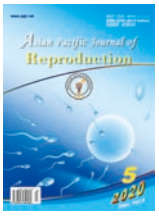

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Protective and therapeutic effect of protocatechuic acid in assessment of letrozole-induced polycystic ovary syndrome in rats

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ABSTRACT

Objective: To investigate the potential activity of protocatechuic acid in female Wistar rats with letrozole-induced polycystic ovary syndrome (PCOS).

Methods: Thirty rats were divided into five groups of six each. Group 1 received 0.5% carboxy methyl cellulose orally and served as the normal control group; group 2 was treated orally with 1 mg/kg of letrozole daily for 21 days and served as the PCOS induced group; group 3 was orally administered with letrozole of 1 mg/kg for 21 days and further administered with standard drug of clomiphene citrate at a dose of 1 mg/kg body weight in 0.5% carboxy methyl cellulose per oral and served as the standard group; groups 4 and 5 were administered with letrozole of 1 mg/kg for 21 days and further treated with protocatechuic acid orally at low dose of 5 mg/kg body weight and high dose of 15 mg/kg body weight respectively for 15 days. At the end of the study period, rats were subjected for the estimation of invasive blood pressure and heart rate, biochemical estimations and antioxidant assay. In addition, ovarian histomorphology was examined.

Results: The PCOS was confirmed in the letrozole induced rats with increased concentration of androgen, abnormal lipid levels, glucose, glycosylated haemoglobin and also depletion of antioxidants. After protocatechuic acid treatment, the increased levels of testosterone due to induction of PCOS were restored to normal levels. Additionally, there was a consistent decrease in luteinizing hormone and follicle stimulating hormone levels in the treatment groups, followed by decrease in the number of cysts after treatment with protocatechuic acid. Histopathological observations showed a remarkable recovery of the ovarian tissue and the presence of normalized structure of antral follicle. Protocatechuic acid treatment restored all the parameters to normalcy and abolished cysts formation in ovaries of female rats.

Conclusions: Protocatechuic acid shows potential protective effects in letrozole-induced PCOS rats. The protective effect is comparable to that of clomiphene citrate and thus shows its potential in the treatment of PCOS.

KEYWORDS: Polycystic ovary syndrome; Letrozole; Protocatechuic acid; Fertility; Ovulation

1. Introduction

Polycystic ovary syndrome (PCOS) is a hormonal disorder common in women during their reproductive age which can lead to infertility. It is also known to be one of the most common endocrine disorders causing infertility among women[1,2]. It is a multifactorial based ovarian disorder accompanied by excess production of androgen and according to the literature the etiology of PCOS has not been fully elucidated.

Women affected with PCOS have impaired metabolism of estrogen and elevated androgens. The typical neuro endocrine feature of PCOS includes increased serum concentration of luteinizing hormone (LH), LH/follicle stimulating hormone (FSH) ratio and increase in the amplitude and frequency of LH secretion[3-6].

PCOS has heterogenous signs and symptoms and in some cases there was severe imbalance of reproductive, endocrine and metabolic functions[7,8]. Among the multi factorial conditions, insulin resistance appears to be a central feature with the increased risk of developing type- Ⅱ diabetes, hyperinsulinemia and central obesity. The risk factors associated with PCOS are cardiovascular disease, endothelial dysfunction and dyslipidemia[9,10]. Hence, an appropriate treatment of individuals is essential and need of the hour due to increased adverse consequences.

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In PCOS, a deficiency in aromatase activity is responsible for the intraovarian disturbance in steroidogenesis is thought to trigger anovulation. Aromatase catalyzes the rate-determining step during the biosynthesis of estrogens from androgens and causes hormonal imbalance due to decreased activity of the enzyme. This in turn leads to circulating hyperandrogenism and intraovarian excess of androgen and results in polycystic ovaries[11,12]. The similar effects of hyper-androgenism during the pre-pubertal stage in a PCOS murine model was obtained by daily administration of letrozole (1 mg/kg) orally for 28 consecutive days in pre-pubertal rats[13]. This dose of letrozole ensures a hyper-androgenized status equivalent to that found in women with PCOS, which is 1 mg/kg letrozole in total per day[14]. Pre-pubertal hyper-androgenism alters ovarian functions since letrozole treated rats showed increased serum estradiol and progesterone levels.

Follicular atresia and abnormal follicular development is observed due to induced elevation of androgen levels in the ovary[15]. Letrozole induction was reported to cause hyperglycemic condition which may contribute to insulin resistance, hyperlipidemia leading to a metabolic syndrome[16,17].

Clomiphene citrate, exogenous gonadotropins, insulin sensitizers, such as metformin, are used to reduce insulin resistance which results in a reduction of ovarian androgen production and a consequent improvement in menstrual cyclicity[18]. Although many drugs have been shown to be effective in the treatment of PCOS, alternatives are continuously being searched because of actual or possible side effects ranging from arthritis, joint or muscle pain, psychological disturbances and lactic acidosis[19,20]. In traditional medicine, there are many exceptional herbal drugs that have the potential competence of preventing and curing PCOS. However, many herbs are not evaluated and extensive research was not done on its mechanism of action.

Protocatechuic acidis one of the widely distributed and commonly found compound in human diet like bran, grain brown rice and onion and also it is found in many fruits, such as plums, grapes and nuts[21]. This bioactive compound is well known for its biological properties and a range of potential pharmacological activities such as anti-oxidant, antibacterial, anticancer, antiulcer, antidiabetic, antiaging, antifibrotic, antiviral, anti-inflammatory, analgesic, antiatherosclerotic, cardiac, hepato-protective, neurological and nephroprotective[22].

In a recent *in vivo* study, it was observed that antioxidant properties and the ability to block oxidative stress signal transduction, the protocatechuic acid showed protective action against hepatic damage induced by tert-butyl hydroperoxide in rats[23].

However, there was no scientific evidence conducted on these lines in the poly cystic ovary syndrome in rats. Hence, the present study was designed to understand and evaluate the effects of protocatechuic acid by using various estimations of hormonal levels and biochemical parameters in normal and letrazole-induced PCOS rat model.

2. Materials and methods

2.1. Experimental animals

The cyclic, adult female Wistar Albino rats aged 16-18 weeks, weighting 160-200 g were used in the study. The rats were procured from Animal House Facility of National Institute of Nutrition, Tarnaka, Hyderabad, India. The rats were housed in an animal house facility approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals and were acclimatized for two weeks. Rats were fed with pellets of feed and drinking water *ad libitum* and were maintained in controlled experimental conditions in standard polypropylene cages. The rats were housed in controlled environment of (22±3) ℃, (55±5)% humidity and a 12 light/dark cycle.

2.2. Drugs and reagents

Protocatechuic acid was procured from Sigma Chemicals Co., St. Louis, MO, USA. Letrozole was purchased from Sun Pharmaceuticals, Mumbai, India. Clomiphene citrate tablets were purchased from Ar-Ex Laboratories Private Limited, Goregaon (E), Mumbai, India. All other chemicals used for the study were of analytical grade reagents. The glucose, cholesterol, triglycerides, high density lipoprotein and glycosylate hemoglobin diagnostic kits were purchased from ERBA Diagnostic, USA.

2.3. PCOS induction

The PCOS in adult female rat was induced by the oral administration of letrozole at a dose of 1 mg/kg body weight using 0.5% carboxy methyl cellulose as vehicle once a day daily for 21 days[24]. The control group received vehicle *i.e.* 0.5% carboxy methyl cellulose. Vaginal smears were collected daily and were observed microscopically by using Giemsa stain to confirm the induction of the PCOS in female rats. The induction of PCOS was confirmed by microscopically observing the persistent vaginal cornification of vaginal smears using Giemsa stain. The irregularity of estrous cycle confirmed PCOS.

2.4. Study design

Thirty female albino Wistar rats with normal estrous cycle were divided into five groups, with six rats in each group. Group 1 was the normal control group, receiving 0.5% carboxy methyl cellulose orally. Group 2 was orally administered with letrozole of 1 mg/kg for 21 days and served as PCOS-induced group without treatment (the positive control group). Group 3 was orally administered with letrozole of 1 mg/kg for 21 days and further administered with standard drug of clomiphene citrate at a dose of 1 mg/kg body weight in 0.5% carboxy methyl cellulose per oral and served as the standard group. Groups 4 and 5 were administered with letrozole

of 1 mg/kg for 21 days; as in estrous cycle, the rats remained static in the dietrus phase, which was predominantly leukocyte cells. Further, the rats were administered by protocatechuic acid with the dose of 5 mg/kg (low dose) and 15 mg/kg (high dose) body weight respectively in 0.5% carboxy methyl cellulose per oral for 15 days *i.e.*, from day 22nd-36th and designated as treatment groups[25].

Estrous cyclicity was monitored by vaginal smears obtained between 08:00 and 12:00 a.m., and was assessed by light microscopy for the relative proportion of leukocytes, epithelial and cornified cells found in daily vaginal lavages, which characteristically changed during different stages of the estrous cycle. Progression of PCOS was monitored by observing for persistent vaginal cornification and establishment of PCOS was considered positive when the cornification persisted continuously for four successive days. The estrous cycle of individual rat was monitored daily by examining vaginal smear. Vaginal smears were observed microscopically by using Giemsa stain to confirm the induction of the PCOS in female rats. The PCOS control group after 21 days and rats from other groups after 36 days were fasted overnight and were anaesthetised. By using retino orbital puncture, blood was collected and serum was separated by centrifugation at 782×*g* for 15 min and was used for estimation of hormones, glucose, glycosylated haemoglobin, lipid parameters and histopathological investigations.

The rats were then sacrificed by using cervical dislocation method. The ovaries and uterus were excised and cleaned of fat and weighed. After excision, ovaries were freed from blood and cleaned with ice cold saline and homogenized by using 10% ice cold potassium chloride for antioxidant estimation.

2.5. Measurement of invasive blood pressure and heart rate

An attempt was made to investigate the blood pressue and heart rate as PCOS was closely related to several cardiometabolic risk factors and had an estimated 4-11 fold increased risk of coronary heart disease[26].

On termination day of the study, four animals from each group were anesthetized with ketamine. Arterial blood pressure was recorded from carotid artery. A polyethylene catheter (PE-50) [1 mm O.D.] for rats was attached to a pressure transducer filled with heparinized saline, and inserted into the carotid artery and tied. Pressure fluctuations in the artery were transmitted along the catheter tubing to the transducer's diaphragm, which moved in response to the blood pressure. The diaphragm movements were converted into a varying electrical signal which were then amplified through a bridge amplifier and measurements were recorded using Power lab system (AD instruments, Australia).

2.6. Biochemical estimations

2.6.1. Hormonal assay

Hormonal analysis were done by using five different kits which were purchased from ADVIA Centaur, Siemens Healthcare Diagnostics Inc., USA. The estimation included testosterone, estrodiol, progesterone, LH and FSH. Hormones were assayed by competitive chemiluminescent immunoassay using automated instrument ADVIA Centaur, Siemens Healthcare Diagnostics Inc., USA.

2.6.2. Measurement of fasting blood glucose and glycosylated hemoglobin (HbA1c) levels in serum

Fasting blood glucose was measured by Trinder's method using a commercial diagnostic kit procured from ERBA Diagnostics, USA[27].

The HbA1c levels were measured by cation-exchange method using a diagnostic kit purchased from ERBA Diagnostics, USA[28].

2.6.3. Assessment of lipid profile

Plasma lipid profiles including triglycerides (TG), total-cholesterol (TC), and high density lipoprotein (HDL)-cholesterol were analysed by using commercially available assay kits from ERBA Diagnostics, USA. All the procedures were carried out by using the manufacturer's labelled instructions. Low density lipoprotein (LDL)-cholesterol was calculated by using Friedewald's formula[29].

2.6.4. Antioxidant assay

2.6.4.1. Catalase

Catalase activity was determined in 50 mL of sample mixed with equal proportion of substrate for 60 s then 100 mL of 32.4 mM ammonium molybdate solution was added and the absorbance was measured at 405 nm wavelength. One unit of the enzyme was defined as μmoles of hydrogen peroxide degraded/min/mg of protein[30].

2.6.4.2. GSH

Glutathione content was estimated according to the method reported by Alam *et al*[30]. Ovarian homogenate of 0.25 mL (10%) was added to equal volume of ice cold 5% trichloroacetic acid. The precipitate was removed by centrifugation at 1 252×*g* for 10 min. To 1 mL aliquot of supernatant, 0.25 mL of 0.2 M phosphate buffer, pH 8.0 and 0.5 mL of 5, 5'-dithio-bis 2-nitrobenzoic acid was added. The absorbance was read at 412 nm using UV visible spectrophotometer. The values were expressed as units/mg protein.

2.6.4.3. Assay of thiobarbituric acid reacting species (TBARS) content

The homogenate (0.25 mL) was pipetted into (15 mm×100 mm) test tubes and incubated at 37 ℃ in a metabolic shaker for 1 h. An equal volume of homogenate was pipetted into a centrifuge tube, placed at 0 ℃ and marked as 0 h incubation. After 1 h of incubation, 0.5 mL of 5% (w/v) chilled trichloroacetic acid, followed by 0.5 mL of 0.67% thiobarbituric acid (w/v) was added to each of the test tube and centrifuged at 1 000×*g* for 15 min. Thereafter, the supernatant was transferred to other test tubes and was placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535 nm in a UV visible spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molar extinction coefficient of (1.56×10^5) M^{-1} cm^{-1} and expressed as nmol of TBARS formed min^{-1} mg^{-1} of protein[30].

2.7. Ovarian histomorphology

Ovary tissues were fixed in 10% buffered formalin for 48 h and were embedded in paraffin wax. Paraffin-embedded tissue sections were de-waxed, rehydrated, stained with hematoxylin and eosin. The stained slides were then mounted by using DPX under glass cover slips. The slides were then observed under light microscope (Olympus, Tokyo, Japan) ×10 magnification connected to a camera for the capturing images of the stained slides.

2.8. Statistical analysis

Statistically analysis was done by using one-way analysis of variance followed by Newman-Keuls multiple comparison tests and data were expressed as mean±standard deviation (mean±SD). $P<0.05$ was considered to be statistically significant. The statistical analysis were performed by using Graph pad prism 5.0 software (Graph Pad Software Inc, California).

2.9. Ethics statement

The study protocol was approved by the Institutions Animal Ethics Committee of Anurag Group of Institutions, Ghatkesar, Hyderabad as per the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals with protocol number (I/IAEC/AGI/015/2018/WR).

3. Results

3.1. Arterial blood pressure and heart rate

No significant difference was found between the control group and the PCOS group in arterial blood pressure and heart rate ($P>0.05$).

3.2. Organ weights

Letrozole treatment in female rats led to increase in ovarian weight and decrease in uterine weight but the increase was not significant as compared to the control group. Repeated administration of low dose and high dose of protocatechuic acid showed no significant changes in the ovarian and uterine weight ($P>0.05$).

3.3. Serum hormonal profile

The serum levels of LH and FSH significantly increased in the positive control group ($P<0.01$). The increased LH and FSH levels were significantly reduced in the standard, low dose and high dose groups when compared with the positive control group (P all<0.01) (Table 1). The ratio of LH and FSH was calculated in each group as shown in Figure 1 as increased serum levels of LH/FSH ratio were a key symptom of PCOS. Figure 1 showed the LH/FSH levels ratio were augmented in the PCOS-induced positive control group which were regulated in the standard and high dose groups.

Reduced estradiol levels were significantly restored ($P<0.05$) in the standard and high dose groups as compared with the PCOS-induced positive control group. Testosterone levels showed a significant decrease ($P<0.001$) in the standard, low dose and high dose groups. Progesterone levels were also increased significantly ($P<0.001$) in the standard, low dose and high dose groups comparing with the positive control group (Table 1).

3.4. Measurement of fasting blood glucose and HbA1c levels in serum

The PCOS-induced group showed a significant increase in glucose level ($P<0.001$) and HbA1c level ($P<0.001$) as compared with the negative control group. All the treatment groups exhibited a significant decrease in glucose level ($P<0.001$) and HbA1c level ($P<0.001$) as compared with the PCOS-induced group (Table 2).

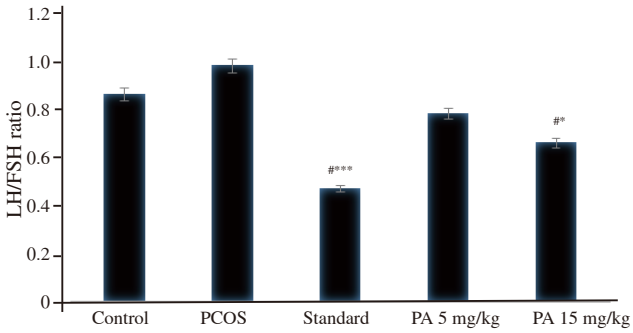

Figure 1. Effect of treatment groups on LH/FSH ratio. #: compared with the PCOS-induced group; *$P<0.05$, ***$P<0.001$, $n=6$ in each group. PA: Protocatechuic acid; LH: luteinizing hormone, FSH: follicle stimulating hormone.

Table 1. Effect of various treatments on serum sex steroids.

Parameters	Control	PCOS (Letrozole)	Standard (Clomiphene citrate)	Low dose (Protocatechuic acid 5 mg/kg)	High dose (Protocatechuic acid 15 mg/kg)
Estradiol (pg/mL)	28.28±9.14	$14.38\pm5.79^{\triangle *}$	$27.88\pm7.50^{\#*}$	20.08 ± 4.98^{ns}	$30.25\pm5.40^{\#*}$
Progesterone (ng/mL)	33.49±5.60	$15.99\pm3.74^{\triangle ***}$	$29.78\pm2.29^{\#***}$	$25.61\pm2.38^{\#***}$	$30.69\pm3.84^{\#***}$
Testosterone (ng/dL)	27.45±3.70	$103.10\pm6.19^{\triangle}$	$22.58\pm2.89^{\#***}$	$26.63\pm1.74^{\#***}$	$25.13\pm6.20^{\#***}$
LH (mIU/mL)	0.59±0.08	$0.96\pm0.29^{\triangle **}$	$0.33\pm0.08^{\#**}$	$0.58\pm0.11^{\#**}$	$0.42\pm0.09^{\#**}$
FSH (mIU/mL)	0.65±0.14	$0.98\pm0.11^{\triangle **}$	$0.70\pm0.08^{\#**}$	$0.74\pm0.06^{\#**}$	$0.64\pm0.04^{\#***}$

$^{\triangle}$: compared with the normal control group; $^{\#}$: compared with the PCOS-induced group (the positive control group); $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, ns: not significant, $n=6$ in each group.

Table 2. Effect of treatment groups on plasma glucose and glycosylated haemoglobinin.

Parameters	Control	PCOS (Letrozole)	Standard (Clomiphene citrate)	Low dose (Protocatechuic acid 5 mg/kg)	High dose (Protocatechuic acid 15 mg/kg)
Glucose (mg/dL)	59.00±3.91	150.2±6.00$^{\triangle ***}$	70.88±1.94$^{\#***}$	65.66±1.75$^{\#***}$	59.85±1.32$^{\#***}$
Glycosylated haemoglobin (%)	7.89±0.24	11.73±0.58$^{\triangle ***}$	8.62±0.34$^{\#***}$	8.08±0.25$^{\#***}$	7.28±0.19$^{\#***}$

$^{\triangle}$: compared with the normal control group; $^{\#}$: compared with the PCOS-induced group (the positive control group); $^{***}P$<0.001, n=6 in each group.

Table 3. Effect of various treatments on serum lipid profile.

Parameters	Control	PCOS (Letrozole)	Standard (Clomiphene citrate)	Low dose (Protocatechuic acid 5 mg/kg)	High dose (Protocatechuic acid 15 mg/kg)
TG (mg/dL)	82.00±2.54	141.40±1.82$^{\triangle ***}$	92.28±2.17$^{\#***}$	93.40±1.84$^{\#***}$	87.51±1.89$^{\#***}$
TC (mg/dL)	78.01±1.33	131.70±2.80$^{\triangle ***}$	96.36±1.78$^{\#***}$	105.00±4.14$^{\#***}$	91.52±1.15$^{\#***}$
HDL (mg/dL)	34.941±1.06	17.95±0.57$^{\triangle ***}$	26.27±1.12$^{\#***}$	22.23±1.09$^{\#***}$	29.05±1.24$^{\#***}$
LDL (mg/dL)	28.38±0.87	86.63±2.18$^{\triangle ***}$	30.76±1.88$^{\#***}$	40.35±2.02$^{\#***}$	30.48±0.53$^{\#***}$

$^{\triangle}$: compared with the normal control group; $^{\#}$: compared with the PCOS-induced group (the positive control group); $^{***}P$<0.001, n=6 in each group. TG: triglycerides; TC: total-cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein.

Table 4. Effect of various treatments on antioxidation and lipid peroxidation.

Parameters	Control	PCOS (Letrozole)	Standard (Clomiphene citrate)	Low dose (Protocatechuic acid 5 mg/kg)	High dose (Protocatechuic acid 15 mg/kg)
GSH (Units per mg protein)	10.88±4.03	3.95±2.82$^{\triangle *}$	6.84±3.90$^{\#ns}$	5.48±3.69$^{\#ns}$	12.32±2.62$^{\#*}$
Catalase (micro moles of H_2O_2 consumed per mg protein)	4.20±2.14	0.37±0.29$^{\triangle *}$	2.88±1.24$^{\#ns}$	2.90±1.54$^{\#ns}$	4.92±2.13$^{\#**}$
TBARS (nmol TBARS formed per minute per mg protein)	56.45±15.47	82.47±13.50$^{\triangle *}$	50.73±14.91$^{\#*}$	51.83±14.03$^{\#*}$	42.50±10.70$^{\#***}$

$^{\triangle}$: compared with the normal control group; $^{\#}$: compared with the PCOS-induced group (the positive control group); $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, ns: not significant, n=6 in each group.

3.5. Measurement of serum lipid profile

Administration of letrozole (1 mg/kg) to female rats caused significant changes in serum lipid levels as compared with the control group. TG, TC and LDL were significantly increased (P<0.001) whereas HDL levels was significantly decreased (P<0.001) in the PCOS-induced group. Clomiphene treatment caused a significant reduction in TG, TC and LDL levels as compared with the PCOS-induced group. Low dose as well as high dose of protocatechuic acid significantly decreased the levels of TG, TC and LDL levels significantly (P<0.001) as compared with the PCOS-induced group. Clomiphene treatment, low dose as well as high dose of protocatechuic acid were effective in increasing the HDL levels significantly (P<0.001) in comparison to the PCOS-induced group.

3.6. Effect of protocatechuic acid on antioxidant activity and lipid peroxidation (TBARS)

The PCOS-induced group caused a reduction in antioxidant enzyme activity of catalase (P<0.05) and GSH (P<0.05) whereas there was an increase in TBARS levels (P<0.05) as compared with the control group. Low dose (5 mg/kg) group showed no significant activity on catalase and GSH. A significant decrease in TBARS was observed (P<0.05) in the low dose group. High dose (15 mg/kg) group potentially augmented the antioxidant enzyme activity of GSH (P<0.05) and catalase (P<0.01) significantly while reduced TBARS level (P<0.001) when compared with the PCOS-induced group (Table 4).

3.7. Effect of protocatechuic acid on histopathology of ovary

The histological changes in PCOS rats were studied and also observations were recorded. In the control animals, the sections of ovaries showed normal healthy follicles in the cortex region. Also, there was no fibrosis in the cortex section of the ovary as depicted in Figure 2A. Letrozole-treated rats exhibited numerous follicular cysts of varying sizes with diminished granulosa cells. Corpora lutea were completely absent, indicating anovulation. There was also an increase in thickness of the antral follicle theca layer and they were accompanied with atretic follicles containing fluid filled antrum and showed higher incidence of pyknotic granulosa cells (Figure 2B). With the clomiphene citrate treatment, no follicular cysts were found in the follicles. Many corpus luteum were found in the cortex region of the ovary, indicating that rats reverted to regular cyclic condition (Figure 2C). Sections from low dose (5 mg/kg) of protocatechuic acid group exhibited mild numbers of cysts and follicles larger in size with few corpora lutea (Figure 2D). Cysts were absent and normal sized healthy follicles at different developmental stages with oocytes were found in section with high dose (15 mg/kg) of protocatechuic acid group (Figure 2E).

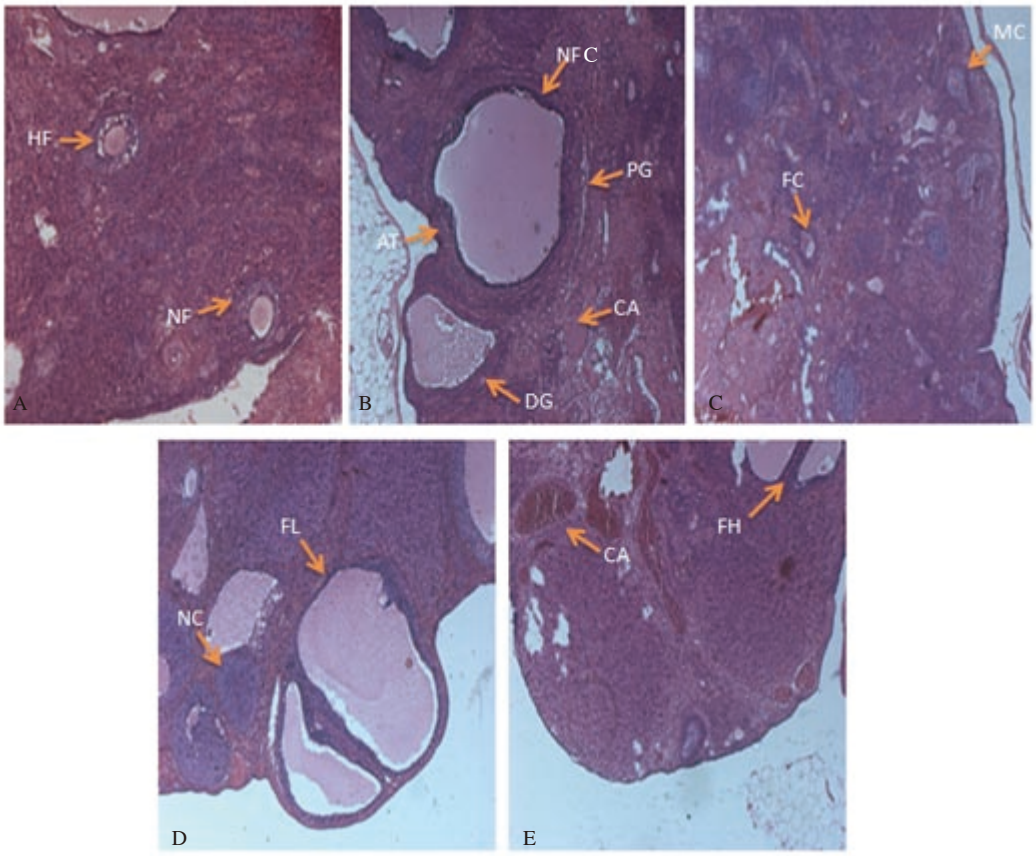

Figure 2. Histopathological sections of female Wistar rats ovaries treated with procatechuic acid (hematoxylin and eosin; ×10 magnification). A: Control ovaries show follicles in the cortex. B: PCOS-induced ovary depicts muiltiple cysts inside the follicles. C: Standard treated ovaries show no follicular cysts in the follicles. D: Protocatechuic acid treated with low dosage of 5 mg/kg shows mild to moderate number of follicles in the cyst. E: Protocatechuic acid treated with high dosage of 15 mg/kg shows follicles at different development stages of corpora lutea. HF: Normal healthy follicles; NF: No fibrosis; NFC: Numerous follicular cysts; DG: Diminished granulosa cells; CA: Corpora lutea absent; AT: Antral follicle theca layer thickness increased; PG: Pyknotic granulosa cells; FC: Follicular cysts absence; MC: Many corpous luteum; NC: No cysts; FL: Follicles larger in size; CA: Cysts absent; FH: Follicles healthy, with oocytes.

4. Discussion

PCOS is one of the most common female endocrine disorders. PCOS is caused due to deficiency in the activity of the aromatase enzyme. It is one of the many intra-ovarian disturbances. In the current study, PCOS rat model was established after treatment with letrozole-aromatase inhibitor and it showed many histological and biochemical findings consistent with human PCOS[24].

The inhibition of aromatase activity caused an increase of ovarian androgens and consequently resulted in hyperandrogenism, which is an indication of PCOS[31]. The plasma levels of androgen and LH were the most consistent hormonal feature of rats with PCOS and low levels of progesterone and estradiol were also observed in rats with PCOS[32].

The augmented estrogen levels have a negative feedback effect and resulted in steady increase in levels of LH and LH: FSH ratio which in turn served as biomarkers for diagnosing PCOS[33]. The same findings of augmented levels of LH and LH: FSH ratio were observed in our study. This hormonal imbalance created by letrozole resulted in irregular and prolonged estrus cycle in PCOS rats.

The previous studies conducted have reported that being a non-steroidal aromatase inhibitor letrozole blocks the conversion of testosterone to estradiol and thus reduces the levels of estrogen[17]. In this present study we found the levels of testosterone were elevated in the letrozole control group and the treatment groups were able to normalize serum testosterone levels similar to that of clomiphene citrate.

Serum levels of progesterone and estrogen were decreased in the PCOS-induced group. The repetitive administration of high dose of protocatechuic acid (15 mg/kg) significantly increased the estrogen concentration due to inhibition of aromatase in the PCOS-induced group. High and low doses of protocatechuic acid significantly inhibited the decrease in progesterone levels, indicating the inhibition in anovulation due to low levels of progesterone[34].

Ovarian weight in PCOS-induced rats was increased and the uterine weight was decreased. However, the repeated administration of low

dose and high dose of protocatechuic acid showed no significant changes in the ovarian weight and uterine weight.

PCOS is also a metabolic disorder associated with type Ⅱ diabetes mellitus and insulin resistance. Further, the developed insulin resistance in diabetes is also the clinical feature of PCOS[35,36] In our study PCOS-induced rats showed marked increase in fasting blood glucose and glycosylated haemoglobin levels. The excess of glucose present in the blood in the hyperglycemic conditions reacts with haemoglobin to form glycosylated haemoglobin[37]. The low dose and high dose of protocatechuic acid significantly prevented the increase in fasting blood glucose and HbA1c, which indicates the potential of protocatechuic acid in preventing insulin resistance and diabetic complication.

One of the consequences of this insulin resistance leads to imbalances in lipid profile and results in dyslipidemia and hyperandrogenemia[38]. In this study the induction of PCOS with letrazole had similar results in changing the lipid profiles, insulin and glucose levels. The PCOS induced group substantially showed the increase in TC, TG, LDL and decrease in HDL levels. The high and low doses of protocatechuic acid significantly contributed to its antihyperlipidemic action by showing the decrease in serum TC, TG, LDL and increase in HDL levels.

Oxidative stress is generally regarded as the phenomena where the generation of ROS causes disruption in the normal functionality of biological system. The ROS level increased significantly in the ovaries of PCOS group which clearly showed an increase in the oxidative stress and further contributed to an increase in androgen production in polycystic ovaries[39]. The increase in ROS level indicates the molecular damage in the cellular structure and the tissue was mainly due to the over production of free oxidative radicals. ROS value was also decreased significantly with the high and low doses of protocatechuic acid groups, which is due to its antioxidant activity. Catalase and GSH activity were significantly diminished in the PCOS group and concomitant treatment with protocatechuic acid restored their activities. The exact mechanism through which protocatechuic acid induces antioxidative action is not clear but it reduces the causative agents of oxidative stress[23].

Lipid peroxidation is a free-radical mediated promulgation of oxidative tissue damage and induces free radical damage to the components of cell membrane. This consequently leads to cell necrosis and the cessation of lipid peroxidation occurs by antioxidants through their enzymatic action or by free radical scavenging activity[30]. TBARS is formed as a by-product of lipid peroxidation. TBARS formation significantly increased in polycystic ovaries. The high and low doses of protocatechuic acid was able to restore the lipid peroxidation levels to its normalcy.

Women with PCOS are prone to higher risk of hypertension as a long term consequence[40]. We measured the parameters studies *i.e* changes in blood pressure and heart rate of letrozole induced PCOS model. The results showed that there were no significant differences in these parameters among the control and PCOS-induced groups.

Histopathological observations of ovaries in letrozole induced PCOS model showed the resemblance to human PCOS, showing sub capsular cysts lined with thin layer of granulosa cells, hyperplasia and abnormal follicular growth. The treatment groups of protocatechuic acid led to the reduction in the number of cysts and as well as decreased incidence of pyknotic granulosa cells. These histological findings were pinpointing towards the normal estrous cyclicity as well as ovulation due to presence of corpora lutea. Normal and visible granulosa cell layer was observed with follicles of different stages of development along with oocytes in histopathological studies.

In the letrazole induced PCOS study, the treatments groups were able to decrease the levels of testosterone and ratio of LH and FSH to normalcy simultaneously. The levels of progesterone and estradiol indicate the promising results of the protocatechuic acid. The study results revealed that the fasting glucose and glycosylated hemoglobin levels were decreased, indicating the potentiality of protocatechuic acid. The treatment groups also exhibited the antihyperlipidemic activity.

The anti-oxidant activity plays an important role in management of PCOS; we have conducted a wide range of anti-oxidant studies for determining its potentiality. Overall, the results showed positive activity towards letrozole induced PCOS model.In our study, protocatechuic acid improved sex hormones in PCOS rats by significantly up-regulating FSH and estradiol concentration and down-regulating the testosterone and LH levels. Additionally, protocatechuic acid exhibited the antiandrogenic effect by depicting the conversion of testosterone into estradiol. This improved estradiol levels, thus leading to decrease in multiple cyst formation and increase in number of corpus luteum. This may be due to potential antioxidant activity shown by protocatechuic acid. Histopathological studies revealed that there is a decrease in number of cysts showing normal estrous cyclicity and ovulation due to corpus luteum[41]. PCOS-affected women showed metabolic disorders due to increased glucose levels and HbA1c levels[42]. Whereas in our study the protocatechuic acid treated groups showed the decrease in glucose levels and HbA1c levels which indicates its prevention insulin resistance.

It is demonstrated that letrozole-induced PCOS model is associated with oxidative stress in ovaries. Based on the results obtained, the antioxidant activity of protocatechuic acid showed protection with improved functionality of ovaries.

In conclusion, the present study presents the potential activity of protocatechuic acid in many beneficial effects similar to clomiphene citrate in treating PCOS condition and inducing ovulation. Protocatechuic acid shows antiandrogenic and antihyperglycemic effects, antioxidant and glycemic status as well as recovered

ovarian cysts in letrozole-induced PCOS rats. Thus, protocatechuic acid shows a blend of pharmacological activities like estrogenic, antihyperlipidmic, antioxidant and hypoglycemic effects which could be useful in managing PCOS condition and preventing ovarian cell dysfunction, ovulation and thereby improving the fertility index. Together, broad spectrum biological effects of protocatechuic acid make it a promising drug for treating clinical and pathological abnormalities in PCOS condition.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

Rupavath Chandrashekhar and Jagruthi Jeripothula contributed in terms of performing the experiment and submitting the data for further analysis. Bakshi Vasudha designed the research work and analyzed the data with inferences. The remaining authors Nelavelli Lakshmi Bhavani and Bhavani Ram contributed in terms of technical support and manuscript preparation.

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