P. Sudhakar et al /J. Pharm. Sci. & Res. Vol. 11(8), 2019, 2827-2832

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Development of an animal model for Polycystic Ovarian Syndrome in relation to reproductive and metabolic phenotype

P. Sudhakar[1*], R. Kothai[2], V. Thenmozhi[1]

[1]*Department of Pharmacology and Toxicology, Swamy Vivekanandha College of Pharmacy, Tiruchengode, Namakkal, Tamil Nadu- 637 205, India*

[2]*Department of Pharmacology, Vinayaka Misson's College of Pharmacy, Vinayaka Missions Research foundation, Salem, Tamil Nadu, India*

Abstract

Aim: This study was aimed to develop the Polycystic ovary syndrome (PCOS) animal model in relation to reproductive and metabolic phenotype using antiepileptic medications by the mechanism of altering hypothalamic-pituitary-ovarian axis.

Materials and Methods: Twenty four female adult virgin rats six per each were used and grouped as letrozole (400 µg/kg/day) was used as control PCOS model. Most commonly used antiepileptic medications Diazepam (4.0 mg/kg/day), Sodium valproate (300 mg/kg/day) and carbamazepine (50 mg/kg/day) were used in this study to develop PCOS model. The occurrence of reproductive phenotypic PCOS was evaluated by estrous cycle, serum sex hormones estimation, morphology and histological changes of ovary, the metabolic phenotypes were evaluated by assessment of OGT and lipid profile estimation.

Results and Conclusion: The results of this study showed weight gain, obesity, estrous irregularity, insulin resistance and hyperandrogenism in all the study groups except diazepam treatment. In conclusion administration of sodium valproate (300 mg/kg/day) for 21 consecutive day induced PCOS in similar to the human being with the same metabolic and reproductive characteristic features with upstream of hyperandrogenemia.

***Keywords*:** Animal model; Carbamazepine; Diazepam; Letrozole; Polycystic ovary syndrome; Sodium valproate.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most widespread female endocrine disorder, affecting 5-10% of women at reproductive age, causing infertility due to dysfunctional follicular maturation and ovulation, distinctive multicystic ovaries and hyperandrogenism. Women with PCOS also often exhibit nonreproductive metabolic abnormalities such as obesity, metabolic syndrome, hyperinsulinemia, insulin resistance, dyslipidemia with an increased risk of cardiovascular disease and type 2 diabetes [1-2].

PCOS is an intricate clinical condition with multifaceted etiology related to primary defects in the hypothalamic-pituitary axis results in increased Luteinizing hormone (LH) secretion, an alteration in the insulin action results in hyperinsulinemia and insulin resistance, a defect in androgen synthesis that results in increased ovarian androgen production. A complete understanding of the underlying pathophysiology of PCOS is still lacking due to the heterogeneity of this disorder [3 - 5].

Current therapeutic approach for PCOS ranges from lifestyle modification to pharmacological interventions. Lifestyle modifications are associated with diet, exercise and weight loss. Pharmacological interventions include; antiandrogens (Spironolactone, Flutamide), insulin lower agents (Metformin and Thiazolidinediones), and estrogen-progestin combination (Oral contraceptives). Though such treatment is associated with substantial cost and may cause various side effects, such as irregular menstruation, gastrointestinal symptoms, weight gain, and increased insulin resistance [6]. Consequently treatment is palliative rather than curative and focuses on symptomatic approaches. However, therapeutic approaches to PCOS remain an ongoing source of debate [7].

The unclear etiology and symptomatic therapeutic approaches rather than systematic curative, are increasing an interest on develop a suitable animal model could provide a valuable means with which to study the pathogenesis of the characteristic reproductive and metabolic abnormalities and thereby identify novel and more effective treatments. There are several animal models have been developed and investigated to determine the etiology of PCOS. So far, the most widely used animal models include steroid induction at different stages of development, pre- or postnatal or in adulthood, to induce different phenotypes of PCOS, and these are predominately associated with the onset of hyperinsulinemia and/or insulin resistance. The ideal rat model treated with Letrozole (400 µg/day for 21 days) shows the many similar features to PCOS in women like increases in body weight/obesity are due to the high lipid and cholesterol content and ovary weight and altered estrus cyclicity, developed insulin resistance with the exception of the hallmark increase in basal LH level. This model is reliant on artificial hyperandrogenemia and therefore does not help to identify abnormalities upstream of hyperandrogenemia [8]. It is a difficult task to develop animal models that mimic both the abnormal hypothalamic–ovarian axis and metabolic factors in PCOS. Hence, certain considerations need to be made when using or establishing an animal model with PCOS-like features: the animal model should not only have a well characterized reproductive cycle, but the anatomical,

biological, and biochemical features of the model should also be similar as possible to the human PCOS phenotype of interest [9], and the most suitable model should be chosen according to study purposes or hypotheses to be tested.

PCOS is more common in women with epilepsy (13 - 25%) than in general female population (4 - 6%), through several mechanisms. The modified function of hypothalamic - pituitary axis (HPA), including production of luteinizing hormone (LH), follicle-stimulating hormone (FSH), gonadotropin-releasing hormone (GnRH), prolactin (PRL) and their end-products (oestrogen, testosterone and dehydroepiandrosterone) in female might be a key mechanism of PCOS with epilepsy [10].

Anticonvulsants and atypical antipsychotics are medications used to treat bipolar disorder (BD). These medications can have deleterious effects on blood levels of reproductive hormones and consequently on the hypothalamic-pituitary gonadal (HPG) axis and reproductive function. Studies that have specifically addressed the association between psychotropic medications, on the one hand, and menstrual abnormalities, polycystic ovary syndrome (PCOS), and overall reproductive endocrine function in women with BD [11-13]. Hence this study was designed to develop the PCOS animal model is an analogous human PCOS phenotype by using anti epileptic agents.

MATERIALS AND METHODS

Materials

All the chemicals used in this study were of analytical grade. The anti epileptic medication like Sodium valproate, Carbamazepine, Diazepam and the non-steroidal aromatase inhibitor Letrozole were purchased from standard market samples.

Experimental Animals and study designing:

Twenty colony inbred female virgin 12-18 weeks aged albino Wistar rats (180-230gm) were obtained from Central Animal house of Swamy Vivekanandha College of Pharmacy, Elayampalayam, Namakkal - 637 205. The animals were kept under standard environmental conditions of 12/12 light/dark rhythm, maintained under controlled room temperature (23 ± 2ºC) and a relative humanity of 60% ± 10% in polypropylene cages. They were fed with standard pellet diet and water *ad libitum.* Each cage contained 3 rats with a bedding of husk. The cages were cleaned daily by changing the husk bedding. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC), care and use of laboratory animals were confirmed to CPCSCEA guidelines.

They were randomly divided into four groups of six per each. The groups and treatment were designed as follows

Group I : Receives Letrozole (400 µg/kg/day), p. o.
Group II : Receives Diazepam (4.0mg/kg/day), p. o.
Group III: Receives Sodium valproate (300 mg/kg/day), p. o.
Group IV: Receives Carbamazepine (50 mg/kg/day), p. o.

Selections of doses are based on the previous studies.

Changes in Body Weight

Body weight of each rats in all the groups were measured daily throughout the study period by using a weighing balance and the changes were recorded.

Examination of Estrous cycle

During the experimental period, every morning, vaginal smear was examined for the determination of estrous cycle. Vaginal fluid was collected by inserting 1-2 inches pipette with a drop of normal saline. The smears were prepared immediately after collecting the vaginal fluid. The prepared smears were dipped 3-5 times with 70% alcohol in order to fix and stained by using 0.5% aqueous solution of methylene blue. After staining, the slides were rinsed in tap water and examined. Using this technique the characterization of each phase of the estrous cycle was done based on the proportion among three types of cells observed in the vaginal smear viz. epithelial cells, cornified cells, leukocytes and keratinocytes. The cells were seen under the light microscope [14].

Oral glucose tolerance test (OGTT)

On day 22 of study period OGTT was performed after 12 hr fasting for all rats using an Accu Chek Active glucometer (Roche Diagnostics Ltd). The fasting glycemia was measured by using blood samples from the tail vein. Each rat received orally 0.5 ml solution containing 400 mg glucose. After 2 hr the glycemia was again assessed [15].

Biochemical Assay

On 22nd day of the study, the animals were anesthetized by using diethyl ether. The blood was drawn through retro orbital plexus and the serum was separated after centrifugation of total blood without anticoagulants, at 3000rp, for 10 min. The lipid profile, HDL, LDL and VLDL [16] were estimated in serum by standard laboratory technique. The Serum Testosterone, Estrogen and Progesterone were measured by using an enzyme immunoassay kits with standard laboratory techniques [17].

Measurement of Ovary Weight

At the end of study on 22 day, all the animals were sacrificed and ovary were removed, cleaned from fats and subjected to gross examination and later weighted.

Histopathological Evaluation

Histopathological evaluation was performed the ovaries of control and experimental groups. The excised ovaries were fixed in 10% neutral formal saline, embedded in paraffin wax, and then sectioned serially at 5-µm thickness. Sections were mounted and stained by the haematoxylin and eosin procedure. Also some sections were stained by Masson's trichrome (M.T.) and under light microscope with 100x magnification for histopathological changes [18].

Statistical analysis

The data represents as mean ± SEM to determine significance and when animals were compared over time or within multiple groups, we used one-way analysis of variance (ANOVA) followed by post hoc Dunnet's test by using Graph Pad Statistical Package software. The values were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

Letrozole is an oral non-steroidal aromatase inhibitor which induced hyperandrogenesim is a most widely used animal model for PCOS. Inhibiting of aromatase prevent the conversion of androgens to estrogen. Rat treated with 7-35 days with 400 µg/day produce the similar features to PCOS in women reliant on artificial hyperandrogenemia and it does not help to identify abnormalities of upstream hyperandrogenemia [19], the level of androgen decline once the letrozole treatment stopped. Hence this model was used as reference model in our study.

Changes in body weight

Obesity is strongly associated with PCOS and maybe present in up to 50% of cases. Obese women with PCOS are to suffer from anovulation [20]. Twenty one days consequent administration of Letrozole shows higher rate of weight gain which was attributable to deposition of abdominal fat, in the mechanism of elevated level of androgen might increase the amount of adipose tissues particularly in abdominal region [21]. Next to Letrozole treatment sodium valproate treated group shows higher rate of weight gain (Table 1).

Table 1: Changes in body weight

Treatment	Initial body weight (g)	Final body weight (g)	Difference in body weight (g)
Group – I (Letrozole)	189.50 ± 6.91	222.30 ± 7.75	32.8 ± 6.06
Group – II (Diazepam)	189.45 ± 6.02	196.32 ± 5.71**	6.70 ± 0.85***
Group – III (Sodium valproate)	189.33 ± 6.42	215.50 ± 6.70	26.25 ± 3.47
Group – IV (carbamazepine)	188.30 ± 4.90	200.00 ± 8.94*	11.75 ± 2.25**

Values are expressed as mean ± SEM, n=6
Comparisons were made between: Group I vs II, III and IV.
Symbols represent statistical significance: *** P<0.001, ** - P <0.01, *- P<0.05

Changes in estrous cycle

Estrous cycle phases are determined based on the presence of four different cell types such as epithelial, cornified, leukocytes and keratinocytes, the cells with nucleus is known as epithelial cell, without nucleus are cornified and small rounded are leukocytes. Proestrous consists of predominance of nucleated epithelial cells; an estrous stage primarily consists of anucleated cornified cells. The diestrous consists of predominant leukocytes [22]. The result in table (2) and figure (1) shows that there is frequent occurrence of diestrous stages in all the treated groups, which indicates the abnormality in estrous cycle. Results of this study support the menstrual abnormalities and more of anovulatory cycles producing ability of selected antiepileptic medication [23].

Oral glucose tolerance test

In table 3 signify OGTT profile showed the hyperglycemia in both fasting and after 2 hrs glucose level in all the treatment except diazepam treatment, it shows the glucose intolerance nature of treatment groups. PCOS is positively correlated with insulin resistance. The finding of this study exhibited hyperglycemic tendencies contributing to insulin resistance, leading to hyperglycemia and metabolic syndrome [24]. Thus, insulin resistance may be a consequence of increased truncal fat and high levels of free fatty acids.

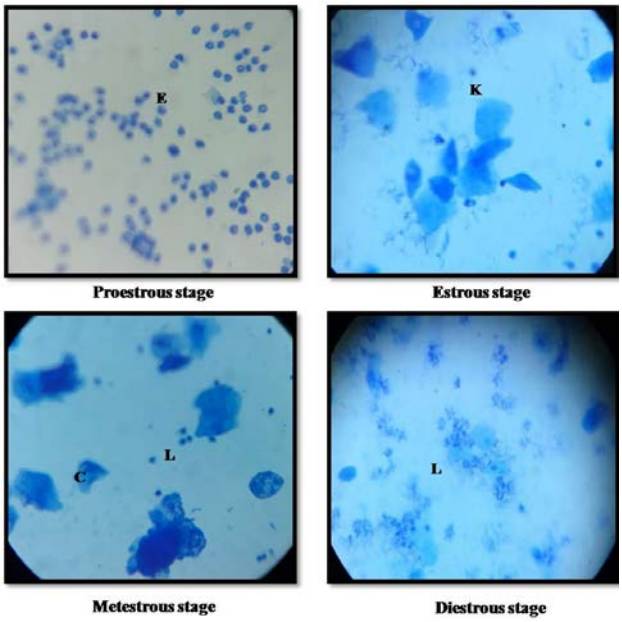

Figure 1: Estrous cycle of Rats showing Keratinocytes (K), Leukocyte (L), Cornified cells (C), Epithelial cells (E)

Table 2: Comparison of Estrous cycle

Days	Group – I (Letrozole)	Group – II (Diazepam)	Group– III (Sodium valproate)	Group – IV (carbamazepine)
1	Estrous	Diestrous	Estrous	Metestrous
2	Estrous	Proestrous	Metestrous	Metestrous
3	Metestrous	Estrous	Diestrous	Diestrous
4	Diestrous	Estrous	Proestrous	Diestrous
5	Diestrous	Metestrous	Estrous	Proestrous
6	Proestrous	Metestrous	Metestrous	Estrous
7	Proestrous	Diestrous	Diestrous	Estrous
8	Estrous	Diestrous	Diestrous	Metestrous
9	Estrous	Proestrous	Diestrous	Diestrous
10	Metestrous	Proestrous	Proestrous	Diestrous
11	Metestrous	Estrous	Estrous	Proestrous
12	Diestrous	Metestrous	Estrous	Estrous
13	Diestrous	Diestrous	Metestrous	Estrous
14	Diestrous	Diestrous	Diestrous	Metestrous
15	**Diestrous**	Proestrous	Diestrous	Metestrous
16	Proestrous	Estrous	Diestrous	Metestrous
17	Metestrous	Metestrous	Diestrous	**Metestrous**
18	Metestrous	Metestrous	**Diestrous**	Diestrous
19	Metestrous	Diestrous	Proestrous	Diestrous
20	Metestrous	Diestrous	Estrous	Diestrous
21	Metestrous	**Diestrous**	Metestrous	Diestrous

Table 3: Oral glucose tolerance test

Treatment	Altered Glucose Metabolism	
	O hr (FBG)	2 hrs
Group – I (Letrozole)	129.50 ± 4.11	178.25 ± 4.77
Group – II (Diazepam)	95.50 ± 6.34***	108.50 ± 7.27***
Group – III (Sodium valproate)	134.50 ± 2.90	180.25 ± 6.41
Group – IV (carbamazepine)	129.00 ± 4.10	166.25 ± 8.91

Values are expressed as mean ± SEM, n=6
Comparisons were made between: Group I vs II, III and IV.
Symbols represent statistical significance: *** P<0.001, ** - P <0.01, *- P<0.05

Table 4: Effect on Lipid profile

Treatment	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Group – I (Letrozole)	13.75 ± 1.75	156.25 ± 16.00	45.50 ± 2.72
Group – II (Diazepam)	41.50 ± 5.12***	74.50 ± 15.17***	23.00 ± 2.54***
Group – III (Sodium valproate)	11.75 ± 1.38	149.50 ± 14.99	44.75 ± 3.90
Group – IV (carbamazepine)	14.50 ± 2.90	123.75 ± 8.64**	35.25 ± 3.86*

Values are expressed as mean ± SEM, n=6
Comparisons were made between: Group I vs II, III and IV.
Symbols represent statistical significance: *** P<0.001, ** - P <0.01, *- P<0.05

Changes in lipid profile and hormones level

Women with polycystic ovary syndrome (PCOS) are dyslipidemic with hypertriacylglycerolemia, reduced high-density lipoprotein (HDL), and increased small dense low-density lipoprotein (LDL) being characteristic feature. The lipid metabolism and insulin resistance are independent in nature but hyperandrogen state with the increased central adiposity is the causative agents for PCOS [25]. Results of this study showed that decreased HDL, Increased LDL, and VLDL in letrozole treatment, which was concur with previous studies [26]. Among the antiepileptic drugs treatments sodium valproate treated group shows the similar altered lipid profile of letrozole treatment (Table 4).

The low estrogen level inhibits the LH and FSH secretions at the pituitary level. High amount of the estrogen exhibits the positive stimulatory feedback with LH by inducing the LH surge at midcycle, whereas the level of estrogen raises steadily leading to a sustained elevated LH secretion [27]. The alteration of estrogen leads to the formation of cyst in the ovary [28]. Low progesterone level acts at the pituitary gland level to enhance the LH response to the GnRH and is responsible for the FSH surge in the midcycle and finally suggested that the abnormalities of sex steroids concentration in the primary level have the stimulatory effect on LH secretion in PCOS. Decreased progesterone production, which reflects anovulation, is a factor depicted in human PCOS [29]. Elevation of estrogen and low level of progesterone was higher in the letrozole and sodium valproate treatment groups which show the endocrinal feature of PCOS in relation to human PCOs (Table 5).

Hyperandrogenism (the level of high testosterone levels), which is evident in human PCOS. The use of non-steroidal aromatase inhibitor letrozole, which shows a marked elevation of testosterone levels, same hyperandrogenism was observed in Sodium valproate and carbamazepine treatment.

Table 5: Effect on Sex hormones

Treatment	Testosterone (ng/ml)	Progesterone (ng/ml)	Estrogen (pg/ml)
Group – I (Letrozole)	125.5 ± 6.61	13.00 ± 1.08	61.75 ± 3.75
Group – II (Diazepam)	92.25 ± 5.06**	17.50 ± 1.04*	46.75 ± 2.75**
Group – III (Sodium valproate)	127.5 ± 8.07	12.75 ± 1.31	62.50 ± 3.07
Group – IV (carbamazepine)	128.25 ± 5.23	12.00 ± 1.08	62.00 ± 3.03

Values are expressed as mean ± SEM, n=6
Comparisons were made between: Group I vs II, III and IV.
Symbols represent statistical significance: *** P<0.001, ** - P <0.01, *- P<0.05

Table 6: Effect of anti epileptic medications on Ovary weight

Treatment	Ovary weight (mg)	
	Right ovary	Left ovary
Group – I (Letrozole)	63.0 ± 0.91	71.75 ± 1.11
Group – II (Diazepam)	52.5 ± 1.55	55.25 ± 1.49***
Group – III (Sodium valproate)	58.8 ± 2.06	62.25 ± 2.39
Group – IV (Carbamazepine)	65.5 ± 1.85	66.00 ± 3.03

Values are expressed as mean ± SEM, n=6
Comparisons were made between: Group I vs II, III and IV.
Symbols represent statistical significance: *** P<0.001, ** - P <0.01, *- P<0.05

Morphological changes in ovary

The results in the table (6) show an increase in the weight of the ovary in the letrozole treated rats, in relation with increased volume of ovary, cortex, and medulla. The letrozole induced rats induce the development of cysts with hyperplasia of internal theca cells and a thickened ovarian capsule, although letrozole treatment also causes the reduction of corpus luteum, changes in the granulose cells and an increase in the number of atretic follicles [30]. Apart from letrozole group Sodium valproate and carbamazepine treatment groups also shows the similar increase in response on ovary weight. Morphology also shown the numbers of cysts are higher in sodium valproate and carbamazepine treatment groups (Figure 2).

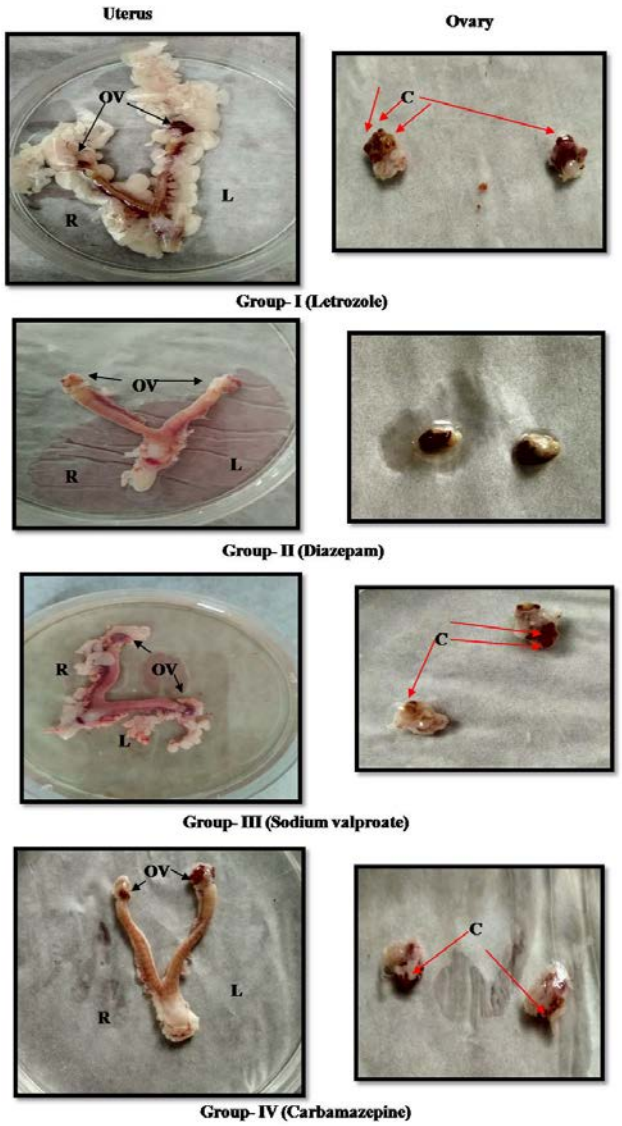

Figure 2: Morphology of reproductive tract OV- Ovary, C- Cyst, R- Right, L- Left

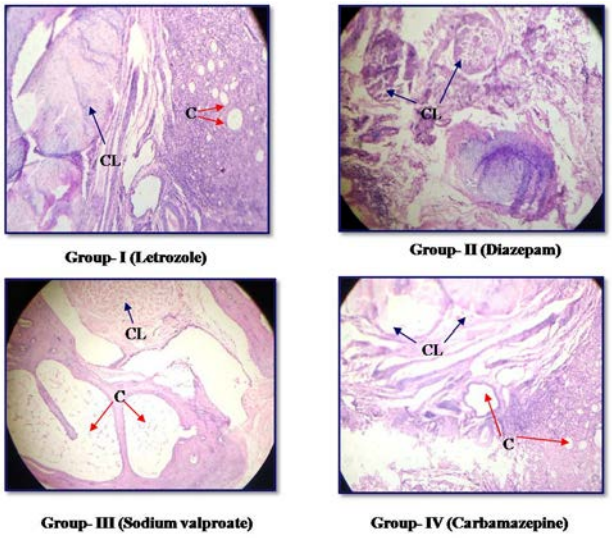

Figure 3: Histological changes on ovary

Histopathological changes

In figure 3 the exhibited histological changes in ovarian morphology are increased size, thickened capsule, cystic graffian follicle. An inner layer of granulosa cells with uniform round nuclei and little cytoplasm lines follicle cysts and an inner layer of theca interna cells, which are small and spindle shaped. These characteristic histological changes were observed in all the study groups of PCOS models except diazepam treatment, it has milder changes. This ovarian histology changes are relatively confers the similar to human PCOS.

CONCLUSION

The rodents are the widely used animal model to study PCOS, with beneficial relating to their size, short lifespan, high reproductive index and the different strains. The most commonly used model letrozole induced PCOS is reliant on artificial hyperandrogenemia and therefore does not help to identify abnormalities upstream of hyperandrogenemia. However at present, a convincing whole animal model representing all feature associated with human PCOS need to be established. In this study we examined the impact of antiepileptic drugs on reproductive functions in female rats.

Among the selected drugs sodium valproate, suggesting that the drug can perturb ovarian function and androgen synthesis, possible as a result of multiple effects on the hypothalamic-pituitary-ovarian axis. Also produced significant reproductive and metabolic abnormalities like weight gain, obesity, menstrual disturbances, insulin resistance and hyperandrogenism are common in women with PCOS. It might be due to its broad spectrum mechanism on hypothalamic-pituitary-ovarian axis.

In conclusion administration of sodium valproate (300 mg/kg/day) for 21 consecutive day induced PCOS in similar to the human PCOS with the same metabolic and reproductive characteristic features. It might be a better model for further development of new pertinent therapeutic procedure and agent to manage the PCOS.

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