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LETROZOLE AND FRUCTOSE-INDUCED POLYCYSTIC OVARIES IN THE RAT: A NOVEL MODEL EXHIBITING BOTH OVARIAN AND METABOLIC CHARACTERISTICS FOR POLYCYSTIC OVARY SYNDROME IN RAT

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ABSTRACT: Introduction: There are numerous animal models available for polycystic ovary syndrome (PCOS), a fully convincing model for PCOS reflecting all pathologic condition of PCOS seen in humans is not available due to difficulties in inducing all pathologies in rat models. If so, it will help to test drug to treat PCOS. The main goal of this study is to create a new model to reflect all pathologic conditions of PCOS seen in humans using non-steroidal aromatase inhibitor letrozole and fructose in rats. **Methods:** Twelve rats were divided into two groups containing 6 rats each. Vehicle control group rats received 1% aqueous solution of carboxmethlycellulose (CMC) once daily orally for 28 days and PCOS group was administered letrozole at a concentration of 1 mg/kg dissolved in 1% CMC p.o. once daily for 28 days. Along with these rats were allowed free access of 10% fructose solution daily. During experimental period, vaginal smears were collected daily for estrus cycle determination. Rats were sacrificed and blood sample were collected for hormonal and other biochemical assays. Ovaries were removed to proceed with histolopathological study. **Results:** When compared to vehicle control group, ovaries from PCOS group showed high incidence of ovarian cyst with incomplete luteinization and decreased number of corpus lutea. Although serum estradiol and progesterone levels were reduced, testosterone levels were elevated, as were levels of luteinizing hormone (LH). Serum follicle-stimulating hormone (FSH) levels were markedly increased in the PCOS group. The PCOS group rats showed significant increase in insulin resistance with compensatory significant hyperinsulinemia than the vehicle control group. **Conclusions:** Our study was completely fulfilling and convincing as in human PCOS, this rat model is fully convincing for studying PCOS and in several ways, it is similar to the human PCOS.

INTRODUCTION: Polycystic ovarian syndrome (PCOS) is a most common heterogeneous endo-crinopathy disorder in women of uncertain etiology with an ovulatory infertility, which affects 5 - 10 % of population of reproductive age.

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The PCOS is characterized as hyper-androgenic disorder associated with chronic oligo-anovulation and polycystic ovary morphology [1]. In women, it is often associated with depression and other mood disorders along with metabolic derangements, chiefly insulin resistance and compensatory hyper-insulinaemia.

This metabolic derangement was recognized as a major factor responsible for altered androgen production and metabolism [2]. In addition to that neuro-endocrine features of PCOS includes increased serum concentration of luteinizing hormone (LH),

increased LH/Follicle stimulating hormone (FSH) ratio, and increase in amplitude and frequency of pulsatile LH secretion [3, 4]. Recent study shows that increased insulin resistance and compensatory hyper-insulinemia play a key role in the pathogenesis of PCOS [5]. In current situation, the understanding of the pathogenesis has improved remarkably and it reflected in the revolution of treatment of PCOS.

There are numerous animal models available for PCOS study. However, fully convincing rat model for PCOS reflecting all pathologic condition of PCOS in women is not available due to difficulties in inducing all pathologies in one rat model. In estradiol valerate rat model anatomy and physiology of the ovary were same as of the human PCOS [6]. However, in this model progressive degeneration of hypothalamus and altered response of pituitary to LHRH made it inappropriate for human PCOS.

Mahajan (1988) studied PCOS in rats after continuous administration dehydroepiandrosterone (DHA) and came out with DHA metabolites interfered in many experimental conditions. In neonatally androgenized female rat model, ovaries were smaller than that of controls and normal tunica albuginea and no hyperthecosis were found. Moreover, because testosterone administered prior to differentiation of hypothalamus and pituitary cells, these organs were rendered nonresponsive to steroids, luteinizing hormone-releasing hormone (LHRH) and FSH [6]. Although anatomic features consequent to PCOS resulted from constant-light similar to human PCOS and ovarian cells still retained the ability to respond to FSH and LHRH, no increment in levels of LH and androgens that have a pivotal role in PCOS rendered this model different from human PCOS [6].

After thoroughly reviewing currently available PCOS rats model study. We proposed a new technique to create both ovarian morphological changes and metabolic hormonal alteration in same rats, which will be almost reflecting most of the pathologic condition of PCOS seen in humans. This forms the main goal of our study. This novel technique will help us to study the drugs can be used for the treatment of PCOS. The same will be targeting both hormonal and morphological changes to resist and normalize in PCOS in woman. In this technique, we used non-steroidal aromatase inhibitor letrozole and fructose to create polycystic ovary in rats.

MATERIALS AND METHODS: Virgin female Wistar albino rats having weight of 110 - 120 g, with four day regular estrous cycles were purchased from TANUVAS, Chennai. All rats were housed at controlled temperature (22 °C ± 2 °C) on a 12 h alternating light-dark cycle with free access to water and a standard pelleted diet. Estrous cycles were monitored daily by vaginal smear examinations. In our study, 12 animals were randomly divided into two groups (6 animals in each group). Experiments were started after getting permission from Institutional animal ethical committee (IAEC No. TMC/IAEC/02/003/2016). Chemicals Letrozole (trade name Femara®) was purchased from sun pharmaceuticals, Andheri road and Mumbai, India. Carboxmethlycellulose (CMC) and fructose were purchased from southern India scientific corporation, Chennai.

Group 1: (Vehicle control group) All rats were given only 1% aqueous solution of carboxy-methlycellulose (CMC) once daily p.o. for 28 days.

Group 2: (PCOS group) All rats were administered with letrozole with a concentration of 1mg/kg dissolved in 1% CMC (2 ml/kg) p.o once daily for 28 days along with free access to 10% Fructose daily.

Throughout the entire study period, vaginal smears were taken daily and evaluated microscopically for estrous cycle determination. The day after 28th day all animals were sacrificed by CO_2 inhalation. Blood and tissue sample were removed for following assay and ovarian morphology.

Hormonal and Biochemical Assay: Blood was quickly collected by cardiac puncture into plain sample bottles, allowed to clot and centrifuged at 3,000 rpm for 15 minutes to get clear serum samples, which were subsequently kept frozen (-20ºC) until LH, FSH, serum insulin, testosterone, and progesterone assay and biochemical parameters like blood glucose and lipid profile study. All hormones were measured using Enzyme-linked immunosorbent assay (ELISA) [16] methods procured from the Diagnostic Automation, Inc. Biochemical assays were done by automated

chemical analyzer. HOMA-IR scores were calculated using fasting serum insulin and CBG concentrations at the end of the experimental period according to the following formula:

HOMA-IR = [Insulin (U/l) × Blood glucose (mmol/l)] / 22.5

Conversion factor: Insulin (1U/l = 7.174 pmol/l) and blood glucose (1 mmol/l = 18 mg/dl)

Ovarian Morphology: The ovaries from the two groups were carefully isolated, washed in buffered saline, fixed in 10 % formalin, passed through ascending series of ethanol baths, embedded in paraffin, sectioned (5 µm thick); mounted on slides and stained with haematoxylin and eosin. This was to observe the various stages of follicular differentiation and to determine the cytoarchitectural changes in cells following the method reported by Pedersen, (1970). The slides were subsequently viewed under the light microscope and photo-micrographs taken at 10X and 45X magnifications.

Statistical Analysis: Statistical analysis was performed with SPSS (version 17.0). Results are expressed as means ± SD. The significance of differences among groups was analyzed statistically using Student's unpaired t - test and test values with $p < 0.05$ were considered statistically significant.

RESULTS: Hormonal assay in both groups have been tabulated in **Table 1**. Serum FSH was found to be remarkably decreased ($p < 0.05$) than the vehicle control group. Estradiol was also found to be remarkably decreased. Progesterone was significantly decreased in study group than vehicle control group. Testosterone and LH were significantly ($p<0.05$) increased in the experimental group.

TABLE 1: HORMONAL ASSAY IN ANIMALS OF VEHICLE CONTROL AND PCOS GROUPS

Groups	FSH (IU/mL)	LH (IU/L)	Testosterone (ng/dL)	Progesterone (ng/mL)	Estradiol (pg/mL)
Vehicle control	0.755 ± 0.163	0.725 ± 0.219	0.700 ± 0.312	8.503 ± 2.018	57.20 ± 10.59
PCOS Group	0.306 ± 0.101*	0.998 ± 0.206*	1.680 ± 0.526*	3.183 ± 1.867*	20.57 ± 10.29*

Ligand: FSH: Follicle Stimulating Hormone; LH: luteinizing hormone
Data were expressed as means ± SD. * $p < 0.05$ compared with the vehicle control group

Biochemical Analysis: Data of Biochemical analysis shows in **Table 2** and in **Table 3**. Serum triglycerides were significantly ($p < 0.05$) increased in the PCOS group when compare to vehicle control group; the serum cholesterol was not statistically significant than that of vehicle control group. Blood glucose and Serum insulin were markedly increased in the PCOS group ($p < 0.05$). HOMA-IR was calculated for both experimental and vehicle control groups using serum insulin and blood glucose level and it is found to be significantly increased in PCOS group.

TABLE 2: SERUM LIPID PROFILE IN DIFFERENT ANIMAL GROUPS AT THE END OF THE EXPERIMENTAL PERIOD

Groups	Triglycerides (mg/ml)	Cholesterol (mg/dl)
Vehicle control	71.66 ± 9.437	92.63 ± 8.787
PCOS control	95.33 ± 11.07*	112.6 ± 18.82

Data are means ± SD. * $p < 0.05$ compared with the vehicle control group

TABLE 3: HISTOLOGICAL CHANGES OF THE OVARY IN BOTH STUDY GROUPS

Histological changes	Vehicle control group						PCOS group					
	CG1	CG2	CG3	CG4	CG5	CG6	EG1	EG2	EG3	EG4	EG5	EG6
Hyperplasia of theca cells	-	-	-	-	-	-	++	++	++	++	+	++
Decreased no.of corpus luteum	-	-	-	-	-	-	++	++	+	++	++	++
Subcpsular follicular cyst	-	-	-	-	-	-	++	++	++	+	++	++

'+' present ; '-' absent; CG - control group and EG - PCOS experiment group

TABLE 4: BLOOD GLUCOSE, SERUM INSULIN, AND CALCULATED HOMA-IR SCORES AT THE END OF THE EXPERIMENTAL PERIOD

Groups	Blood glucose (mg/dl)	Serum insulin(pmol/l)	HOMA-IR
Vehicle control	99.83 ± 23.74	114.4 ± 14.80	3.965 ± 14.80
PCOS control	371.33 ± 19.835*	150.6 ± 6.991*	8.987 ± 1.466*

HOMA: homeostatic model assessment, IR: insulin resistance
Data are means ± SD. * $p < 0.05$ compared with the vehicle control group

Ovarian Morphology: All vehicle control group rat ovaries show normal histology and exhibited follicles in various stages (Secondary follicle, Graafian follicle and Corpus luteum) in the cortex region **Fig. 1** and **2**.

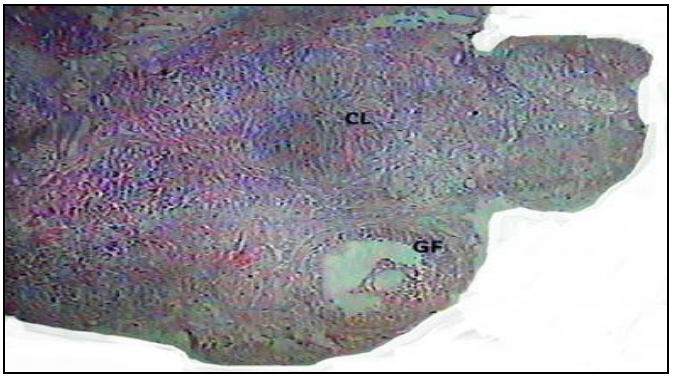

FIG. 1: MAGNIFICATION - 10X, SECTION OF OVARY FROM CONTROL RAT SHOWS NORMAL SECONDARY FOLLICLE WITH OOCYTE AND CORPUS LUTEA. GF – GRAAFIAN FOLLICLE, CL- CORPUS LUTEUM

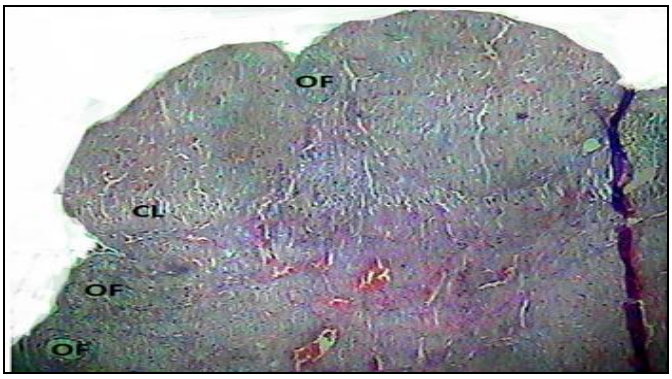

FIG. 2: MAGNIFICATION - 10X SECTION OF OVARY FROM VESICAL CONTROL RAT SHOWS FOLLICLES IN VARIOUS STAGE OF DEVELOPMENT: OF – OVARIAN FOLLICLE, CL- CORPUS LUTEUM

Most of the follicles in the experimental group were found to have large cysts without or scanty granulosa layer **Fig. 3** and showed evidence of atresia. Also, the ovaries from experimental group showed high incidence of subcapsular ovarian cyst and decreased number of corpus luteum in the cortex giving the ovary a nodular appearance **Fig. 4**. Histological changes of the ovary in both groups have been tabulated in **Table 3**.

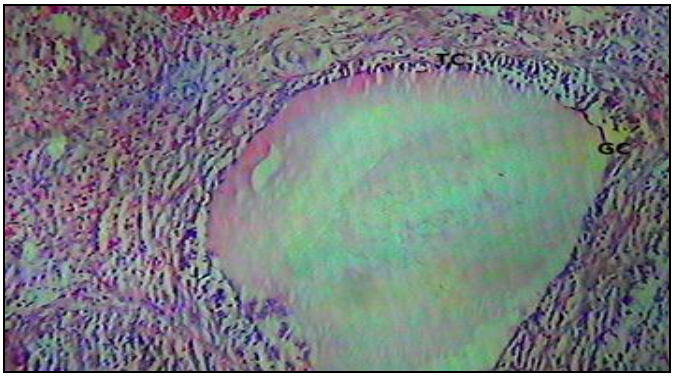

FIG. 3: MAGNIFICATION - 40X, SECTION OF OVARY FROM PCOS GROUP SHOWS A SUBSCAPULAR CYSTS WITH SCANTY GRANULOSA CELLS AND HYPERPLASIA OF THECA CELLS: GC - GRANULOSA CELLS

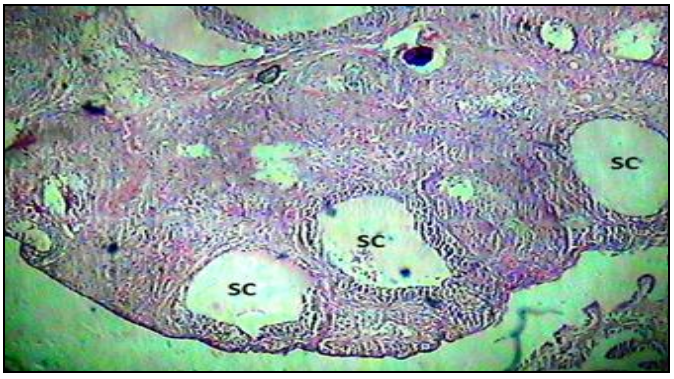

FIG. 4: MAGNIFICATION - 10X, SECTION OF OVARY FROM PCOS GROUP SHOWING NUMEROUS SUB CAPSULAR CYSTS AND ATRETIC SECONDARY FOLLICLE. SC - SUBSCAPULAR CYST

DISCUSSION: After administration of letrozole and fructose for 28 days to rats which shows multiple cyst in ovary with altered hormone like FSH, LH, estirol, progesterone and insulin levels. A deficiency in activity of aromatase was one reason for the intraovarian disturbance in steroid-ogenesis to cause PCOS. Because it catalyzes the rate-limiting step in biosynthesis of estrogens from androgens [7], decrease in activity of this enzyme will result in increased ovary-an androgen production and development of PCOS.

In light of these facts, letrozole, a non-steroidal aromatase inhibitor, was administered to virgin female rats along with fructose which induces increased insulin resistance resulting in hyper-insulinemia.

This Compensatory hyper-insulinemia plays an important role in the development of metabolic abnormalities and high androgen levels observed in women with PCOS [8]. This new animal model showed many histologic and biochemical findings consistent with human PCOS. Rats treated with letrozole plus 10% fructose showed significant increase in body weight, ovarian weight, and irregular estrus cycles compared to vehicle control group.

Histopathology of ovaries taken from study group showed very striking similarity to human PCOS. In our PCOS group animals were shows less corpus luteum when compare to vehicle control group which indicates disturbances in ovulation and decreased frequency of estrous cyclicity. This is one of the important characteristic features of PCOS.

It was also observed that there is abundance of subcapsular cysts lined with a thin layer of granulosa cells and hyperplasia of theca interna cells. These findings show the presence of active levels of FSH, increased LH, and lack of interplay between granulosa and theca cells, which would otherwise lead to ovulation. The number of graafian follicles and corpus luteum were markedly reduced in study group. As graafian follicle is indicative of active folliculogenesis, we suggest that it might be arrested in preantral stage during follicular development. The excess amount of fructose might have affected it through alterations in hormonal balance *via* insulin resistance that which would have led to ovarian dysfunctions [9].

PCOS is positively correlated with insulin resistance. Testosterone levels were markedly higher in the PCOS group rats than in vehicle control group rats, presumably because insulin resistance might block the conversion of androgen substrates to estradiol[10]. Androgens are synthesized in theca cells and then transported to the granulosa cells where P450 aromatase converts the androgens to estrone and estradiol [11].

Theca cell hyperplasia in vehicle control group suggests greater androgen production and a decreased granulosa cells suggests conversion of androgen to estrogen is reduced. It is in agreement with previous studies showing that androgen treatment in rats resulted in a pronounced thickening of the theca cell layer [12]. Irrespective of the model's clinical presentation, excess production of androgen and elevated LH were considered the most consistent biochemical feature of PCOS [13]. In this PCOS group, both testosterone and LH levels were markedly elevated as compared with vehicle control groups. Increased testosterone levels reflects accumulation of androgens as conversion of androgen substrates into estrogens were blocked and elevation of LH presumably due to stimulation of theca interna cell. Decrease in progesterone reflects anovulation, another biochemical finding of this model, consistent with human PCOS. In women with PCOS, the predominant reason for high serum LH concentrations was abnormal negative feedback on LH secretion mediated by either estradiol or progesterone [14].

PCOS is associated with features of the metabolic syndrome, a most important pathophysiological factor which affects 70% of PCOS women [15]. The clinical and metabolic changes of polycystic ovary syndrome are mainly related to hyper-androgenism and insulin resistance with compensatory hyper-insulinemia. The Compensatory hyper-insulinemia is important in the development of metabolic abnormalities and also contributes to the high androgen levels observed in women with PCOS [16]. The rats in our study groups showed marked increase in insulin resistance with compensatory significant hyper-insulinemia than the vehicle control groups.

CONCLUSION: In conclusion, this animal model, which shows both hypera-ndrogenism and increase in insulin resistance with compensatory significant hyper-insulinemia, the most characteristic feature of PCOS in woman, is a fully convincing rat model for studying PCOS and in several ways is similar to the human PCOS.

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CONFLICT OF INTEREST: No conflict of interests.

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