### Novel therapeutic insights of Alpha lipoic acid: antioxidant, and steroidogenesis regulatory role in letrozole induced PCOS rat model

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#### 1. Abstract

Oxidative stress is widely identified as a critical etiological player in several diseases, including PCOS as it is a multifaceted endocrinological disorder affecting females during their reproductive life. Alpha lipoic acid is widely known for its role as a potent antioxidant and energy metabolism regulator. However, its mechanistic pathway in regulating PCOS hasn't been elucidated yet. For this aim, 24 adult female rats were allocated into 4 groups (n=6). The first group is the control group, the second group is the PCOS group that received letrozole orally (1mg/kg bwt) to induce PCOS, third and fourth groups are PCOS groups that were treated orally with alpha lipoic acid (ALA) (100 mg/kg bwt) and clomiphene citrate (CC) (2mg/kg bwt) respectively. After 35 days, intraovarian redox status was assessed via malondialdehyde (MDA) levels, catalase (CAT) enzymatic activity, and reduced glutathione (GSH) levels measurement. Also, serum metabolic profile and hormonal analysis were determined. Ovarian relative expression of CYP11A1, CYP17A1, and STAR genes involved in the steroidogenesis pathway was evaluated. Furthermore, ovarian histomorphometric analysis besides Caspase 3 (casp3) immune expression of granulosa cells was performed. ALA treatment revealed significant improvement in the intraovarian redox status, metabolic profile, hormonal analysis as well as relative gene expression of the steroidogenesis pathway. In addition, significant improvement in the ovarian histomorphometric analysis. Also, ALA treatment reduced granulosa cells' immune expression of casp 3. So, ALA is considered a promising adjunct therapy for PCOS owing to its antioxidant and steroidogenesis regulatory role.

Keywords: Antioxidant; Alpha lipoic acid; PCOS; Steroidogenesis

#### 2. Introduction

Alpha lipoic acid (ALA) is an organosulfur fatty acid with eight carbons produced from caprylic acid (octanoic

acid) [1]. Animals generate ALA in trace amounts, and it is present in microbes, plants, and animals [2]. ALA is commonly recognized as a universal



antioxidant and a metabolism regulator [3]. Its antioxidant potential has recently been established [2, 4–6] as well as a high scavenging action against the hydroxyl radical (HO) [7]. In addition, ALA has anti-inflammatory and anti-aging properties [8]. ALA is an essential dithiol molecule that occurs naturally. It has become a popular constituent in many pharmaceutical and food supplement products utilized in oxidative stress-dependent diseases [1]. In physiological conditions, ROS (reactive oxygen species) and antioxidant defense mechanisms are balanced. When oxidative stress (OS) occurs, the balance is disrupted by an overproduction of ROS [9]. The existing literature provides evidence of the beneficial effects of ALA in various processes, like fertilization, oocyte maturation, embryogenesis, and many other reproductive functions [10, 11].

OS affects the whole reproductive life of a woman [12]. PCOS is a complex endocrine condition that affects about 7% of females during reproductive period [13]. The common PCOS features are oligo- or anovulation, obesity, and hyperandrogenism. Additionally, PCOS has been linked to several metabolic conditions. including cardiovascular events, dyslipidemia, insulin sensitivity reduction. diabetes. type 2 and hypertension [13, 14].

Even though PCOS has been studied extensively throughout the years, its pathophysiology remains unclear. OS is

widely recognized as a critical player in the etiology of a wide range of diseases, including PCOS [15]. According to research, OS appears to be implicated in PCOS pathophysiology via steroidogenesis disruption in the ovaries, which contributes to elevated levels of androgens, folliculogenesis impairment, and infertility [16, 17]. However, the exact underlying molecular mechanism remains imprecise. Therefore, the main goal of our study is to assess the impact of ALA on letrozole-induced polycystic ovarian syndrome animal model on oxidative stress, metabolic profile, hormonal analysis, relative expression of certain genes involved in steroidogenesis mechanism (StAR, CYP11A1, and *CYP17A1*), as well as histomorphometric analysis.

#### **3. Materials and Methods**

### 3.1. Chemicals

All chemicals were of high analytical grade such as Letrozole (LTZ) from Novartis Pharma (Femara ®), Alphalipoic acid (ALA) from Sigma-Aldrich and Clomiphene citrate (CC) from Novartis Pharma (Clomid®).

#### 3.2. Animals

Twenty-four adult female Wistar rats weighing  $(200 \pm 20 \text{ g})$  were obtained from the animal house at faculty of Veterinary Medicine Cairo University.

The animals were kept at room temperature (25°C) with 12 hours of light



and 12 hours of darkness and they had unrestricted access to a standard diet and water. Female rats with successive three regular estrus cyclicity were used in this Throughout the experiment, study. animals were weighed weekly and vaginal smears were examined microscopically to detect the estrus phase. The whole experiment was validated presented and by the Institutional Animal Care and Use Committee (IACUC), Cairo University (CUIIF921) Egypt that have been formed in accordance with the guidelines for the Care and Use of Laboratory Animals 8th Edition 2011 (the Guide).

## 3.3. Establishment of the PCOS rat model

#### I. Control group (n=6):

Rats daily received 0.5% CMC carboxymethylcellulose (1 mL/day) oral solution by intragastric administration for 21 days and after that received vehicle for 14 days with normal diet and free water.

#### II. PCOS group (n=18):

Polycystic ovarian syndrome was induced using Letrozole (1mg/kg bwt) dissolved in 0.5 % CMC solution by intragastric administration for 21 days which is a well-established PCOS animal model [18]. Vaginal smears were applied every day. Animals showing prolonged diestrus stage assured induction of PCOS rat model.

### III. After 21 days, PCOS-induced rats were randomly allocated into three groups (n=6):

- **1.** The PCOS group did not receive any treatment except for the tween for 14 days.
- 2. The PCOS+ALA group that received alpha lipoic acid (ALA) for 14 days orally at a dose (100 mg/kg bwt) dissolved in a vehicle (tween solution 0.2%) [19].
- **3.** PCOS+CC group that received Clomiphene citrate (CC) for 14 days (2mg/kg bwt) as a standard drug for the treatment anovulation.

At the end of the experiment serum samples and ovarian tissue were taken for biochemical, molecular, and histological evaluation

#### 3.4. Evaluation of the estrous cycle

Α pipette containing normal physiological saline was used to flush the vagina to retrieve the epithelial cells. Subsequently, the last flush was applied to a glass slide and allowed to fully dry at room temperature. Following a oneminute crystal violet staining, the slide was rinsed twice in distilled water for one minute each time. Finally, the slide was examined using an Olympus IX71 microscope. Leukocytes, nucleated epithelial cells, and cornified epithelial cells were identified for determining the stage of the estrous cycle (proestrus, estrous, metestrus, or diestrus) [20].

### 3.5. Metabolic profile

Measurement of serum glucose, total cholesterol (TC), high-density lipoproteincholesterol (HDL-C), and triglycerides



(TG) were done using standardized kits purchased from Spectrum, Egypt. Lowdensity lipoprotein cholesterol (LDL-C) was calculated as follows: LDL-C (mg/dl) = total cholesterol - (HDL-C + VLDL-C) [21].

#### 3.6. Hormonal assay

The ELISA kits purchased from (Diagnostic Biochem Canada) was used to measure total testosterone and a rat specific ELISA purchased from (Sun Long Biotech Co., LTD) was used to measure luteinizing hormone (LH) respectively according to the manufacturer's instructions.

#### 3.7. Oxidative stress evaluation

Ovarian reduced glutathione (GSH) level was assessed [22], malondialdehyde (MDA) concentration was measured [23], and catalase (CAT) enzymatic activity was evaluated [24]. All parameters were done using specialized standardized kits purchased from Biodiagnostic Company Egypt.

#### 3.8. Molecular study

Determination of *CYP17A1*, *CYP11A1*, and *StAR* mRNA relative gene expression analysis was done by realtime PCR. Total RNA Extraction Kit (Vivantis, Malaysia) was used for total RNA extraction from the ovarian tissue. A Reverse Transcriptase reaction was performed on purified mRNA using M-MuLV (NEB#M0253). using the primers shown in Table (1). All samples were used in duplicates for each experiment.

### 3.9. Histopathology

#### 3.9.1. Histopathological and Histomorphometric analysis

Right and left ovaries were dissected out, fixed in 10% neutral buffered formalin (10 % NBF), embedded in paraffin wax, and stained with H&E for general histological structure [26]. Measurements of the granulosa and theca cells of the ovarian cysts were made for histomorphometric investigations, including their number, diameter, and thickness [27].

# 3.9.2. Immunohistochemistry for activated Caspase 3

To measure apoptosis in granulosa cells, caspase-3 in its active form was evaluated using the avidin-biotinperoxidase methodology [28]. The activated caspase 3 was detected as an indicator of apoptosis. Ovarian sections were treated to retrieve the antigen, block endogenous peroxidase, and minimize nonspecific background. The sections were then incubated with a primary antibody against caspase-3, followed by secondary antibody treatment and visualization 3.3'using diaminobenzidine tetrachloride. Hematoxylin stain used for was counterstaining. Negative control slides used only the secondary antibody IgG,

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while palatine tonsils sections served as positive controls.

#### 3.10. Statistics

Statistical analysis was conducted on the data, and the results were expressed as means  $\pm$  standard errors. The SPSS software (Version 20) was utilized for the analysis, and significant variations between groups were determined through a one-way ANOVA. To further examine the differences, the Tukey test was employed as a post hoc test. Statistical significance was defined as having p values  $\leq 0.05$ .

#### 4. Results

### 4.1. Evaluation of estrus cycle

Data presented in table (2) revealed estrus significant irregularity and complete cessation of the estrus cycle at the diestrus stage after 21 days of letrozole daily oral administration at P < 0.05. On the other side, treatment with either ALA or CC after induction leads to normalization of the estrus cycle and return to its normal cyclicity when compared to the letrozole-induced untreated group.

### 4.2. Metabolic profile

Table (3) illustrates that the letrozole-induced PCOS untreated group exhibited a significant increase in serum glucose, total cholesterol (TC), lowdensity lipoprotein cholesterol (LDL-C), and triglyceride (TG) levels, as well as a significant decrease in high-density lipoprotein cholesterol (HDL-C) level. Conversely, treatment with ALA significantly reduced serum glucose, TC, LDL-C, and TG levels, while increasing HDL-C levels compared to the untreated PCOS group. Treatment with CC also significantly reduced serum glucose, TC, and TG levels similar to the untreated PCOS group at P < 0.05.

### 4.3. Hormonal assay

Figure (1) shows that the PCOS untreated group had significantly higher levels of testosterone and LH compared to the control group. Furtherly, treatment with either ALA or CC significantly lowered the levels of testosterone and LH compared to the untreated PCOS group at P < 0.05.

#### 4.4. Oxidative stress evaluation

The results of the Ovarian redox status evaluation are demonstrated in Table. 4 shows a significant reduction of ovarian GSH levels and CAT enzymatic activity as well as a significant elevation of ovarian MDA levels in the untreated PCOS-induced group in contrast to the control group. On the contrary, treatment with either ALA or CC led to significant elevation of ovarian GSH levels and CAT enzymatic activity in addition to significant reduction to ovarian MDA levels when compared to untreated PCOS group (P < 0.05).

### 4.5. Molecular study

The current data revealed a significant elevation in the relative transcription levels of the ovarian



CYP17A1 gene of the PCO group by 2.8fold compared to the control group (P < 0.05). On the other hand, the ovaries of the PCO group exhibited a significant decrease in the genetic expression of CYP11A1 and StAR genes by 0.43-fold and 0.06-fold respectively in contrast to the control group (P<0.05). Both PCO treatments with either ALA or CC. succeeded in significantly alleviating LTZ-induced PCOS's drastic effect on expression of ovarian *CYP17A1*. CYP11A1, and StAR genes (Figure. 2).

#### 4.6. Histopathological and histomorphometry

Table (5)reveals а histomorphometric analysis of the ovary. Normal ovarian follicles with normal diameter and thickness of granulosa and theca cell layers were visible upon microscopical examination of the control group's ovary. No cysts and corpus luteum at various stages of development were observed (Fig. 3IA, B). On the other hand, the PCOS group revealed a significant increase in the ovarian follicular cysts number. These ovarian cysts' diameter was significantly larger than normal follicles observed in the control group. The thickness of the granulosa cell layer was lower, while that of the theca cell layer was higher than that of normal follicles observed in the control group. The ovarian cysts of the PCOS appeared thin-walled with group degenerated granulosa cell layer and oocyte, in addition to the presence of pale acidophilic residues inside these ovarian

cysts (Fig. 3II A, B). Interestingly, treatment of PCOS with ALA revealed a significant decrease in the number of ovarian cysts, when compared with the control group (Fig. 3A). The granulosa cell layer was significantly thicker, while the theca cell layer of the same group was significantly thinner than untreated PCOS group. On the contrary, PCOS+CC treatment showed a higher number of ovarian cysts with larger diameter and thinner granulosa cell layer as well as thicker theca cell layer (Fig. 3IIIB), when compared with the control group and PCOS+ alpha lipoic acid treatment.

### 4.7. Immunohistochemistry

Immunohistochemical analysis of the ovary of the control group revealed mild expression of caspase-3 (Fig. 4A), while PCOS group showed intense positive immune reactivity to caspase-3 (Fig. 4B). Interestingly, treatment of PCOS group with alpha lipoic acid exhibited low expression of caspase-3 (Fig. 4C), while its treatment with CC revealed moderate positive immune expression of caspase-3 (Fig. 4D) (Table.5).

#### 5. Discussion

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PCOS involves a complex interplay of hormonal, metabolic, and endocrine disruptions. A possible pathophysiology of hormonal disruption in intraovarian steroidogenesis that could contribute to polycystic ovary syndrome (PCOS) is a deficiency in aromatase activity. Aromatase is an enzyme that plays a



critical role in androgens aromatization to estrogens, and a decrease in its activity may lead to increased production of ovarian androgens, potentially leading to the development of PCOS [29, 30]. To investigate this further, researchers administered letrozole to female rats. designing a rat PCOS model that exhibited histological and pathophysiological characteristics consistent with PCOS in humans. In the rat model. current the excessive production of androgens and elevated luteinizing hormone (LH) were considered the most consistent biochemical features of PCOS [31]. PCOS-induced animal model in the present study showed significantly elevated levels of Testosterone and LH hormones as well as estrus irregularity which are indicators of successful induction of hyperandrogenic letrozoleinduced PCOS animal model as previously reported [32, 33].

The initial stage of steroidogenesis entails pregnenolone synthesis from cholesterol, which is aided by the P450 cytochrome side chain cleavage enzyme encoded by the CYP11A gene [27]. Variations in the number of tandem repeats in the CYP11A gene have been linked to fluctuations in serum testosterone levels [34]. In the subsequent phase of steroidogenesis pregnenolone and progesterone are converted into 17hydroxypregnenolone and 17hydroxyprogesterone, respectively. This conversion is facilitated by the P450c17a enzyme encoded by the CYP17 gene [35].

One of the most prevalent signs of PCOS is hyperandrogenism, which can be caused by a shift in the StAR protein's gene expression. The StAR protein is responsible for mitochondrial cholesterol transfer and steroidogenesis initiation reported [36]. We significant deterioration and alterations in the expression of StAR, CYP11A1, and CYP17A1 in the PCOS group. It has been reported that using letrozole to induce PCOS in rats showed significant dysregulation of the steroidogenesis signaling pathway which is speculated to be a direct consequence of intraovarian redox alteration associated with PCOS [27].

Recently, oxidative stress (OS) is considered to be a key player in the pathogenesis of numerous conditions, including PCOS. OS is a generic term used to describe the loss of the body's ability to combat free radicals' adverse effects, which include DNA damage, cell death, as well as various dysregulations, and the generation of free radicals [37]. Specifically, we have looked at intraovarian oxidative stress biomarkers like MDA as well as anti-oxidative biomarkers like CAT activity and reduced glutathione levels to investigate how the OS is involved in the pathogenesis of PCOS. Antioxidant biomarkers and the assessment of OS have been recommended as an effective approach for determining the likelihood of oxidative damage and related disorders [37, 38].



MDA, which is a stable compound that is produced by the polyunsaturated fatty acids lipid peroxidation, can be used as a beneficial biomarker. We reported a significant elevation of ovarian MDA levels in PCOS-induced groups when compared to the control group. MDA levels in PCOS have been previously observed and showed elevation in all PCOS subjects [37, 39, 40]. GSH is required for regulating the protein disulfide bonds as well as the elimination of electrophiles and peroxides. Redoxactive thiol groups of GSH are oxidized when GSH depletes target molecules, mediating GSH's oxidative activity [41]. Data presented in the current study revealed a significant depletion of ovarian GSH content. Our findings are consistent with studies reporting a substantial reduction in the GSH antioxidant effect in PCOS [40, 42, 43].

Catalase (CAT) plays a key player in cytoprotection from the harmful effects of hydrogen peroxide and similar hydrogen compounds. It converts peroxide (H2O2) into harmless substances like molecular oxygen and water, without generating free radicals. This reaction generates oxygen, which is utilized in other metabolic processes [44]. In conditions characterized bv inflammation, CAT enzymatic activity in the plasma of the blood increases. Most likely due to CAT release from damaged cells [44]. Conversely, individuals with atherosclerosis and diabetes have been found to exhibit low CAT activity in their blood. This suggests that there is persistent oxidative stress in the cells of these individuals [45].

This supports the growing body of evidence showing a substantial link between OS and PCOS [46]. There is a strong relationship between altered redox status and steroidogenesis signaling [47]. Previous studies have concluded that the pathogenic development of PCOS's hyperandrogenism is highly related to reactive oxygen species (ROS). Hyperandrogenism PCOS in is substantially linked to oxidative stress [48]. ROS buildup in the mitochondria leads to the upregulation of steroidogenic enzymes, which hypercauses androgenism [49].

A relatively prevalent metabolic disorder associated with PCOS is dyslipidemia. Since insulin resistance is a significant contributor to the pathophysiology of PCOS, dyslipidemia in PCOS patients may be consistent with that who are insulin resistant [50]. Insulin resistance as well as dysregulation of lipid metabolism has been correlated to ROS overproduction and OS. In addition, OS inhibits glucose absorption in muscle and adipose tissue as well as reduces insulin production from pancreatic beta cells. Furthermore, OS can lead to the activation of stress-sensitive intracellular signaling pathways resulting in insulin resistance decreased and insulin production, both in vitro [51, 52] and in vivo [53, 54].

Several extensive therapeutic approaches using several antioxidants

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have been done to ameliorate the metabolic, oxidative stress, and steroidogenesis dysregulation associated with PCOS [27, 35, 55]. However, there is still a need for innovative, safe, and effective therapeutic adjuncts to control PCOS and the related endocrine and metabolic disorders associated with it. Many of the current drugs used to manage this syndrome were reported to have side effects and many patients developed a resistance to some of them [56–59].

ALA a naturally occurring is molecule that is crucial for the proper action of many enzymatic actions involved in mitochondrial oxidative respiration. It is thought that ALA and its reduced form, dihydro lipoic acid (DHLA), have many physiological roles, including acting as potent antioxidants, metal chelators, reducing the oxidized forms of many antioxidants like vitamin C, E, and glutathione as well as a modulator of several signaling pathways [60]. ALA's potent antioxidant potential has been previously discussed [2, 6, 60]. In parallel to such previous findings, the current studv reported significant intraovarian redox modulation manifested by lowered ovarian MDA and elevated GSH as well as CAT enzymatic activity. We speculated that ALAinduced antioxidant and cytoprotection are associated with the Nrf2 mediate pathway in vivo as well as GSH regeneration via the Nrf2-downstream genes,  $\gamma$ -glutamate-cysteine ligase, and Glutathione reductase elevation, both of which are key enzymes for the synthesis of GSH [61]. Furthermore, ALA was able to restore the cellular antioxidant potential and lowered lipid peroxidation [62].

Furthermore. ALA has a lipidlowering impact that has been previously reported [63, 64]. In line with the present study, Alpha lipoic acid administration lowered significantly serum total cholesterol, triglycerides, and LDL-C as well as significantly elevated HDL-C in PCOS-induced rats when compared to untreated PCOS rats. This could be due to its role in activating Sirtuin1 (SIRT1) AMP-activated protein kinase and (AMPK) genes which have lipidlowering effects [63]. However. uncovering the precise lipid-lowering mechanism of ALA in the animal model of PCOS will require further investigation.

ALA Interestingly, showed а significant favorable modulatory role to genes involved in the steroidogenesis pathway (StAR, CYP11A1. and ALA's significant *CYP17A1*). steroidogenesis regulation could be due to its metabolic and redox signaling modulation [65–67]. Also. its steroidogenesis regulatory role in male rats has been previously reported [68]. This steroidogenesis modulation via ALA supplementation is reflected in the reversal of estrus irregularity induced by letrozole and a significant reduction in testosterone and LH hormones.

Upon histopathological investigation, PCOS induced group showed several



ovarian cysts that appeared with degenerated and thin wall granulosa cell layer. In addition, immunohistochemical staining with caspase-3(casp-3) showed significant elevation in the granulosa cell layer that was significantly improved with ALA administration. It has been previously reported that increased apoptosis of granulosa cells induces premature follicular atresia [69, 70]. On the other hand. ALA treatment led to a significant reduction of immune reaction to ALA. We speculated this antiapoptotic effect of casp-3 was due to its antioxidant potential. as it has been documented that ALA led to inhibition of oxidative stressinduced apoptosis in the brain via Nrf2 signaling pathway modulation [71].

### 6. Conclusion

ALA is considered a promising treatment and adjunct therapy for PCOS. Treatment significantly with ALA improved intraovarian redox status through reduction of MDA as well as replenishment of glutathione content and activation of CAT enzymatic activity. Furthermore, it regulates steroidogenesis pathways via adjustment of relative gene expression of ovarian (StAR, CYP11A1 and CYP17A1). This was reflected in hormonal levels of testosterone and LH and normalization of estrus cyclicity.

### Conflict of interest

The authors declare no conflict of interest

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Gene symbol	Primer Sequence	Product size (bp)	Accession number
Cytochrome P450 11 A1 (CYP11A1)	F :- 5'- GCT GGAAGG TGT AGC TCA GG -3' R:- 5'- CAC TGG TGT GGA ACA TCT GG -3'	224 bp	NM_017286.3
Cytochrome P450 17 A1 (CYP17A1)	F: -5'- ACT GAG GGT ATC GTG GAT GC -3' R:-5'- CCG TCA GGC TGG AGA TAG AC -3'	187 bp	NM_012753.3
Steroidogenic Acute Regulatory (StAR) protein	F: -5'- GCC TGA GCA AAG CGG TGT C -3' R:-5'- CTG GCG AAC TCT ATC TGG GTCTGT -3'	180 bp	NM_031558.3
Glyceraldehyde3-phosphate dehydrogenase (GAPDH)	F:- 5'-ACCACAGTCCATGCCATCAC-3' R:- 5'-TCCACCACCCTGTTGCTGTA-3'	452 bp	NM_017008.4

#### Table 1: Primers sequences used for qRT-PCR analysis

#### Table 2: Duration estrus cycle in four different groups during the experimental period

Groups	С	PCOS	PCOS+ALA	PCOS+CC
<b>Before PCOS induction (days)</b>	$4.5 \pm 0.22$ <sup>a</sup>	$4.41 \pm 0.20$ <sup>a</sup>	<b>4.67</b> ± <b>0.16</b> <sup>a</sup>	4.33 ± 0.16 <sup>a</sup>
After PCOS induction (days)	$4.42 \pm 0.20$ <sup>a</sup>	$0.00 \pm 0.00$ b	$0.00 \pm 0.00$ b	$0.00 \pm 0.00$ b
After treatment (days)	$4.50 \pm 0.22$ <sup>a,c</sup>	$0.00 \pm 0.00$ <sup>b</sup>	$4.42 \pm 0.20$ <sup>c,a</sup>	$4.5 \pm 0.18$ <sup>c,a</sup>

Values presented as means $\pm$  SD (n=5/6). Means denoted with different superscript letters in the same row are statistically significant at P < 0.05.

#### Table 3: Effect of alpha lipoic acid on metabolic profile in letrozole-

### induced PCOS rat model.

Groups	Glucose	TC	HDL	LDL	TG
Control	91.72 ± 8.38 <sup>a</sup>	89.00 ± 7.59 <sup>a</sup>	$10.20 \pm 1.92$ <sup>a</sup>	$26.20 \pm 4.82$ <sup>a</sup>	60.00± 4.18 <sup>a</sup>
PCOS	184.4 ± 9.71 <sup>b</sup>	$184.00 \pm 12.94$ <sup>b</sup>	$4.50 \pm 1.12^{\text{ b}}$	$67.00 \pm 5.70^{\text{ b}}$	<b>91.44</b> ± 5.52 <sup>b</sup>
PCOS+ALA	112.00 ± 7.58 °	119.00 ± 14.32 °	$7.40 \pm 0.96$ <sup>c</sup>	33.50 ± 4.58 <sup>c,a</sup>	69.60 ± 3.65 °
PCOS+CC	$136.00 \pm 7.41$ <sup>d</sup>	$150.00 \pm 13.22$ <sup>d</sup>	$4.10 \pm 0.89^{\text{ b}}$	58.40 ± 3.85 <sup>b</sup>	74.60 ± 5.18 °

Values presented as means± SD (n=5/6). Means denoted with different superscript letters in the same column are statistically significant at P < 0.05. TC: Total Cholesterol, TG: Triglycerides, HDL-C: High Density Lipoprotein Cholesterol, LDL-C: Low Density Lipoprotein Cholesterol.



## Table 4: Effect of alpha lipoic acid (ALA) on oxidative stress parameters in letrozole induced PCOS animal model.

Groups	GSH	CAT	MDA
Control	71.00 ± 3.81 <sup>a</sup>	10.20 ± 1.92 <sup>a</sup>	26.20 ± 4.82 <sup>a</sup>
PCOS	$19.40 \pm 3.65^{\text{b}}$	4.50 ± 1.12 <sup>b</sup>	67.00 ± 5.70 <sup>b</sup>
PCOS+ALA	44.20 ± 3.19 °	7.40 ± 0.96 °	33.50 ± 4.58 <sup>c,a</sup>
PCOS+CC	$36.80 \pm 4.32$ d	$4.10 \pm 0.89$ <sup>b</sup>	58.40 ± 3.85 <sup>b</sup>

Values presented as mean± SD (n=5/6). Means denoted with different superscript letters in the same column are statistically significant at P < 0.05. MDA: <u>Malondialdehyde</u>, GSH<u>: Glutathione reduced</u>, and CAT: <u>Catalase</u>.

# Table 5: Histomorphometric analysis of letrozole (LTZ) induced polycystic ovary co-treated with alpha lipoic acid and clomiphene (CC)

Parameters	С	PCOS	PCOS +ALA	PCOS +CC
Number of ovarian cysts/follicles	<b>3.33±1.53</b> <sup>a</sup>	17.67±1.45 <sup>b</sup>	<b>3.33±0.58</b> <sup>ac</sup>	8.00±1.00 <sup>d</sup>
Diameter of ovarian cysts/follicles	244.42±38.97 <sup>a</sup>	453.25±80.61 <sup>b</sup>	258.10±28.21 <sup>ac</sup>	438.55±168.24 <sup>db</sup>
Granulosa cell layer thickness	70.42±29.72 <sup>a</sup>	35.22±4.83 <sup>b</sup>	53.14±11.25 <sup>abc</sup>	31.36±1.04 <sup>dbc</sup>
Theca cell layer thickness	21.93±5.65 <sup>a</sup>	16.02±0.63 <sup>b</sup>	11.26±1.45 <sup>abc</sup>	14.83±0.24 <sup>dbc</sup>
Caspase-3	5.12±0.88 <sup>a</sup>	27.53±3.92 <sup>b</sup>	6.60±1.91 <sup>ac</sup>	15.47±0.50 <sup>d</sup>

a,b,c,d Means with different superscripts within the same row differ significantly at P < 0.05. Caspase-3 immunostaining (mean area%  $\pm$ SD).





Fig. 1: Effect of alpha lipoic acid on hormonal levels in letrozole-induced PCOS rat model. Values are presented as mean ± SD (n=5/6). Means denoted with different superscript letters are statistically significant at P < 0.05. LH: Luteinizing hormone.



Fig. 2: The effect of ALA treatment on the relative mRNA ovarian expression of *CYP17A1*, *CYP11A1*, and *StAR* genes. Data are represented as mean ± SD. Different letters indicate significant differences from the corresponding groups at p≤ 0.05.





Fig. (3 III): A photomicrograph of H&E-stained sections (40X) of the ovary of female albino rat showing: A) PCOS+ alpha lipoic acid treated group revealing ovary with fewer ovarian cysts and increased granulosa cell layer. B) PCOS+CC exposed group exhibiting numerous ovarian cysts. Notice few cysts appeared smaller in size with a thickened granulosa layer.

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**Fig. 4:** A photomicrograph of caspase-3-immunostained sections (400X) of the ovary of a female albino rat showing: A) Control group with a mild expression of caspase-3. B) PCO group with intense positive immunoreactivity to caspase-3. C) PCO+ ALA treated group with low reaction to caspase-3. D) PCO+CC exposed group with moderate expression of caspase-3

