High-fat diet exposure from pre-pubertal age induces polycystic ovary syndrome (PCOS) in rats

Roshni Patel^{1,*} and Gaurang Shah^{2,*}

¹Department of Pharmacology and Pharmacy Practice, K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India and ²Department of Pharmacology, L. M. College of Pharmacy, Ahmedabad, Gujarat, India

Correspondence should be addressed R Patel: roshnidutta174@yahoo.co.in

*(R Patel and G Shah contributed equally to this work)

Abstract

Polycystic ovary syndrome (PCOS) is associated with hyperandrogenism, oligo-anovulation, polycystic ovaries and metabolic syndrome. Many researchers reported that PCOS often starts with menarche in adolescents. Presently available animal model focuses on ovarian but not metabolic features of PCOS. Therefore, we hypothesized that high-fat diet feeding to pre-pubertal female rats results in both reproductive and metabolic features of PCOS. Pre-pubertal female rats were divided into two groups: group I received normal pellet diet and group II received high-fat diet (HFD). In the letrozole study, adult female rats were divided into two groups: group I received 1% carboxy methyl cellulose and group II received 1 mg/kg letrozole orally. Oral glucose tolerance test, lipid profile, fasting glucose, insulin, estrus cycle, hormonal profile, ovary weight, luteinizing hormone (LH) receptor and follicle-stimulating hormone receptor expression were measured. Polycystic ovarian morphology was assessed through histopathological changes of ovary. Feeding of HFD gradually increase glucose intolerance and fasting insulin levels. Triglyceride level was higher in HFD study while total cholesterol level was higher in the letrozole study. Alteration in testosterone and estrogen levels was observed in both studies. LH receptor expression was upregulated only in HFD study. Histopathological changes like increase cystic follicle, diminished granulosa cell layer and thickened theca cell layer were observed in letrozole as well as HFD study. High-fat diet initiated at pre-puberty age in rats produces both metabolic disturbances and ovarian changes similar to that observed clinically in PCOS patients. Letrozole on the other hand induces change in ovarian structure and function.

Reproduction (2018) 155 139–149

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder encountered in premenopausal women, with estimated prevalence of about 5–10% (Diamanti-Kandarakis 2008). The symptoms of PCOS were first described by Stein and Levanthal in 1935 (Stein & Leventhal 1935). It is characterized by clinical or biochemical hyperandrogenism, oligoanovulation and polycystic ovarian morphology (Franks 1995, Azziz *et al.* 2009). Along with this, metabolic abnormalities such as hyperinsulinemia, impaired glucose tolerance and resulting type II diabetes mellitus (Ehrmann *et al.* 1999, Park *et al.* 2001), obesity, dyslipidemia (Moran *et al.* 2010) and cardiometabolic syndrome (Hoffman & Ehrmann 2008) are quite frequently observed in PCOS patients.

Currently, three different criteria for diagnosis of PCOS exist. According to Androgen excess-PCOS society 2006, essential components for diagnosis of PCOS are hyperandrogenism along with chronic

anovulation or polycystic ovaries (Azziz *et al.* 2006). In contrast, Rotterdam criteria 2003 suggests the presence of any two of hyperandrogenism, chronic anovulation and polycystic ovaries (No authors listed 2004). Earlier criteria provided by The National Institute of Child Health and Human Development conference, 1990, include only hyperandrogenism and chronic anovulation (Zawadzki & Dunaif 1992).

Since the 1960s, a range of animal models including rodents, sheep and non-human primates have been developed. The prenatal exposure of androgen to sheep and rhesus monkey shows striking similarities with human PCOS (Abbott *et al.* 1998, Recabarren *et al.* 2005). However, long developmental line (menarche occurs at 2.5 years of age) and high cost limits the practical utility of these large animal models. On the other hand, rodent models are inexpensive, easy to handle, have short estrous cycles and generation times. Androgens (Pinilla *et al.* 1993, Wu *et al.* 2010), estrogens (Brawer *et al.* 1986, Pinilla *et al.* 1993) anti-progesterone (Sanchez-Criado *et al.* 1993) and letrozole (Kafali *et al.* 2004) are used

to induce reproductive and metabolic characteristics of PCOS in rats. However, cyclicity and ovarian functions are inconsistently affected in prenatal androgenized animals (Walters *et al.* 2012). In contrast to enlarged ovaries observed in PCOS patients, postnatal treatment with most androgens show inconsistent increase in luteinizing hormone (LH) and androgen levels and also decreased ovary weight (Walters *et al.* 2012). Estradiol administration develops cystic follicles, but in most cases, without testosterone overproduction (Paixao *et al.* 2017). Letrozole, an aromatase inhibitor, frequently used to induce PCOS, produces endogenous hyperandrogenism, disrupts estrous cycle and increases ovarian weight with multiple cysts (Kafali *et al.* 2004). Producing hormonal imbalances using letrozole develops only ovarian features of PCOS but not the metabolic features (Mannerås *et al.* 2007). In rodent models, induced hyperandrogenism and resulting imbalanced hormonal milieu leads to development of PCOS. Many studies have determined both the reproductive and metabolic features in hyperandrogenized rodents. The presence of metabolic disturbances in such animals seems to be dependent on the androgen used and also the timing of the treatment.

The prevalence of IR in PCOS patients ranges from 50 to 70% (Dunaif 1997, Park *et al.* 2001, Carmina & Lobo 2004) and occurs independently of obesity (Dunaif *et al.* 1989). The effect of obesity can aggravate insulin resistance (Dunaif *et al.* 1989). High insulin levels increase androgen production by augmenting LH secretion from the pituitary gland, acting synergistically with LH on thecal cells and reducing hepatic synthesis of serum sex hormone-binding globulin (Nestler *et al.* 1991, Balen 2004, Sheehan 2004). Insulin exposure to cultured rat anterior pituitary cells cause dose-dependent increase in basal and gonadotropin-releasing hormone-stimulated LH and follicle-stimulating hormone release (Adashi *et al.* 1981, Soldani *et al.* 1994, 1995). Long-term hyperinsulinemia and IR leads to an increase in serum total and free testosterone levels (Adashi *et al.* 1981, Declue *et al.* 1991). Feeding of high-fat diet (HFD) for 120–180 days to an adult rats produce IR, hyperinsulinemia, increased serum progesterone levels, extended estrus cycle and altered ovarian morphology in rats. Moreover, the increase in progesterone levels occur together with enhancement of LH levels (Akamine *et al.* 2010). Pre-pubertal administration of andronate in HFD-fed rats showed ovarian as well as metabolic features of PCOS such as reduced insulin sensitivity, hyperandrogenism, irregular cycle and increased LH levels (Zhai *et al.* 2012). Similarly, administration of dehydroepiandrosterone in HFD-fed C57BL/6 pre-pubertal mice also induces ovarian as well as metabolic features of PCOS (Lai *et al.* 2014). A significant increase in LH along with infertility by either GnRH or insulin stimulation was observed in diet-induced obese wild-type female mice (Brothers *et al.* 2010). Considering the

controversy regarding fundamental pathophysiologic defect of PCOS and parallel growing consensus that development of insulin resistance precedes androgen excess and abnormal gonadotropin dynamics (Guzick & York 1998), it is worthwhile to study the effect of HFD to pre-pubertal rats. Such study can throw light on possible role of insulin resistance on the development of PCOS. Along with this, pre-pubertal administration of letrozole for 90 days and post-pubertal administration of letrozole for 21 days have shown similarity in generation of PCOS-related ovarian dysfunction and hyperandrogenism. However, insulin resistance was not observed in both cases.

In the present study, we observed the effect of HFD to pre-pubertal rats on ovarian functions, insulin sensitivity and hormonal levels and compared the same with letrozole-induced PCOS in rats. The results of the study can suggest possible use of pre-pubertal HFD as alternative animal models of PCOS.

Materials and methods

Materials

The normal pellet diet (NPD) was purchased from VRK Nutritional solutions, Pune, India. The other feed ingredients such as whey protein (Balaji Nutraceuticals, Ahmedabad, India) and choline bitartrate (Chemdyes Corporation, Rajkot, India; Cat. No. CH0171) were procured from commercial sources. Letrozole was purchased from West-coast pharmaceutical works Ltd., Ahmedabad, India.

Animals

The animals were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) with 12:12-h light and darkness cycle. All the rats were provided with commercially available rat NPD and water *ad libitum*, prior to the dietary manipulation. All experimental protocols (KBIPER/14/481 and KBIPER/16/574) were approved by the Institutional (K. B. Institute of Pharmaceutical Education and Research) Animal Ethics Committee (IAEC) under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guideline before carrying out the project.

High-fat diet study protocol

Female Sprague-Dawley rats (3–4 weeks of age, weighing 45–55 g at the beginning of the experiments) were randomized into two matching weight groups. Rats are allocated into two dietary regimens, Group I ($n=9$): control group (NPD: 65% carbohydrate, 21.5% protein and 4% fat and 9.5% other; 314 kcal) and Group II ($n=15$): HFD group (HFD: 36% carbohydrate, 20% protein, 40% fat and 4% other; 530 kcal) for 105 days. The composition of HFD is given in Table 1. For adapting the animals to HFD (40%), the fat content was gradually increased over 2 weeks

Table 1 Composition of the high-fat diet.

Ingredients	Percentage (%)
Normal pellet diet	55.8
Whey protein	6
Soyabean oil	19
Coconut oil	19
Choline bitartrate	0.2

from 20% to 40%. Later, the animals were maintained on 40% HFD throughout the study. Diet was freshly prepared every 3–4 days and stored at 2–8°C till used. Oral glucose tolerance test (OGTT), blood samples for lipid profile and insulin was collected in overnight fasted rats on day 49, 77 and 105. However, blood samples for hormone estimation were collected during diestrus phase.

Letrozole protocol

Female SD rats (8–10 weeks of age, weighing 200–250g at the beginning of the experiment) were randomized into two groups. Rats displaying normal estrus cycle were selected for study. Group I ($n=9$): Control group (1% CMC) and Group II ($n=12$): Letrozole group (letrozole 1 mg/kg, p.o. in 1% CMC) for 21 days. The letrozole dose was selected based on the previous study (Kafali *et al.* 2004). OGTT, blood sample for lipid profile and insulin were collected in overnight fasted rats on day 21. However, blood samples for hormone estimation were collected during diestrus phase.

Oral glucose tolerance test (OGTT) and insulin sensitivity indices

After an overnight fast, rats were given a glucose load (2 g/kg) by oral gavage. Blood glucose was estimated using a glucometer (Dr Morepen Glucometer, Delhi, India) at 0, 30, 60 and 120 min. after glucose administration.

The homeostasis model assessment of insulin resistance (HOMA-IR) and β -cell function (HOMA- β) were calculated using the equations, $HOMA-IR = FI \times FG / 22.4$ and $HOMA-\beta = FI \times 20 / (FG - 3.5)$, where FI is fasting insulin (in $\mu U/mL$) and FG is fasting glucose (in mmol/L). Quantitative insulin sensitivity check index (QUICKI) was calculated as the inverse log sum of fasting insulin (in $\mu U/mL$) and fasting glucose (in mg/dL) using the equation, $QUICKI = 1 / (\log(FG) + \log(FI))$. Finally, fasting glucose-to-insulin (GI) ratio was calculated as the ratio of FG (in mg/dL) to FI (in $\mu U/mL$).

Determination of estrus cycle

A vaginal smear was performed 8 days before the day of blood collection. Cells on vaginal walls were collected by washing vagina with normal saline (0.9% NaCl) and smeared on glass slides. The slides were examined under microscope for the relative abundance of nucleated epithelial cell (E), cornified epithelial cell (CE) and leucocytes (L). The four stages of cycle estrus (CE), meta-estrus (L & CE), diestrus (L & E) and proestrus (E) were determined.

Biochemical analysis

Blood was collected from retro-orbital plexus of the rats under light ether anesthesia into Eppendorf tubes. The blood samples were kept at room temperature for 45 min. The serum was separated by centrifugation at 12000g for 10 min at 4°C (REMI, Mumbai, India). The serum was analyzed for triglyceride (TG) level (GPO-PAP) and total cholesterol (TC) level (CHOD-POD) using commercially available colorimetric diagnostic kits (Span Diagnostic, Surat, India). Serum insulin, LH, FSH, estrogen, progesterone and testosterone levels were estimated by ELISA kits (Genxio Health Sciences, Delhi, India).

Ovarian gene expression analysis

At the end of experiment, ovaries were collected from all groups during diestrus phase and homogenized in RiboEx™ (GeneAll Biotechnology, Seoul, South Korea). Total cellular RNA was extracted from ovary using RNA isolation kit (GeneAll biotechnology) in accordance with the manufacturer's instructions. Total RNA from each sample was reverse transcribed into cDNA using PrimeScript Reverse Transcriptase. An equal amount of cDNA from each sample was taken for quantitative real-time PCR using MX3005P qPCR system (Agilent Technologies). Oligonucleotide primers (FP: forward primer; RP: reverse primer) used for qRT-PCR are listed below.

LH receptor – FP: (5'-3') TCCGTGGACTCCCAAACA,
RP: ATCGTG GCGATCAGCGTA;

FSH receptor – FP: (5'-3') ACTGTGCATTCAACGGAA,
RP: GCCTCC ATGAGGGTGACA;

β -actin – FP: (5'-3') CTGGAGAAGAGCTATGAGCTGCCTG,
RP: AGGAAGGAAGGCTGGAAGAGAGCCT

Differences in gene expression, expressed as fold-change, were calculated, where β -actin was used as the reference gene (Livak & Schmittgen 2001).

Ovarian weight and histopathology of ovary

At the end of the study, during diestrus phase, rats were killed and ovaries were isolated. Afterward, ovaries were weighted and transferred to 10% formalin (formaldehyde in saline). Formalin-fixed ovarian tissue was dehydrated, embedded in paraffin and sectioned at 4–5 μm . Sections were mounted on glass slide and stained with hematoxylin-eosin for light microscopy. The slides were scanned with trinocular microscope Primostar (Zeiss, Germany) and analyzed with Magnus Pro 3.0. The number of follicles, diameter of largest follicle and thickness of follicular wall were measured by two persons blinded to the origin of sections.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. Statistical difference between two groups was tested by unpaired t -test, and $P < 0.05$ was considered significant. Only the effect of letrozole or HFD on body weight and

glucose level during OGTT were assessed by two-way ANOVA and Bonferroni's *post hoc* test. All data were expressed as mean $\pm$ S.E.M.

Results

Body weight and food intake

The body weight is represented in Fig. 1A and B. Twenty one days after the letrozole administration, significant increase in the body weight was observed in LET group as compared to CL group (CL: 252 ± 5.03 g vs LET: 295 ± 4.00 g, $P < 0.05$). However, the feeding of HFD for 105 days did not significantly increase body weight in HFD group as compared to CH group (CH3: 235 ± 7.63 g vs HFD3: 241 ± 2.46 g, $P < 0.05$). Feeding of HFD reduces the food intake in HFD group as compared to control group (CL3: 12.69 ± 0.95 g/day/animal vs HFD3: 9.01 ± 1.12 g/day/animal). Contrary to this, letrozole administration increases food intake in letrozole group as compared to control group (CL: 15.56 ± 0.67 g/day/animal vs LET: 18.11 ± 0.88 g/day/animal).

Fasting glucose level, oral glucose tolerance test (OGTT) and area under curve (AUC)

In the letrozole study, significant difference was observed regarding 2-h glycemia (CL: 127 ± 7.3 vs LET:

171 ± 17.48 , $P < 0.05$) and AUC of OGTT (CL: 322 ± 3.50 vs LET: 347 ± 4.10 , $P < 0.05$). In HFD study, there was no significant change in 2-h glycemia (CH1: 137 ± 5.09 vs HFD1: 144 ± 3.12) and AUC of OGTT (CH1: 336 ± 5.32 vs HFD1: 318 ± 5.65) between groups on day 49. Additionally, on day 77, significant change was observed in 2-h glycemia (CH2: 127 ± 3.92 vs HFD2: 157 ± 3.82 , $P < 0.05$) and AUC of OGTT (CH2: 300 ± 10.33 vs HFD2: 333 ± 6.79 , $P < 0.05$) in HFD2 group as compared to CH2 group. On day 105, significant change was observed in

Figure 1 Body weight of animals in the letrozole study (1A) and HFD study (1B); Data represented as mean $\pm$ S.E.M.; * $P < 0.05$ compared to control group (unpaired *t*-test); (A) CL-control group; LET-Letrozole group. (B) CH-control group; HFD-High-fat diet group.

Figure 2 Oral glucose tolerance test and Area under curve of letrozole study (A and B), HFD study on day 49 (C and D), HFD study on day 77 (E and F) and HFD study on day 105 (G and H); Data represented as mean $\pm$ S.E.M.; * $P < 0.05$ compared to control group (data of OGTT was analyzed by two-way ANOVA and Bonferroni's *post hoc* test and data of AUC was analyzed by unpaired *t*-test); CL-control group; LET-Letrozole group. CH1-control group on day 49; HFD1-HFD group on day 49; CH2-control group on day 77; HFD2-HFD group on day 77; CH3-control group on day 105; HFD3-HFD group on day 105.

Table 2 Fasting glucose level, fasting insulin level and insulin sensitivity indices of different animal groups in the letrozole study and HFD study.

Groups	Day of measurement	Fasting glucose (mg/dL)	Fasting insulin (mU/L)	HOMA-IR	HOMA-β	QUICKI	GI ratio
Letrozole study							
CL	Day 21	130.5±3.25	2.49±0.55	0.86±0.1	11.68±1.68	0.4±0.007	56.44±6.44
LET	Day 21	128.17±6.14	4.65±0.88	1.58±0.14	17.88±1.03	0.36±0.005	29.12±3.10
HFD study							
CH1	Day 49	129.5±1.52	1.99±0.22	0.69±0.05	9.27±0.41	0.42±0.005	66.22±3.29
HFD1	Day 49	130.17±2.08	3.12±0.06	1.11±0.03	13.92±0.68	0.38±0.001	42.94±1.15
CH2	Day 77	109.33±3.89	2.05±0.37	0.58±0.04	13.41±1.44	0.43±0.050	56.15±4.02
HFD2	Day 77	115.25±4.04	10.41±0.93*	3.27±0.27*	61.09±5.49*	0.32±0.004*	11.34±0.57*
CH3	Day 105	131.5±1.66	1.87±0.89	0.7±0.04	9.06±0.6	0.41±0.005	67.39±3.84
HFD3	Day 105	133.71±3.31	12.14±1.44*	4.41±0.38*	53.08±3.13*	0.31±0.005*	11.35±0.7*

Data are represented as mean±S.E.M.; * $P<0.05$ compared to respective control group (unpaired t -test); In the letrozole study: CL, control group; LET, Letrozole group. In HFD study: CH1, control group on day 49; HFD1, HFD group on day 49; CH2, control group on day 77; HFD2, HFD group on day 77; CH3, control group on day 105; HFD3, HFD group on day 105.

2-h glycemia (CH3: 133 ± 2.27 vs HFD3: 159 ± 2.41 , $P<0.05$) and AUC of OGTT (CH3: 292 ± 3.81 vs HFD3: 335.97 ± 3.08 , $P<0.05$) and in HFD3 group as compared to CH3 group (Fig. 2). The significant change was not observed in fasting glucose level in both studies at any time point (Table 2).

Fasting insulin level and insulin sensitivity indices

In the letrozole study, there was no significant change in fasting insulin level, HOMA-IR, HOMA-β, QUICKI and GI ratio. In HFD study, on day 49, there was no significant change in fasting insulin levels and insulin sensitivity indices. However, feeding of HFD leads to significant change in fasting insulin level, HOMA-IR, HOMA-β, QUICKI and GI ratio in HFD2 and HFD3 groups as compared to respective control group on day 77 and day 105. (Table 2).

Table 3 Lipid profile of different animal group in the letrozole study and HFD study.

Groups	Day of measurement	TG level (mg/dL)	TC level (mg/dL)
Letrozole group			
CL	Day 21	97.39±3.6	94.20±2.59
LET	Day 21	135.22±7.12*	95.18±3.69
HFD group			
CH1	Day 49	86.49±1.47	92.87±2.8
HFD1	Day 49	82.72±1.97	106.43±6.08
CH2	Day 77	97.49±4.25	99.22±3.56
HFD2	Day 77	87.27±1.47	98.34±2.78
CH3	Day 105	95.79±2.69	98.95±3.53
HFD3	Day 105	89.00±1.28	118.74±2.36*

Data are represented as mean±S.E.M.; * $P<0.05$ compared to respective control group (unpaired t -test); In the letrozole study: CL, control group; LET, Letrozole group. In HFD study: CH1, control group on day 49; HFD1, HFD group on day 49; CH2, control group on day 77; HFD2, HFD group on day 77; CH3, control group on day 105; HFD3, HFD group on day 105.

Lipid profile

In the letrozole study, TG level was significantly higher (39%) in LET group than observed in control animals. However, TC levels remain unaffected. High-fat diet for 105 days (HFD3) caused a small but statistically significant increase in TC levels, but not TG levels, as compared to control animals (Table 3).

Estrus cycle

Estrus cycle was assessed by vaginal smears. Control animals of letrozole study as well as HFD study exhibited regular estrous cycle of 4–5 days. Vaginal smear of LET group showed only leucocytes, the dominant cell type of the diestrus phase indicating complete acyclicity. However, HFD groups showed prolonged diestrus phase indicating irregular estrous cycles. As the duration of the study increases, duration of diestrus phase gradually increases in HFD group as compared to CH group (Table 4).

Hormonal profile

In the letrozole study, significantly lower estrogen level (27%) and higher testosterone level (87%) was observed in LET group as compared to CL group. However, progesterone levels were similar in both groups. Animals subjected to HFD showed significantly lower estrogen and progesterone levels and higher testosterone levels as compared to control animals. This effect was independent of days of HFD administration. Furthermore, LH, FSH levels and LH/FSH ratio were not different in both letrozole and HFD study. Table 5 shows the hormonal profile of different animal groups in the letrozole study and HFD study.

Table 4 Percentage of diestrus phase of different animal groups in the letrozole study and HFD study.

Groups	Day of measurement	Diestrus phase (% of total)
Letrozole study		
CL	Day 14–21	45.83 ± 6.97
LET	Day 14–21	100 ± 0.00*
HFD study		
CH1	Day 42–49	52.08 ± 2.08
HFD1	Day 42–49	61.46 ± 3.91
CH2	Day 70–77	54.17 ± 2.64
HFD2	Day 70–77	67.71 ± 2.41*
CH3	Day 98–105	52.08 ± 3.84
HFD3	Day 98–105	70.36 ± 2.18*

Data are represented as mean ± s.e.m.; * $P < 0.05$ compared to respective control group (unpaired t -test); In the letrozole study: CL, control group; LET, Letrozole group. In HFD study: CH1, control group on day 49; HFD1, HFD group on day 49; CH2, control group on day 77; HFD2, HFD group on day 77; CH3, control group on day 105; HFD3, HFD group on day 105.

Ovarian mRNA expression

A 2.68 ± 0.24-fold increases in mRNA expression of LHR with no effect (1.07 ± 0.14) on *Fshr* mRNA expression was observed in HFD-fed rats. However, such change in *Lhr* (1.15 ± 0.64) and *Fshr* (0.72 ± 0.16) receptor expression was not observed in letrozole-treated animals.

Ovary weight and histopathology of ovary

Ovary weights of letrozole-treated animals were significantly higher than those in control group animals (CL: 54.96 ± 3.16 mg vs LET: 71.94 ± 5.53 mg, $P < 0.05$). In contrast, in HFD-fed animals, the ovary weight remained unaffected (CH3: 38.94 ± 1.65 mg vs HFD3: LET: 48.69 ± 1.22 mg).

Multiple ovarian follicles and less numbers of corpus luteum were observed in letrozole-treated and HFD-fed rats as compared to few ovarian follicles and large number of corpus luteum in control rats. Follicular diameters were also higher in both test group animals as compared to control animals. However, letrozole-treated animals had greater follicular diameter than HFD-fed rats. Additional changes observed were thin layer of granulosa cells, hyperplasia of theca cells and

atresia of follicles. During atresia, ovum degenerates and granulosa cells undergo apoptosis, which are replaced by fibrous material (Figs 3 and 4 and Table 6).

Discussion

Insulin resistance, androgen excess and abnormal gonadotropin dynamics are the key elements in pathophysiologic defect of PCOS. Controversy regarding fundamental element in pathophysiology of PCOS exists (Guzick & York 1998). However, growing evidences suggesting insulin resistance precedes all others (Geffner *et al.* 1987, Nestler *et al.* 1989). Both, hyperandrogenism and hyperinsulinemia are frequently observed in clinical PCOS and the underlying treatment majorly targets hyperinsulinemia and insulin resistance (Azziz *et al.* 2004). Insulin's co-gonadotropin-like activity modulates ovarian steroidogenesis (Balen 2004). Thus, disrupting insulin signaling in the brain affects both ovulation and body weight regulation.

The animal models used for PCOS involve administration of estrogens, anti-progesterone or androgens (Brawer *et al.* 1986, Pinilla *et al.* 1993, Sanchez-Criado *et al.* 1993, Wu *et al.* 2010). Even letrozole that produce androgenic effects by inhibiting aromatase enzyme has been used to generate PCOS like features in rats (Kafali *et al.* 2004). Estrogen valerate but not letrozole administration is reported to produce higher fasting glycemia (Daneasa *et al.* 2016). Several studies have analyzed both the reproductive and metabolic features of hyperandrogenized rodents. The presence of metabolic phenotype seems dependent on the androgen used and the timing of the treatment. Andronate with HFD in rat and dehydroepiandrosterone with HFD in mice shows both ovarian and metabolic features of PCOS which resulted in polycystic ovaries, obesity, irregular cycles of vaginal smear, increased plasma insulin levels, decreased insulin sensitivity, hyperandrogenism and increased LH concentrations (Zhai *et al.* 2012, Lai *et al.* 2014). HFD is known to induce various metabolic changes in animals. We attempted to study the effects of HFD feeding to pre-pubertal animals on both ovarian and metabolic functions. By not administering the

Table 5 Hormonal profile in different animal group in the letrozole study and HFD study.

Groups	FSH (mIU/L)	LH (mIU/L)	LH/FSH ratio	Estrogen (ng/L)	Progesterone (ng/ml)	Testosterone level (mIU/L)
Letrozole group						
CL	10.26 ± 0.89	8.13 ± 0.35	0.80 ± 0.05	59.44 ± 6.98	126.84 ± 5.05	4.67 ± 0.42*
LET	9.48 ± 0.53	7.56 ± 0.31	0.80 ± 0.06	43.39 ± 0.51*	128.17 ± 4.96	8.77 ± 0.36
HFD group						
CH2	14.92 ± 0.66	12.12 ± 0.9	0.82 ± 0.1	58.56 ± 3.36	331.45 ± 5.47	19.40 ± 0.39
HFD2	12.90 ± 0.83	10.08 ± 0.23	0.77 ± 0.06	39.64 ± 2.09*	162.18 ± 33.17*	20.89 ± 0.92
CH3	13.25 ± 0.47	11.67 ± 0.87	0.81 ± 0.04	59.22 ± 4.11	311.46 ± 23.56	17.85 ± 0.38
HFD3	14.31 ± 1.07	13.00 ± 1.22	0.96 ± 0.11	43.53 ± 7.32*	205.09 ± 13.03*	28.09 ± 2.26*

Data are represented as mean ± s.e.m.; * $P < 0.05$ compared to respective control group (unpaired t -test); In the letrozole study: CL, control group; LET, Letrozole group. In HFD study: CH2, control group on day 77; HFD2, HFD group on day 77; CH3, control group on day 105; HFD3, HFD group on day 105.

Figure 3 Histological sections of ovaries at different magnification (10 $\times$, 100 $\times$) from control rats and letrozole administered rats. Scale bar 1 mm. (—) shows thickness of granulosa cell layer (GC) and Theca layer (T) Control rats: Growing follicles in various stages of development and numerous corpus luteum (CL) are seen. Magnified view demonstrates follicle with normal follicular wall (FW) thickness. Letrozole treated rats: Numerous large cystic follicles (arrows) and least number of corpus luteum are seen. Higher magnification shows increased follicular wall thickness (Granulosa cell layer (GC) and Theca layer (T)).

androgens, we wished to observe the development of hyperandrogenism by HFD feeding alone.

The HFD fed rats were divided into three subgroups depending on the duration of HFD feeding. Various parameters for detecting, insulin resistance, glucose intolerance, hormonal imbalance and hyperandrogenism were measured in each of these animals. The study

Figure 4 Histological sections of ovaries at different magnification (10 $\times$, 100 $\times$) from control rats and HFD fed rats. Scale bar 1 mm control rats: Ovary shows follicles at different stages of development and several corpus luteum (CL). Oocyte (O), and normal follicular wall thickness are visible upon magnification. HFD fed rats: Multiple follicles (arrows) are seen. Arrows show follicles in the early process of atresia with apoptotic granulosa cells. Follicles have thickened follicular wall.

design helped us to observe time-dependent changes happening in the rats and different stages of disease progression. A separate group of adult rats were administered letrozole to act as positive control.

Since HFD was introduced at pre-pubertal age and letrozole group were introduced in to the study in adult age, the comparison between both the groups is difficult. However, the age of the animals when the final parameters were measured was similar in both study.

Various pilot studies were conducted for optimization of HFD in pre-pubertal rats. In initial pilot study, HFD was prepared simply by addition of fat in normal pellet diet. Nonetheless, addition of high fat replaces the percentage of protein, an important nutrient for the growth of pre-pubertal rats. Thereafter, protein supplements were added to compensate the need of protein and HFD was prepared with 20, 30 and 40% of fat. On the basis of the results of glucose intolerance and hyperinsulinemia, 40% HFD was selected for further study (results not shown here). However, to accustom the animals with such high fat, we gradually increased the fat content in the diet over two weeks from 20 to 40% level.

Twenty-one-day letrozole administration resulted in approximately 30% increase in body weight in contrast to just 10% increase in control animals. This is in accordance with reports of previous studies (Mannerås *et al.* 2007, Maharjan *et al.* 2010). Lai and coworkers reported that the body weight was increased in DHEA +HFD-treated C57BL/6 mice (Lai *et al.* 2014). In our study, HFD-fed animals failed to show such rise in body weight. Ramirez and coworkers hypothesized that HFD promotes hyperphagia in rodents (Ramirez 1990). In contrast, we observed approximately 25% reduction in food intake in HFD-fed rats. In terms of calorie intake, both the normal and high-fat diet-fed animals showed similarity. Chen and coworkers and Picchi and coworkers also reported reduced food intake in HFD-fed rats (Chen *et al.* 2008, Picchi *et al.* 2011). In clinical situation too, obesity and hyperphagia are not observed in more than 50% of the PCOS patients (Yen 1980, Franks 1989, Morales *et al.* 1996).

It is well-known that hyperinsulinemic IR and glucose intolerance plays a prominent role in PCOS. Reduction of insulin sensitivity by 50% in non-obese PCOS patients and still further reduction in obese PCOS patients well-known (Morales *et al.* 1996, Holte *et al.* 2014). This suggest that obesity contributes an additional component to IR (Angioni *et al.* 2008). Though, elevated fasting insulin level is representing the insulin resistance, simple insulin sensitivity indices like HOMA-IR (ratio indicating insulin resistance), HOMA- β (ratio indicating the insulin-secretory capacity of islet β -cells), QUICKI (log transform of the insulin glucose product) and FGIR are also used to assess insulin sensitivity. FGIR is a useful measure of insulin sensitivity in women with PCOS (Legro *et al.* 1998). Furthermore, glucose intolerance also can be better evaluated by OGTT rather than alone

Table 6 Histopathological changes of ovary in the letrozole study and in HFD study.

	CL (n=9)	LET (n=10)	CH (n=9)	HFD (n=12)
Number of follicles (n)	2.12 ± 0.03	7.89 ± 0.06*	1.54 ± 0.07	9.34 ± 0.13*
Number of corpus lutea (n)	10.26 ± 0.04	4.05 ± 0.05*	10.78 ± 0.04	7.22 ± 0.03*
Diameter of largest follicle (µm)	268.65 ± 33.1	867.32 ± 34.78*	233.65 ± 29.12	478.32 ± 21.22*
Thickness of follicular wall (µm)	58.34 ± 2.67	158.34 ± 7.43*	71.45 ± 1.97	145.23 ± 10.23*

Data are represented as mean ± S.E.M.; * $P < 0.05$ compared to control group (unpaired *t*-test).

CH, control group for HFD group; CL, control group for LET; HFD, high-fat diet group; LET-Letrozole group.

fasting glucose level in PCOS patients (Legro *et al.* 1998, Mastuda & Defronzo 1999). Except for the glucose intolerance reflected in OGTT test, no other parameters which can reflect IR showed any changes due to letrozole administration. Similar observations have been reported in earlier studies (Mannerås *et al.* 2007, Maharjan *et al.* 2010, Reddy *et al.* 2016). Feeding of HFD for 120 days to an adult female rats produced IR and ovulatory infertility (Akamine *et al.* 2010). Impaired glucose tolerance and IR was also observed in DHEA+HFD treated C57BL/6 mice (Lai *et al.* 2014). Similarly, we also observed a gradual increase in fasting insulin levels and glucose intolerance after 49 days of HFD feeding which became more prominent with further continuation of HFD. This suggests that after 49 days of HFD feeding, metabolic disturbances becomes observable. The development of insulin resistance and increased glucose intolerance in HFD fed animals was not associated with increase in body weight. A more detailed study which can look in to the changes in liver and adipose tissue may help in understanding the possible cause of insulin resistance without developing obesity.

According to the National Cholesterol Education Program (NCEP) guidelines, approximately 70% of PCOS women exhibit abnormal serum lipid levels. As PCOS women are also insulin resistant, it would be expected that IR is responsible for reduction in the activity of lipoprotein lipase and resulting dyslipidemia (De Fronzo & Ferrannini 1991, Krauss & Siri 2004). HFD gradually increased TC level but not TG level. Increased TC level was also reported in DHEA+HFD treated C57BL/6 mice (Lai *et al.* 2014). In letrozole treated rats, TG levels were higher and TC levels remained unaffected. In one study, letrozole caused increase in both TG and TC level (Reddy *et al.* 2016). The other study, reported no effect on lipid profile (Mannerås *et al.* 2007).

Letrozole, a non-steroidal aromatase inhibitor reduces conversion of androgens to estrogens in the ovary (Corbin *et al.* 1999). This is reflected as lower estrogen and higher testosterone levels in letrozole treated rats. Additionally, progesterone levels are also reduced (Kafali *et al.* 2004, Baravalle *et al.* 2006, Mannerås *et al.* 2007). Our observations in letrozole treated animals are also similar. The hormonal changes in HFD fed rats could be as a consequence to insulin resistance and hyperinsulinemia. Insulin mediates hyperandrogenism by decreased hepatic production of SHBG (Nestler *et al.* 1991), by activation

of inositol phosphoglycan pathway in theca cell (Medina & Nestler 1998) and amplification of LH response on theca cells (Krauss & Siri 2004).

Pulsatile GnRH stimulation is required to maintain ratio of gonadotropins secretion which is modified by hyperinsulinemia (Dunaif 1997). Along with this, reduction in estrogen level weakens the negative feedback on LH production in pituitary (Ajika *et al.* 1972). This is reflected by increased LH level, increase/no change in FSH levels or altered LH/FSH ratio (>2:1) in PCOS patients. Both LH and FSH levels and their ratio remained unaltered by HFD. However, LH receptor expression got up-regulated by 2.6 folds with little effect on FSH receptor expression. The lack of effect on LH levels in HFD fed rats could probably be because of serum sampling in the morning which missed the high-amplitude LH pulses during night (Venturoli *et al.* 1988). Though, altered LH/FSH ratio is present in a significant proportion of women with PCOS and is suggestive of PCOS but is not specific for diagnosis (Zawadzki & Dunaif 1992).

The increased hyperinsulinemia increases the expression of LH receptor, which in turn can lead to potentiation of ovarian steroidogenic response to gonadotropins, premature granulosa differentiation, arrest of follicular growth and resulting anovulation (Hegsted *et al.* 1974, Thiruppathi *et al.* 2001, Stracquadanio & Ciotta 2015). The absence of hyperinsulinemia could at least partly explain unaltered LH receptor expression in letrozole-treated rats.

A gradual increase in diestrus phase duration is suggestive of disrupted estrus cycle in HFD fed rats. Letrozole administration also produced complete acyclicity in rats. Thus, fertility is significantly impaired in both cases. Imbalance in sex hormone levels also produced structural changes in ovary. The number of cystic follicles was increased and cystic wall was thickened characterized by attenuated or diminished granulosa cell layer and thickened theca cell layer in letrozole-treated as well as HFD-fed rats. Along with this, the ovary also showed increase in atretic antral follicles and decrease in corpus luteum. These histopathological changes in rat ovaries, resembles with earlier studies (Kafali *et al.* 2004, Mannerås *et al.* 2007).

PCOS, as earlier stated, is characterized by hyperandrogenism, polycystic ovaries and oligo and/or anovulation and endocrine disorder with numerous

metabolic consequences, prominent being insulin resistance (Oakley & Lin 2011). In the present study, the initiating HFD in pre-pubertal rats and continuing it for 105 days resulted in all of the above mentioned disturbances. However, LH levels remained unaffected in this study. Thus, inducing HFD at early age in rats has special merits over many other available animal models for PCOS.

Conclusion

HFD initiated at pre-puberty age in rats produces both metabolic disturbances and ovarian changes like increase number of cystic follicles and increase follicular wall thickness similar to that observed clinically in PCOS patients. The developments of these features are slow and progressive and mimics closely to clinical PCOS. Letrozole on the other hand induces change in ovarian structure and function without producing any metabolic disturbances. Also, these changes are quite rapidly developed as compared to those observed with HFD model. As the HFD-induced model produces hyperinsulinaemia-induced hyperandrogenism, it may become helpful in understanding the pathogenesis of PCOS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grant from the Gujarat council on science and technology, under the minor research project scheme (Grant No.: GUJCOST/MRP/2015-16/2689).

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Received 23 September 2017

First decision 16 October 2017

Revised manuscript received 30 October 2017

Accepted 9 November 2017