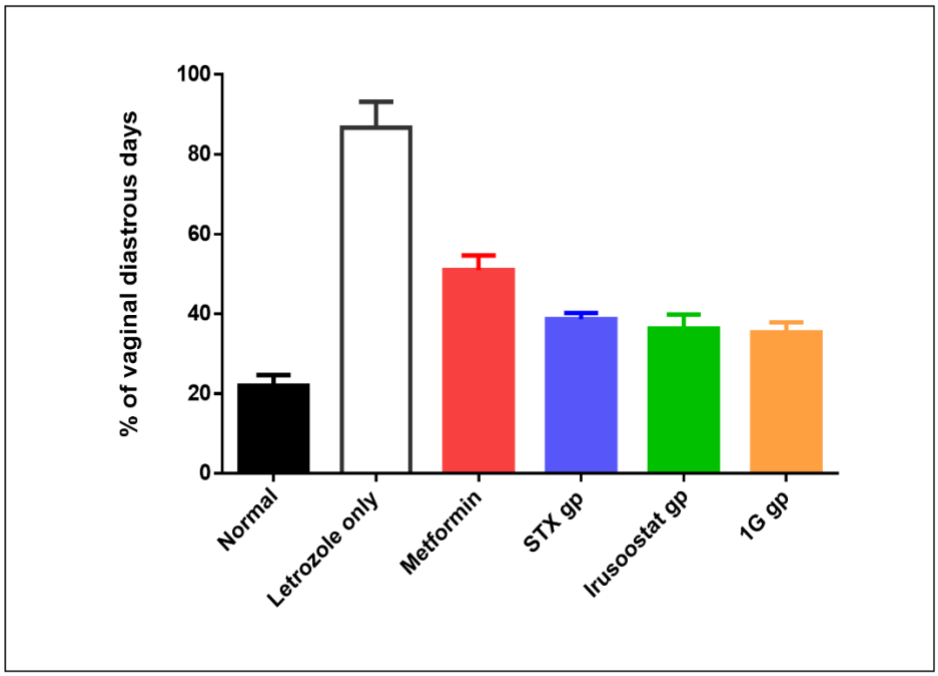

Figure 2B. Effect of drug candidates treatment in letrozole-induced PCOS rats on the percentage of vaginal diastrous days out of the whole estrous period.

2.5. Termination of the procedure

2.5.1. Collection of Ovaries and Blood Samples

At the end of treatment, the animals were fasted for 12 hours. The blood was then collected by retro-orbital puncture in blood-collecting tubes specific for serum or plasma. Plasma was centrifuged immediately at 1000 rpm at -2 to -8 °C for 10 minutes for the determination of oxidative stress and hormonal levels. After clotting for one hour, the serum was centrifuged for 3000 rpm at room temperature for 10 minutes. The clear supernatant was separated using a micropipette into Eppendorf tubes and was stored at -20 °C for biochemical measurements of lipid profiles, blood glucose levels, and total protein in serum. Immediately after the blood collection as mentioned above, the animals were sacrificed by cervical dislocation then the animals were dissected. The ovaries were removed, weighed and either preserved in 10% formalin for histopathology or stored at -80 °C for western blotting tests (Shomer *et al.*, 2020).

.

2.6. Western blot analysis

Western blot analysis was performed to determine the effect of the treatment on protein expression levels of Akt, P-Akt, AMPK-α, P-AMPK-α, mTOR, and P-mTOR. In brief, proteins were extracted from ovaries using RIPA lysis buffer and sonicated 3x for 15 s, followed by boiling at 95^{o}C for 5 min. Per the manufacturer's direction, protein concentrations were determined using the DC protein assay kit (Bio-Rad, USA). Then, proteins were prepared for loading by adding 1× of the 4× laemmli sample buffer (Biorad, USA). Proteins were allowed to run in 12 or 8% SDS polyacrylamide gels, then using a semi-dry transfer cell (Biorad, USA) at 25 v, proteins were transferred onto nitrocellulose membranes. Membranes were then blocked using 5% skimmed milk / 5% Bovine Serum Albumin (Sigma-Aldrich) by incubating for 1 h at room temperature. After that, membranes were incubated with the following primary antibodies at 4^{o}C: anti-β-actin (#4970), anti-Akt (Serine) (9272S), anti-phospho-Akt (4060), anti-AMPK-α (2532S), anti-phospho-AMPK-α (4188S), anti-mTOR (2983), anti-phospho-mTOR (5536s) (Cell Signaling Technologies, USA). All primary antibodies were prepared at a dilution of 1:1000 in 5% BSA TBS-T. After that, membranes were incubated with a secondary antibody (anti-rabbit IgG) at a dilution of 1:2000 (Cell Signaling Technologies, USA). Membranes were washed 3× with TBS-T. Immunoreactive proteins were visualized using ECL chemiluminescence substrate (Bio-Rad, USA) by ChemiDoc Imaging System (Bio-Rad, USA). When necessary, blots were cut horizontaly and stripped with mild stripping buffer (1.5% w/v Glycine, 1% w/v SDS and 1% v/v Tween20 pH 2.2) for 35 min at RT on shaker.

2.7. Measurement of lipid profile

Serum samples were kept in the freezer (-23 °C) until used. Total cholesterol, LDL, HDL and TGL were detected by lipid profile test kits using the manufacturer's guidelines. The test kits were purchased from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). The machine used was Pchem 2: Fully Automated Clinical Chemistry Analyser manufactured by Adaltis (Rome, Italy), along with its accompanying software.

2.7.1. Cholesterol

Serum was analyzed as reported in the literature (Warnick and Remaley, 2001). CHOD PAP: the enzymatic photometric test was the method used. Cholesterol was determined after enzymatic hydrolysis by cholesterol esterase and enzymatic oxidation by cholesterol oxidase. Hydrogen peroxide acts on 4-aminoantipyrine and phenol under the catalytic action of peroxidase (Trinder's reaction) to produce the colorimetric indicator quinoneimine.

2.7.2. Triglyceride (TGL)

Serum was analyzed as reported in the literature (McGowan *et al.*, 1983). The method used was a colorimetric enzymatic test using glycerol-3-phosphate-oxidase. Triglycerides were determined after enzymatic splitting with lipoprotein lipase, followed by further conversion to dihydroxyacetone phosphate by the enzymes glycerokinase and glycerol-3-phosphate-oxidase. Hydrogen peroxide acts on 4-aminoantipyrine and phenol under the catalytic action of peroxidase (Trinder's reaction) to produce quinoneimine.

2.7.3. Low Density Lipoprotein (LDL)

Serum was analyzed as reported in the literature (Nauck *et al.*, 2002). LDL-C Select FS is a homogenous method that directly measures LDL-cholesterol without centrifugation. In the first step, non-LDL-lipoproteins were processed enzymatically by cholesterol esterase and cholesterol oxidase. Meanwhile, the LDL was selectively protected. In the second step, LDL was released, and LDL-cholesterol was selectively determined by a reaction between hydrogen peroxide, 4-aminoantipyrine and *N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline) catalyzed by peroxidase, generating a detected color.

2.7.4. High Density Lipoprotein (HDL)

Serum was analyzed as reported in the literature (Borgel *et al.*, 2006). HDL-C Immuno FS is a homogenous method that measures HDL-cholesterol without centrifugation. Anti-human β-lipoprotein antibodies bound with LDL, VLDL and chylomicrons to form antigen-antibody complexes. Only HDL cholesterol underwent a reaction catalyzed by cholesterol esterase and cholesterol oxidase to produce hydrogen peroxide, so it was selectively determined. Hydrogen

peroxide reacted with 4-aminoantipyrine and *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4-fluoroaniline in sodium salt form under the catalytic action of peroxidase to form a blue complex.

2.8. ELISA Assay

Plasma samples were kept in the freezer (-23 °C) until used. The FSH, LH, testosterone, progesterone, estradiol, TNF-alpha, nitric oxide, and rat superoxide dismutase 1 were detected by ELISA test kits using the manufacturer's guidelines. Rat test kits for FSH, LH, testosterone, progesterone and superoxide dismutase 1 were purchased from Cusabio (Wuhan, China). The test kits for total nitric oxide and nitrate/nitrite assay and rat TNF-α Immunoassay were purchased from R&D Systems (Minnesota, USA). The machine and accompanying software used were Readwell Touch Elisa Plate Analyzer, manufactured by Robonik, (Ambarnath, India). The color change was detected by a spectrophotometric method at 450 nm +/- 2 nm.

2.8.1. Measurement of oxidative stress markers

2.8.1.1. TNF-α Level

Plasma was analyzed as reported in the literature (El-Ansary *et al.,* 2012)**.** The microplates were pre-coated with a monoclonal antibody specific for rat TNF-α by quantitative sandwich enzyme immunoassay. The wells were filled with standard, control and samples. Any TNF-α present bound to the monoclonal antibody that coated the microplate. The excess was washed off using wash buffer. Next, an enzyme-linked polyclonal antibody specific for rat TNF-α was added to the wells. The plate was incubated for 2 hours and washed. Addition of a substrate solution and incubation for another 30 minutes resulted in a blue color. When stop solution was added, the yellow color that appeared was proportional to the amount of TNF-α bound to the monoclonal antibody on the plate.

2.8.1.2. Superoxide dismutase (SOD)-1 Activity

Plasma was analyzed as reported in the literature (Patel *et al.*, 2014). First, the reagents, samples and standards were added to the plate and incubated for 2 hours. The microplate was pre-coated with SOD1 specific antibody that binds any SOD1 present. The excess was then removed and biotin-antibody specific for SOD1 was added to each well. After another hour of incubation, the wells were washed and TMB substrate was added to each well. Another final incubation of 15 to 30 minutes was followed by the addition of stop solution. The color that developed was proportional to the amount of SOD1 bound initially.

2.8.2. Total nitric oxide and nitrate/nitrite Assay

Plasma was analyzed as reported in the literature (Berkels *et al.*, 2004). Enzymatic conversion of nitrate to nitrite by nitrate reductase is used to determine the concentration of nitric oxide. It is followed by the colorimetric detection of nitrite as an azo dye product of a reaction known as Griess Reaction. First, the reaction diluent was added to the blank well of the microplate. The standards and samples were used to fill the rest of the wells, followed by adding the reaction diluent to all the wells. This was followed by adding Griess Reagent I and Griess Reagent II to all wells and mixing. After incubation for 10 minutes, the plate was read at 540 nm.

2.8.3. Measurement of PCOS related hormones

The measurement was done as reported in the literature (Ghazal *et al.*, 2022). The microplates were pre-coated with goat-anti-rabbit antibodies. The addition of standards and plasma samples to the plate resulted in competitive inhibition between HRP labeled according to the test by testosterone, progesterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) or estradiol and unlabeled hormone with the antibody specific for them. A blank well remained without any solution. After an hour of incubation and washing the wells, substrate A and B were added to each well. Incubation for another 15 minutes was followed by the addition of stop solution. The developed color was inversely proportional to the amount of the hormone in the sample.

2.9. Histological examination of ovaries

All ovaries of the rats were fixed for 24 hours in 10% formalin, and after standard procedures, paraffin-embedded ovaries at 56 °C were cut into 5 μm of thickness using a microtome (Shandon Cat. No. 0525, England) and were stained by Haematoxylin and Eosin (H&E). Then, tissue sections were microscopically examined (Leica ICC50 E, MD 500, Germany).

2.9.1. Morphometric analysis

Primary follicles (PF) are identified by the presence of one or more granulosa cell layers surrounding a primary oocyte, secondary follicles (SC) are identified by the presence of an antrum, and tertiary follicles (TF) are identified by the completion of antrum development and the association of the primary oocyte with the cumulus oophorus. Atretic follicles (AF) have a nuclei, oocyte degeneration,

and granulosa cell layer degeneration and separation from the basement membrane as its morphological traits. Cystic follicles (C) are defined as those with four or five granulosa cell layers around a very big antrum or huge fluid-filled follicles with attenuated granulosa cell layers thickened theca layer.

2.10. Statistical analysis

Data were analyzed with GraphPad Prism Software, version 9.3.1 (San Diego, CA, USA). Statistical analysis among groups were performed using one-way analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparison test.

3. Results

3.1. Morphological changes in the ovary and uterus

The letrozole group showed a significant increase in average ovary weight ($P<0.001$) with a marked reduction in the uterus weight ($P<0.01$) compared to the normal group, as in Figures 3A and B. All the groups that received the STX64, STX140 and **1G** were like metformin, the reference drug, in attenuation of the average ovary weight compared to PCOS-group. **1G** showed a significant reduction ($P<0.01$) in ovary weight compared to normal, which needs more investigation. The drug candidates showed no significant difference ($P>0.05$) with normal and letrozole groups in restoring the uterus weight, as shown in Figure 3B.

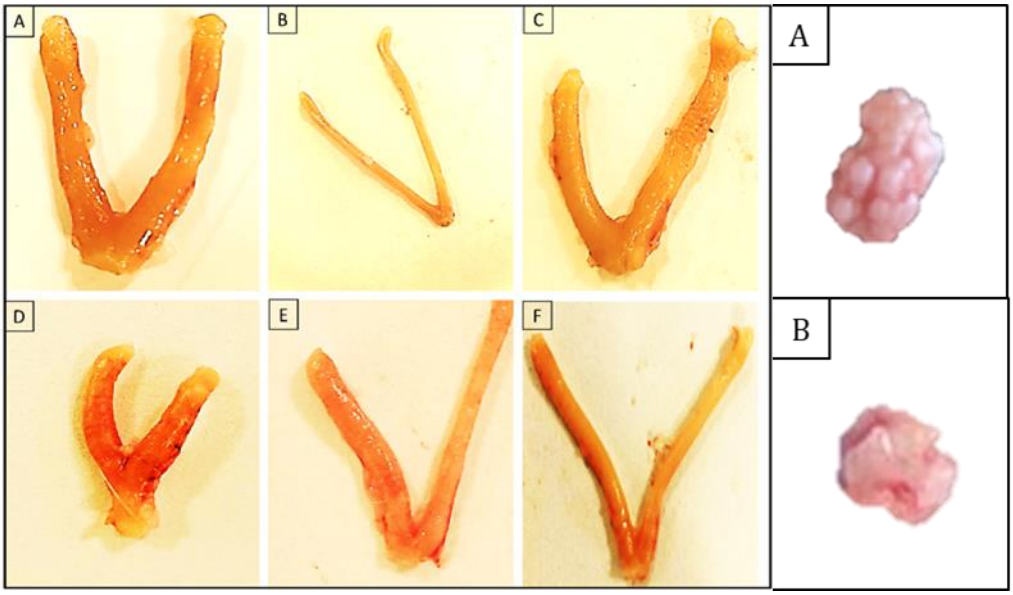

Figure 3A.Morphological change in the uterus and ovary in various groups A) Normal control, B) Letrozole only, C) Metformin, D) STX140, E) STX64, F) **1G**.

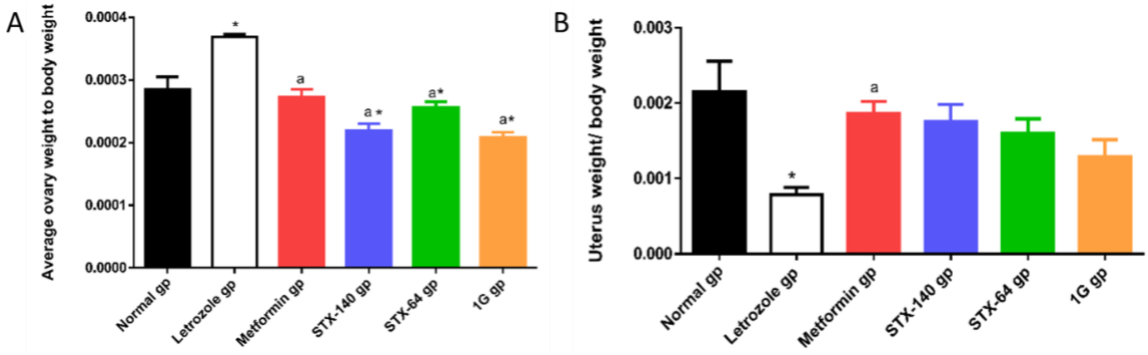

Figure 3B. Average ovary and uterus weight to the body weight compared between Control, Diseased, Standard and Various Treatment groups (A. Measurement of the average ovary weight to body weight, B. Measurement of the average uterus weight to body weight).

* Significant difference when compared with normal control.

[a] Significant difference when compared with letrozole only treated animals.

Data are presented as Statistical Mean ± Standard error of mean (SEM).

3.2. Lipid profile and glucose level measurement

As shown in Figure 4 and in Table S1 (Supplementary File), significant increase in triglycerides ($P<0.001$), cholesterol ($P<0.001$), and LDL ($P<0.01$) levels was observed in the PCOS group in

comparison to the normal control group. The cholesterol level was significantly decreased in **1G** (75.93 ± 3.70), (P<0.05) compared to PCOS group (100.59 ± 4.46). Triglyceride levels decreased significantly in the treated groups to near the normal value (P>0.05) compared to Letrozole's group (*P<0.001*). **1G** (34.65 ± 5.19) was superior to Metformin's group (65.767 ± 4.528) (P<0.05) in the reduction of elevated TGs levels, as shown in Figure 4.

HDL-c level was significantly reduced, while the total protein level in serum was significantly elevated in the PCOS groups (P<0.01**) compared to the normal group. The treated groups showed some increase in the HDL-c level with some reduction in the total protein level compared to the letrozole group.

The treatment with STX140 (P<0.05*) (13.47 ± 1.89) and compound **1G** (P<0.01**) (12.65 ± 1.09) showed a significant reduction in the elevated LDL-C level compared to PCOS group (21.575 ± 1.97). The blood glucose level increased significantly in the PCOS group (*P<0.01**) compared to the normal group. Treatment with STX140, STX64, and 1G showed *a* significant reduction in blood glucose levels compared to rats with PCOS, near the normal value (*P<0.001*), as represented in Figure 4E.

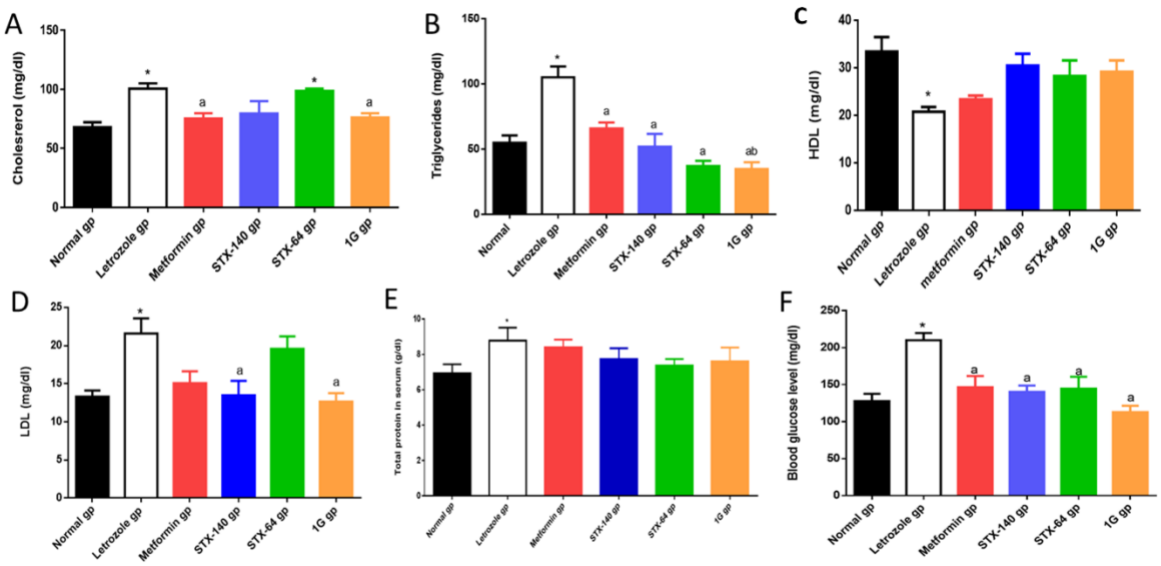

Figure 4. Lipid profile parameters determined and compared between Control, Diseased, Standard

and the Various Treatment groups. Measurement of A. Cholesterol; B. Triglycerides; C. High-density lipoproteins; D. Low-density lipoproteins; E. Serum total protein level; F. Blood glucose level.

*Significant difference when compared with normal control.

[a] Significant difference when compared with letrozole-only treated animals.

[b] Significant difference compared with metformin group.

Data are presented as Statistical Mean ± Standard error of mean (SEM).

3.3. Measurement of the hormonal levels

As represented in Figure 5 and Table S2 (Supplementary File), the progesterone level (*P<0.05*) was significantly decreased while the level of testosterone (P<0.001) was remarkably elevated in letrozole-treated groups compared to the normal control group. STX140 (P<0.001), STX64 (P<0.05), **1G** (P<0.001) significantly decreased the level compared to the letrozole group. It was superior to metformin (P>0.05) in the amelioration of the testosterone level, as shown in Figures 5A and 5B as well as Table S2.

As represented in Figure 5C, there is a significant increase in LH level in PCOS group (P<0.001) compared to the normal one. The treated group markedly ameliorated the LH level (P<0.01) to near normal value.

The FSH and estradiol levels were significantly decreased in letrozole group (P<0.05 and P<0.01, respectively) compared to normal control rats. There was a significant increase in FSH and estradiol levels in animals receiving STX140 (P<0.01), and STX64 (P<0.05). However, **1G** (P<0.05) increased the estradiol but not the FSH levels, as represented in Figures 5D and 5E.

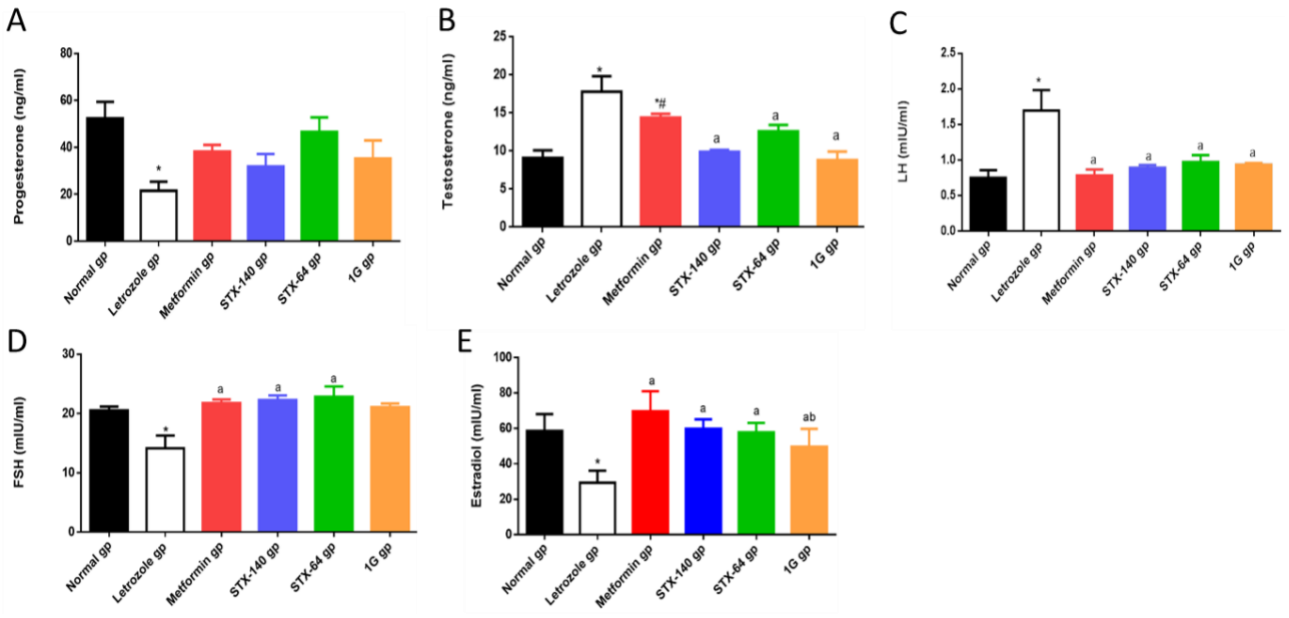

Figure 5. Hormonal Test results comparison between Control, Diseased, Standard and Various Treatment groups. Measurement of: A. Testosterone; B. Luteinizing Hormones; C. Progesterone; D. Follicle Stimulating Hormones; E. Estradiol level.

* Significant difference compared with normal control.

[a] Significant difference compared with Letrozole only treated animals.

[b] Significant difference compared with metformin group.

Data are presented as Statistical Mean ± Standard error of mean (SEM).

3.4. Measurement of oxidative stress markers

A marked increase of 79.52 % in TNF-α level in the letrozole-treated group ($P<0.001$) was observed compared to normal. The drug candidates in the current study, STX140 ($P<0.01$), STX64 ($P<0.05$), and 1G ($P<0.001$) as similarly to metformin ($P<0.05$), reduced the elevated TNF-α level by 36.99%, 35.82%, 47.17%, and 32.35%, respectively compared to the PCOS group (Figure 6A and Table S2). Superoxide Dismutase (SOD) activity and nitric oxide (NO) levels were significantly decreased in the letrozole group ($P<0.01$), ($P<0.05$) compared to normal rats. STX140 ($P<0.05$), STX64 ($P<0.01$), and **1G** ($P<0.05$) managed to increase the SOD activity compared to the untreated group. STX140, similarly to metformin ($P<0.01$), was superior to the other groups in the significant increase in the NO level compared to PCOS as represented in Figures 6B and 6C as well as Table S3 (Supplementary File).

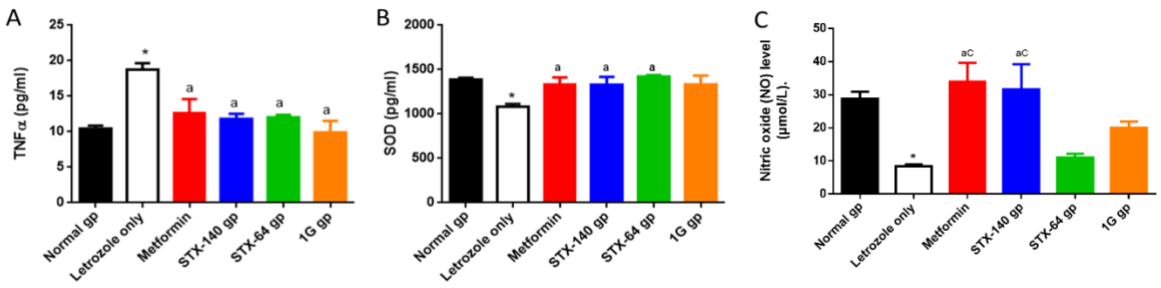

Figure 6. Oxidative Stress Markers tests comparison between Control, Diseased, Standard and Various Treatment groups. A. Measurement of TNF-α, B. Measurement of Superoxide Dismutase (SOD), C. Measurement of NO level.

* Significant difference when compared with normal control.

[a] Significant difference when compared with letrozole only treated animals.

[c] Significant difference when compared with STX64.

Data are presented as Statistical Mean ± Standard error of mean (SEM).

3.5. Histopathological examination results

The control group showed normal histological patterns after staining the ovarian sections with H&E stain, primary follicles at different stages of progress were observed in the normal structure and many corpora lutea (CL). The ovarian sections from the PCOS group showed many cystic follicles with a very thin granulosa layer. These multiple dilated follicular cysts had thinner granulosa cell layers, enlarged antrum, and no oocytes. Corpora lutea was absent in the vaginal slides, which is considered an important characteristic of anovulation and PCOS. The drug candidates managed to reduce cystic follicles and increase corpora lutea compared to PCOS, as shown in Figure 7.

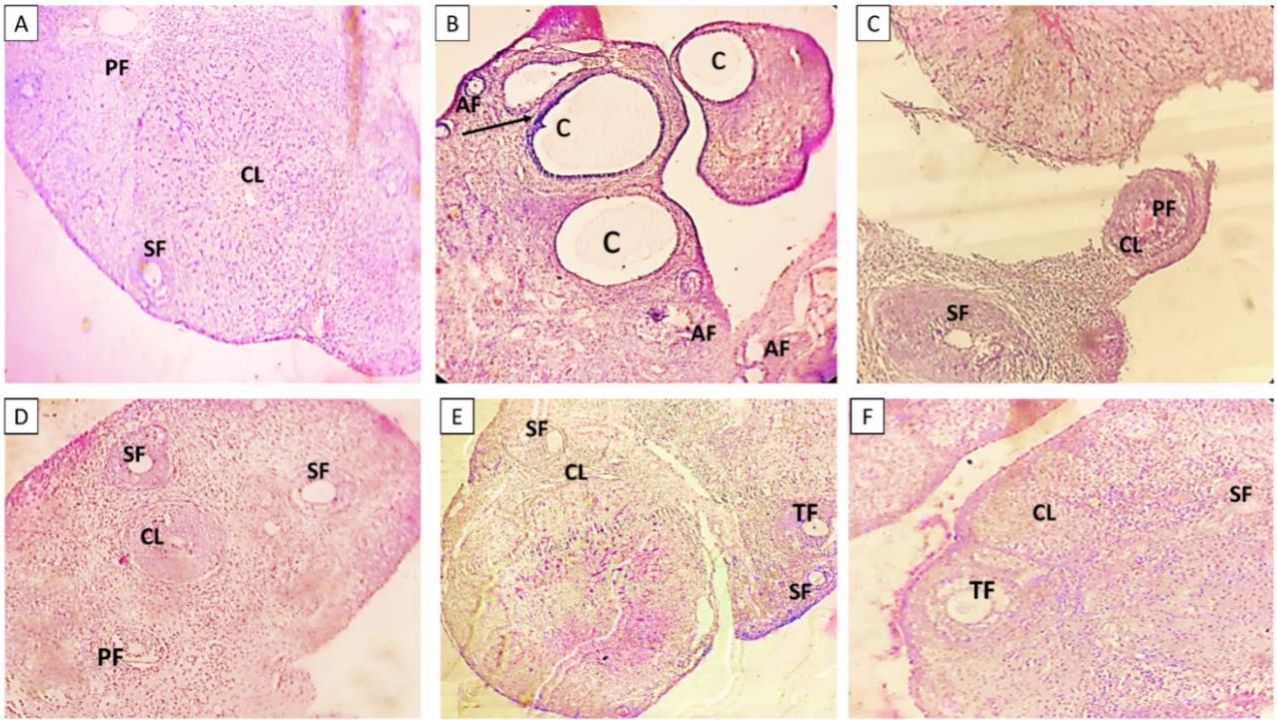

Figure 7. Histopathological result Ovarian sections of different group of rats. A; normal control group, B; PCOS group, C; PCOS-metformin, D; PCOS-STX64, E; PCOS-1G group, F; PCOS-STX140. Some of them showing (EP); surface epithelial (TF); atretic follicle, (C), Large cyst follicle with thinner granulosa cell layer, (CL); corpora lutea, (SF); Secondary follicle, and (PF); Primary follicle.

3.6. Elevated expression levels of ovarian p-AKT, p-AMPK, and p-mTOR in treated rats with PCOS

The PI3K-Akt-mTOR and AMPK signaling pathway is not only involved in the development of IR in patients with PCOS, but is considered to be associated with the progression of PCOS. Therefore, we examined the effect of different treatments on the expression levels of p-Akt, p-AMPK, and p-mTOR in rats with letrozole-induced PCOS. The results showed that STX140 and compound **1G** stimulated the phosphorylation of p-Akt, p-AMPK and p-mTOR in comparison to PCOS group and control group (Figure 8).

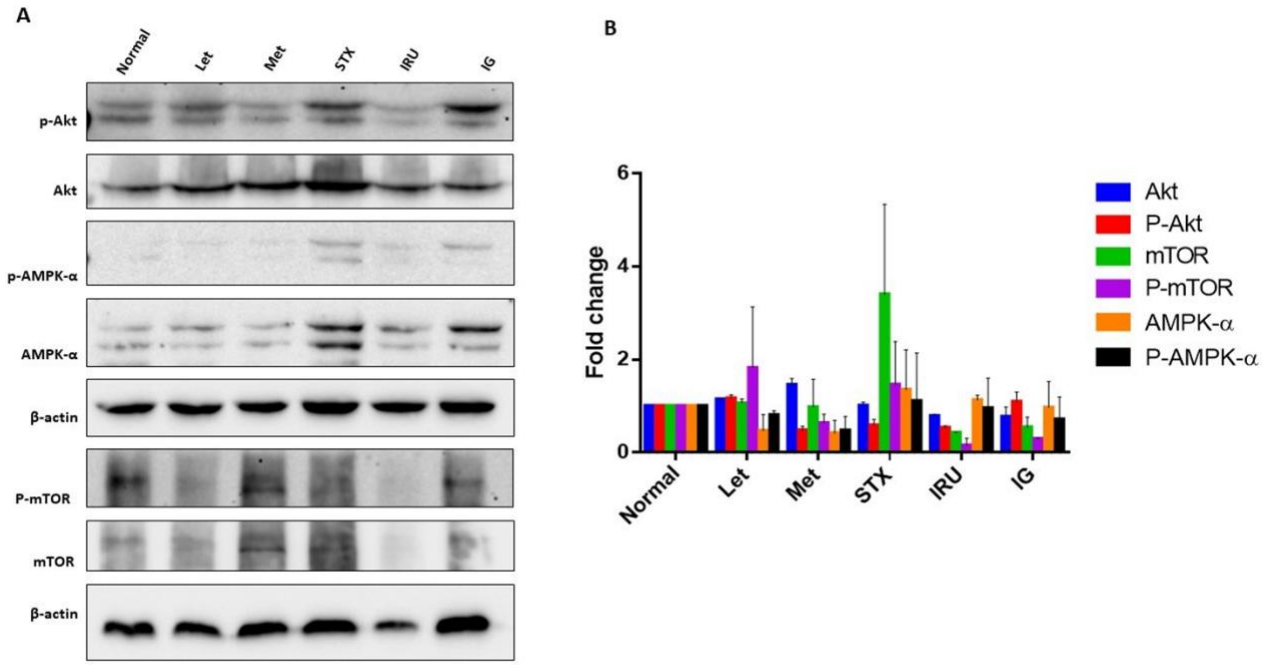

Figure 8. A) The protein expressions of Akt, AMPK and mTOR and corresponding phosphorylated forms in the ovarian tissue of the rats with PCOS treated with metformin, STX140, STX64, **1G**, and control non-PCOS group and untreated PCOS control group. β-actin is used as the loading control; B) Densitometric analysis of the results.

4. Discussion

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder that affects a considerable number of women. Common symptoms of PCOS include irregular menstrual cycles, weight gain, subfertility, and infertility. In addition to anovulation, PCOS is characterized by elevated androgen levels and a higher incidence of ovarian cysts (Rosenfield and Ehrmann, 2016). An intraovarian disturbance resulting in ovarian failure can be attributed to a deficiency in aromatase enzyme activity. Aromatase converts testosterone and androstenedione into estradiol and estrone, respectively. The inhibition of this enzyme by nonsteroidal aromatase inhibitors such as letrozole can result in hormonal imbalance and hyperandrogenism, leading to the development of polycystic ovaries (Caldwell *et al.*, 2014).

The study of Maliqueo et al., 2013, revealed that the estrus cycle was completely disrupted. Ovarian morphology was similar to that in women with PCOS, whose ovaries were enlarged with multiples

cyst (Manneras *et al.*, 2007; Maliqueo *et al.*, 2013). Androgens are required in the early stage of follicular development for normal folliculogenesis. However, the increase in intraovarian androgen leads to follicular atresia (Walters *et al.*, 2008). In their study, Yang et al., concluded that testosterone promotes the activation of primordial follicles by the phosphatidylinositol-3-kinase/Akt/Foxo3a pathway (Yang *et al.*, 2010). In addition, it arrests the pre-antral follicle development by inhibiting the growth differentiation factor 9 expression in oocytes. Moreover, an increase in the ovary weight with the development of fluid-filled cysts was observed. These ovaries contained atretic antral follicles and several numbers enlarged cystic follicles compared to normal control rats. Letrozole-treated rats in our study also showed decreased uterus weight, possibly due to reduced oestradiol levels. The treatment groups, especially Irosustat, STX140 and **1G,** showed a decrease in the ovary weight, indicating that the fluid-filled cysts were disappearing. On the other hand, the uterus weight was normalized among the treatment groups; the most promising results were for STX140 and Irosustat, indicating that the hormonal level of estrogen was returning to normal (Bray *et al.*, 1976).

In the present study, the group with letrozole-induced PCOS exhibited elevated levels of testosterone and LH in the bloodstream. In contrast, a significant decrease in progesterone, estradiol, and FSH STS inhibitor estrone 3-O-sulfamate was used in this stuidlevels was observed when compared to the normal control group. This may be attributed to the disruption of the conversion cycle of free testosterone to estradiol, leading to a buildup of serum testosterone. The exact cause of the cycle interruption remains unknown. Previous research suggests that hyperinsulinemia, caused by insulin resistance in women with PCOS, may also contribute to the development of hyperandrogenism. This effect may be due to the insulin's capability to increase cytochrome P450c17a enzyme activity, which plays a critical role in the biosynthesis of ovarian and adrenal steroid hormones. (la Marca *et al.*, 2000; Balen, 2004).

High LH levels in the blood could be due to the abnormal negative feedback on LH secretion mediated by estrogen-reduced production in the hypothalamus and pituitary. All the treated groups had almost equally reduced testosterone (superior to metformin), reduced LH levels, and increased progesterone and FSH levels, the sa m,oreme as metformin, thhere standard treatment group for PCOS. Similarly, Valigora et al., reported in their research on hypertensive rats that steroid sulfatase inhibitors lowered BP, body weight, and serum testosterone and increased serum estrogen levels (Valigora *et al.*, 2000).

However, the original sulfamate-based inhibitor estrone 3-*O*-sulfamate was used in this study, not the more developed Irosustat, and is known to be highly estrogenic.Those results, however, match ours and may explain the results of the STS inhibitors used in the current study. Reduction in testosterone and LH levels indicates that the hormonal cycle is returning to normal, which helps normalize the follicle development and thus results in normal ovulation, confirmed with the histopathological data (Kim *et al.*, 2018).

Wade et al., and Romanelli et al., revealed that numerous COX products stimulate testosterone production with the relative potency of PGE2 = PGE1 > PGI2 > PGF2α while PGD2 did not show effects (Wade and Van der Kraak, 1993; Romanelli *et al.*, 1995). Their study suggested that PGE2, as a COX metabolite of arachidonic acid, plays a role in controlling steroidogenesis in the testis of goldfish. This may explain the effect of compound **1G** as a prostaglandin E2 inhibitor on the reduction of the elevated testosterone level and the consequences of its effect.

After analyzing the oxidative markers, it was found that the untreated group had elevated levels of TNF-α, while the treated groups exhibited lower levels of this inflammatory cytokine. Specifically, the drug candidates STX140, STX64, and **1G** demonstrated significantly lower oxidative stress markers levels than metformin. This suggests that these drug candidates may be more capable of reducing inflammation and oxidative stress. In addition, SOD, an antioxidant, was found to be at its lowest in the untreated PCOS group. However, the treated groups showed a marked increase in SOD levels, with the STX64 group showing the greatest improvement. This indicates that these groups had a higher level of antioxidant activity, which is beneficial in reducing oxidative stress. Previous research has demonstrated similar findings with metformin treatment. (Buldak *et al.*, 2014; Araujo *et al.*, 2017).

PCOS is associated with several forms of dyslipidemia, with low levels of HDL-C, and high levels of LDL-C, triglycerides, and total cholesterol compared with the treatment groups. Androgens may be a key factor in hyperlipidemia. However, recent research has indicated that hypomethylated genes involved in synthesizing lipids and steroids may encourage the manufacture of androgen and other steroid hormones, which may help partially explain the causes of hyperandrogenism in PCOS (Pan *et al.*, 2018). Abnormal lipid metabolism can promote the pathophysiology of hyperandrogenism, insulin resistance, oxidative stress, and infertility in PCOS, and similarly, insulin resistance can exaggerate disturbance in lipid metabolism (Liu *et al.*, 2019). In our study, STX140 and **1 G-**treated

rats showed significantly lower levels of triglycerides and LDL-C levels, and **1G** was superior to metformin (Valigora *et al.*, 2000). According to Kourounakis et al., most COX-2 inhibitors can lower lipid profiles to different degrees, which matches our result of **1G** as a COX-2 inhibitor (Kourounakis *et al.*, 2002). On the other hand, HDL-C levels were lower in the PCOS group than normal. HDL-C levels were higher in STX140, STX64, and **1G** treatment groups than in metformin-treated rats with PCOS. This may be attributed to hormonal regulation affecting the lipid profile in the treated groups (Kim and Choi, 2013).

In the current study, the blood glucose level was elevated in the diseased group compared to the normal control group. This may be due to insulin resistance, one of the root causes of PCOS. Multiple studies indicate that elevated androgen levels lead to insulin resistance and metabolic syndrome in females. Testosterone leads to insulin resistance and reduces glucose uptake by skeletal muscle in letrozole-treated female rats(Corbould, 2008). In other studies, they indicated that the activation of the PI3K/AKT signaling pathway has a vital role in insulin resistance (Li *et al.*, 2017). PI3K signaling cascades regulate cellular activities such as proliferation, differentiation, survival, apoptosis, and glucose homeostasis (Yu *et al.*, 2021). Upon activation of insulin receptors, they phosphorylate the insulin receptor substrate, which binds to the PI3K protein (Li *et al.*, 2013). The insulin signaling pathway depends upon signaling through several intermediaries, including IRS, PI3K, and AKT, thereby regulating cellular glucose intake (Dupont and Scaramuzzi, 2016). Insulin resistance thus leads to higher blood sugar levels, increasing the risk of developing prediabetes and type 2 diabetes. However, the treated groups showed reduced blood glucose levels, indicating that the blood glucose levels were returning to normal. This may be due to the termination of the hormonal disturbance as one cause of insulin resistance (Joham *et al.*, 2022). Studies have demonstrated the protective function of PI3K-AKT-mTOR and AMPK signaling pathways in response to apoptosis induction via oxidative stress (Wang *et al.*, 2019; Tong *et al.*, 2022). Upon treatment with STX140 and **1G**, AMPK – a key regulator of cellular metabolism – exhibited enhanced relative phosphorylation compared to other conditions. Additionally, a similar pattern of upregulated phosphorylation of AKT and mTOR was observed, indicating the intricate interplay of these distinct signaling pathways and their potential role in mitigating the emergence of IR and, ultimately, PCOS. Nonetheless, additional investigations are required to precisely identify the interaction of these various pathways.

5. Conclusion

The drug candidates STX64, STX140, and **1G** all exhibit potential efficacy in managing PCOS. This is attributed to their mechanisms of action as anti-inflammatory and/or hormonal regulating agents, which are significant factors in the development of PCOS. The results of the study indicate that these drug candidates hold great promise in treating PCOS, particularly in cases where Metformin is contraindicated or not well-tolerated by patients.

Acknowledgments

The authors would like to thank Prof. Saeed Ahmed Khan, Dean of Dubai Pharmacy College for Girls, Dubai, United Arab Emirates, for his continuous support in all the research steps. We are also grateful to Dubai Pharmacy College for Girls and the University of Sharjah for the financial support of this study.

Conflict of Interest Statement

The authors declare no conflict of interest.

References

Ajayi, A.F., Akhigbe, R.E., 2020. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. Fertil Res Pract. 6, 5. https://doi.org/10.1186/s40738-020-00074-3.

Anbar, H.S., Isa, Z., Elounais, J.J., Jameel, M.A., Zib, J.H., Samer, A.M., Jawad, A.F., El-Gamal, M.I., 2021. Steroid sulfatase inhibitors: the current landscape. Expert Opin Ther Pat. 31, 453-472. https://doi.org/10.1080/13543776.2021.1910237.

Araujo, A.A., Pereira, A., Medeiros, C., Brito, G.A.C., Leitao, R.F.C., Araujo, L.S., Guedes, P.M.M., Hiyari, S., Pirih, F.Q., Araujo Junior, R.F., 2017. Effects of metformin on inflammation, oxidative stress, and bone loss in a rat model of periodontitis. PLoS One. 12, e0183506. https://doi.org/10.1371/journal.pone.0183506.

Atef, M.M., Abd-Ellatif, R.N., Emam, M.N., Abo El Gheit, R.E., Amer, A.I., Hafez, Y.M., 2019. Therapeutic potential of sodium selenite in letrozole induced polycystic ovary syndrome rat model: Targeting mitochondrial approach (selenium in PCOS). Arch Biochem Biophys. 671, 245-254. https://doi.org/10.1016/j.abb.2019.06.009.

Azziz, R., Carmina, E., Chen, Z., Dunaif, A., Laven, J.S., Legro, R.S., Lizneva, D., Natterson-Horowtiz, B., Teede, H.J., Yildiz, B.O., 2016. Polycystic ovary syndrome. Nat Rev Dis Primers. 2, 16057. https://doi.org/10.1038/nrdp.2016.57.

Balen, A., 2004. The pathophysiology of polycystic ovary syndrome: trying to understand PCOS and its endocrinology. Best Pract Res Clin Obstet Gynaecol. 18, 685-706. https://doi.org/10.1016/j.bpobgyn.2004.05.004.

Berkels, R., Purol-Schnabel, S., Roesen, R., 2004. Measurement of nitric oxide by reconversion of nitrate/nitrite to NO. Methods Mol Biol. 279, 1-8. https://doi.org/10.1385/1-59259-807-2:001.

Borgel, J., Sanner, B.M., Bittlinsky, A., Keskin, F., Bartels, N.K., Buechner, N., Huesing, A., Rump, L.C., Mugge, A., 2006. Obstructive sleep apnoea and its therapy influence high-density lipoprotein cholesterol serum levels. Eur Respir J. 27, 121-127. https://doi.org/10.1183/09031936.06.00131304.

Bray, G.A., Saiduddin, S., York, D.A., Swerdloff, R.S., 1976. Effect of estradiol on uterine weight, thyroid function food intake, and pituitary weight of genetically obese (fatty-Zucker) and lean rats. Proc Soc Exp Biol Med. 153, 88-91. https://doi.org/10.3181/00379727-153-39486.

Buldak, L., Labuzek, K., Buldak, R.J., Kozlowski, M., Machnik, G., Liber, S., Suchy, D., Dulawa-Buldak, A., Okopien, B., 2014. Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages. Pharmacol Rep. 66, 418-429. https://doi.org/10.1016/j.pharep.2013.11.008.

Caldwell, A.S., Middleton, L.J., Jimenez, M., Desai, R., McMahon, A.C., Allan, C.M., Handelsman, D.J., Walters, K.A., 2014. Characterization of reproductive, metabolic, and endocrine features of polycystic ovary syndrome in female hyperandrogenic mouse models. Endocrinology. 155, 3146-3159. https://doi.org/10.1210/en.2014-1196.

Corbould, A., 2008. Effects of androgens on insulin action in women: is androgen excess a component of female metabolic syndrome? Diabetes Metab Res Rev. 24, 520-532. https://doi.org/10.1002/dmrr.872.

Costello, M., Shrestha, B., Eden, J., Sjoblom, P., Johnson, N., 2007. Insulin-sensitising drugs versus the combined oral contraceptive pill for hirsutism, acne and risk of diabetes, cardiovascular disease, and endometrial cancer in polycystic ovary syndrome. Cochrane Database Syst Rev. CD005552. https://doi.org/10.1002/14651858.CD005552.pub2.

Dupont, J., Scaramuzzi, R.J., 2016. Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle. Biochem J. 473, 1483-1501. https://doi.org/10.1042/BCJ20160124.

El-Ansary, A.K., Ben Bacha, A., Kotb, M., 2012. Etiology of autistic features: the persisting neurotoxic effects of propionic acid. J Neuroinflammation. 9, 74. https://doi.org/10.1186/1742-2094-9-74.

Ghazal, K., Brabant, S., Prie, D., Piketty, M.L., 2022. Hormone Immunoassay Interference: A 2021 Update. Ann Lab Med. 42, 3-23. https://doi.org/10.3343/alm.2022.42.1.3.

Goodarzi, M.O., Dumesic, D.A., Chazenbalk, G., Azziz, R., 2011. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. Nat Rev Endocrinol. 7, 219-231. https://doi.org/10.1038/nrendo.2010.217.

Guzick, D.S., 2004. Polycystic ovary syndrome. Obstet Gynecol. 103, 181-193. https://doi.org/10.1097/01.AOG.0000104485.44999.C6.

Jang, H.L., El-Gamal, M.I., Choi, H.E., Choi, H.Y., Lee, K.T., Oh, C.H., 2014. Synthesis of tricyclic fused coumarin sulfonates and their inhibitory effects on LPS-induced nitric oxide and PGE2 productions in RAW 264.7 macrophages. Bioorg Med Chem Lett. 24, 571-575. https://doi.org/10.1016/j.bmcl.2013.12.018.

Joham, A.E., Norman, R.J., Stener-Victorin, E., Legro, R.S., Franks, S., Moran, L.J., Boyle, J., Teede, H.J., 2022. Polycystic ovary syndrome. Lancet Diabetes Endocrinol. 10, 668-680. https://doi.org/10.1016/S2213-8587(22)00163-2.

Karateke, A., Dokuyucu, R., Dogan, H., Ozgur, T., Tas, Z.A., Tutuk, O., Agturk, G., Tumer, C., 2018. Investigation of Therapeutic Effects of Erdosteine on Polycystic Ovary Syndrome in a Rat Model. Med Princ Pract. 27, 515-522. https://doi.org/10.1159/000494300.

Kim, E.J., Jang, M., Choi, J.H., Park, K.S., Cho, I.H., 2018. An Improved Dehydroepiandrosterone-Induced Rat Model of Polycystic Ovary Syndrome (PCOS): Post-pubertal Improve PCOS's Features. Front Endocrinol (Lausanne). 9, 735. https://doi.org/10.3389/fendo.2018.00735.

Kim, J.J., Choi, Y.M., 2013. Dyslipidemia in women with polycystic ovary syndrome. Obstet Gynecol Sci. 56, 137-142. https://doi.org/10.5468/ogs.2013.56.3.137.

Kourounakis, A.P., Victoratos, P., Peroulis, N., Stefanou, N., Yiangou, M., Hadjipetrou, L., Kourounakis, P.N., 2002. Experimental hyperlipidemia and the effect of NSAIDs. Exp Mol Pathol. 73, 135-138. https://doi.org/10.1006/exmp.2002.2449.

la Marca, A., Egbe, T.O., Morgante, G., Paglia, T., Cianci, A., De Leo, V., 2000. Metformin treatment reduces ovarian cytochrome P-450c17alpha response to human chorionic gonadotrophin in women with insulin resistance-related polycystic ovary syndrome. Hum Reprod. 15, 21-23. https://doi.org/10.1093/humrep/15.1.21.

Li, T., Mo, H., Chen, W., Li, L., Xiao, Y., Zhang, J., Li, X., Lu, Y., 2017. Role of the PI3K-Akt Signaling Pathway in the Pathogenesis of Polycystic Ovary Syndrome. Reprod Sci. 24, 646-655. https://doi.org/10.1177/1933719116667606.

Li, Y., Kuang, H., Shen, W., Ma, H., Zhang, Y., Stener-Victorin, E., Hung, E., Ng, Y., Liu, J., Kuang, H., Hou, L., Wu, X., 2013. Letrozole, berberine, or their combination for anovulatory infertility in women with polycystic ovary syndrome: study design of a double-blind randomised controlled trial. BMJ Open. 3, e003934. https://doi.org/10.1136/bmjopen-2013-003934.

Liu, Q., Xie, Y.J., Qu, L.H., Zhang, M.X., Mo, Z.C., 2019. Dyslipidemia involvement in the development of polycystic ovary syndrome. Taiwan J Obstet Gynecol. 58, 447-453. https://doi.org/10.1016/j.tjog.2019.05.003.

Maliqueo, M., Sun, M., Johansson, J., Benrick, A., Labrie, F., Svensson, H., Lonn, M., Duleba, A.J., Stener-Victorin, E., 2013. Continuous administration of a P450 aromatase inhibitor induces polycystic ovary syndrome with a metabolic and endocrine phenotype in female rats at adult age. Endocrinology. 154, 434-445. https://doi.org/10.1210/en.2012-1693.

Manneras, L., Cajander, S., Holmang, A., Seleskovic, Z., Lystig, T., Lonn, M., Stener-Victorin, E., 2007. A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology. 148, 3781-3791. https://doi.org/10.1210/en.2007-0168.

Marcondes, F.K., Bianchi, F.J., Tanno, A.P., 2002. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol. 62, 609-614. https://doi.org/10.1590/s1519-69842002000400008.

McGowan, M.W., Artiss, J.D., Strandbergh, D.R., Zak, B., 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin Chem. 29, 538-542.

Mesbah, F., Moslem, M., Vojdani, Z., Mirkhani, H., 2015. Does metformin improve in vitro maturation and ultrastructure of oocytes retrieved from estradiol valerate polycystic ovary syndrome-induced rats. J Ovarian Res. 8, 74. https://doi.org/10.1186/s13048-015-0203-x.

Nauck, M., Warnick, G.R., Rifai, N., 2002. Methods for measurement of LDL-cholesterol: a critical assessment of direct measurement by homogeneous assays versus calculation. Clin Chem. 48, 236-254.

Ndefo, U.A., Eaton, A., Green, M.R., 2013. Polycystic ovary syndrome: a review of treatment options with a focus on pharmacological approaches. P T. 38, 336-355.

Newman, S.P., Foster, P.A., Stengel, C., Day, J.M., Ho, Y.T., Judde, J.G., Lassalle, M., Prevost, G., Leese, M.P., Potter, B.V.L., Reed, M.J., Purohit, A., 2008. STX140 is efficacious in vitro and in vivo in taxane-resistant breast carcinoma cells. Clin Cancer Res. 14, 597-606. https://doi.org/10.1158/1078-0432.CCR-07-1717.

Norman, R.J., Dewailly, D., Legro, R.S., Hickey, T.E., 2007. Polycystic ovary syndrome. Lancet. 370, 685-697. https://doi.org/10.1016/S0140-6736(07)61345-2.

Oner, G., Ozcelik, B., Ozgun, M.T., Serin, I.S., Ozturk, F., Basbug, M., 2010. The effects of metformin and letrozole on endometriosis and comparison of the two treatment agents in a rat model. Hum Reprod. 25, 932-937. https://doi.org/10.1093/humrep/deq016.

Pan, J.X., Tan, Y.J., Wang, F.F., Hou, N.N., Xiang, Y.Q., Zhang, J.Y., Liu, Y., Qu, F., Meng, Q., Xu, J., Sheng, J.Z., Huang, H.F., 2018. Aberrant expression and DNA methylation of lipid metabolism genes in PCOS: a new insight into its pathogenesis. Clin Epigenetics. 10, 6. https://doi.org/10.1186/s13148-018-0442-y.

Patel, P., Kriz, J., Gravel, M., Soucy, G., Bareil, C., Gravel, C., Julien, J.P., 2014. Adeno-associated virus-mediated delivery of a recombinant single-chain antibody against misfolded superoxide dismutase for treatment of amyotrophic lateral sclerosis. Mol Ther. 22, 498-510. https://doi.org/10.1038/mt.2013.239.

Purohit, A., Fusi, L., Brosens, J., Woo, L.W., Potter, B.V.L., Reed, M.J., 2008. Inhibition of steroid sulphatase activity in endometriotic implants by 667 COUMATE: a potential new therapy. Hum Reprod. 23, 290-297. https://doi.org/10.1093/humrep/dem308.

Reed, M.J., Purohit, A., Woo, L.W., Newman, S.P., Potter, B.V.L., 2005. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev. 26, 171-202. https://doi.org/10.1210/er.2004-0003.

Romanelli, F., Valenca, M., Conte, D., Isidori, A., Negro-Vilar, A., 1995. Arachidonic acid and its metabolites effects on testosterone production by rat Leydig cells. J Endocrinol Invest. 18, 186-193. https://doi.org/10.1007/BF03347801.

Rosenfield, R.L., Ehrmann, D.A., 2016. The Pathogenesis of Polycystic Ovary Syndrome (PCOS): The Hypothesis of PCOS as Functional Ovarian Hyperandrogenism Revisited. Endocr Rev. 37, 467-520. https://doi.org/10.1210/er.2015-1104.

Shomer, N.H., Allen-Worthington, K.H., Hickman, D.L., Jonnalagadda, M., Newsome, J.T., Slate, A.R., Valentine, H., Williams, A.M., Wilkinson, M., 2020. Review of Rodent Euthanasia Methods. J Am Assoc Lab Anim Sci. 59, 242-253. https://doi.org/10.30802/AALAS-JAALAS-19-000084.

Soares, G.M., Vieira, C.S., de Paula Martins, W., Dos Reis, R.M., de Sa, M.F., Ferriani, R.A., 2009. Metabolic and cardiovascular impact of oral contraceptives in polycystic ovary syndrome. Int J Clin Pract. 63, 160-169. https://doi.org/10.1111/j.1742-1241.2008.01877.x.

Stang, M., Wysowski, D.K., Butler-Jones, D., 1999. Incidence of lactic acidosis in metformin users. Diabetes Care. 22, 925-927. https://doi.org/10.2337/diacare.22.6.925.

Tong, C., Wu, Y., Zhang, L., Yu, Y., 2022. Insulin resistance, autophagy and apoptosis in patients with polycystic ovary syndrome: Association with PI3K signaling pathway. Front Endocrinol (Lausanne). 13, 1091147. https://doi.org/10.3389/fendo.2022.1091147.

Valigora, S.D., Lib, P.K., Dunphy, G., Turner, M., Ely, D.L., 2000. Steroid sulfatase inhibitor alters blood pressure and steroid profiles in hypertensive rats. J Steroid Biochem Mol Biol. 73, 113-122. https://doi.org/10.1016/s0960-0760(00)00062-5.

Wade, M.G., Van der Kraak, G., 1993. Arachidonic acid and prostaglandin E2 stimulate testosterone production by goldfish testis in vitro. Gen Comp Endocrinol. 90, 109-118. https://doi.org/10.1006/gcen.1993.1065.

Walters, K.A., Allan, C.M., Handelsman, D.J., 2008. Androgen actions and the ovary. Biol Reprod. 78, 380-389. https://doi.org/10.1095/biolreprod.107.064089.

Wang, W., Zheng, J., Cui, N., Jiang, L., Zhou, H., Zhang, D., Hao, G., 2019. Baicalin ameliorates polycystic ovary syndrome through AMP-activated protein kinase. J Ovarian Res. 12, 109. https://doi.org/10.1186/s13048-019-0585-2.

Warnick, G.R., Remaley, A.T., 2001. Measurement of cholesterol in plasma and other body fluids. Curr Atheroscler Rep. 3, 404-411. https://doi.org/10.1007/s11883-001-0079-7.

Witchel, S.F., Oberfield, S.E., Pena, A.S., 2019. Polycystic Ovary Syndrome: Pathophysiology, Presentation, and Treatment With Emphasis on Adolescent Girls. J Endocr Soc. 3, 1545-1573. https://doi.org/10.1210/js.2019-00078.

Xu, B., Dai, W., Liu, L., Han, H., Zhang, J., Du, X., Pei, X., Fu, X., 2022. Metformin ameliorates polycystic ovary syndrome in a rat model by decreasing excessive autophagy in ovarian granulosa cells via the PI3K/AKT/mTOR pathway. Endocr J. 69, 863-875. https://doi.org/10.1507/endocrj.EJ21-0480.

Yang, J.L., Zhang, C.P., Li, L., Huang, L., Ji, S.Y., Lu, C.L., Fan, C.H., Cai, H., Ren, Y., Hu, Z.Y., Gao, F., Liu, Y.X., 2010. Testosterone induces redistribution of forkhead box-3a and down-regulation of growth and differentiation factor 9 messenger ribonucleic acid expression at early stage of mouse folliculogenesis. Endocrinology. 151, 774-782. https://doi.org/10.1210/en.2009-0751.

Yang, Y.M., Choi, E.J., 2015. Efficacy and safety of metformin or oral contraceptives, or both in polycystic ovary syndrome. Ther Clin Risk Manag. 11, 1345-1353. https://doi.org/10.2147/TCRM.S89737.

Yu, J., Ding, C., Hua, Z., Jiang, X., Wang, C., 2021. Protective effects of berberine in a rat model of polycystic ovary syndrome mediated via the PI3K/AKT pathway. J Obstet Gynaecol Res. 47, 1789-1803. https://doi.org/10.1111/jog.14730.

Zhang, Y., Hu, M., Meng, F., Sun, X., Xu, H., Zhang, J., Cui, P., Morina, N., Li, X., Li, W., Wu, X.K., Brannstrom, M., Shao, R., Billig, H., 2017. Metformin Ameliorates Uterine Defects in a Rat Model of Polycystic Ovary Syndrome. EBioMedicine. 18, 157-170. https://doi.org/10.1016/j.ebiom.2017.03.023.