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Research Article

The Therapeutic Effects of Juglan Regia Linn (Walnut) Oil on Letrozole Induced Polycystic Ovaries in Rats

Zoha Khan*, Lubna Naz, Sakina Shabbir and Amber Ayaz Memon

Department of Physiology, University of Karachi, Karachi, Pakistan

*Corresponding Author E-mail: lunaz@uok.edu.pk

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ABSTRACT

BACKGROUND

Juglans regia L. (Walnut) is a plant of significant economic and medicinal importance. It is a source of phytosterol, omega-3 fatty acids, vitamin E and K, melatonin, antioxidants, monounsaturated fats (MUFAs) and polyunsaturated fats (PUFAs) and lowers cholesterol by reducing low-density lipoprotein concentration. It is a phytoestrogen that is seen to increase sex hormone binding globulin, improve insulin sensitivity and androgen levels. Walnut with these nutritional properties ameliorates the hormone profile in polycystic ovary syndrome (PCOS) women and can be considered as a positive treatment regime. This study was aimed to determine the antiandrogenic effect of Juglans regia L. on letrozole induced PCOS rats, a cure that is on hand for the victims, less detrimental and economically accessible.

METHOD

Age matched Female Wistar rats were allotted into 3 groups (n=6). Control remained untreated while PCOS was induced in both letrozole treated and letrozole + walnut treated groups by administering a dose of 1mg/kg body weight of letrozole per day for 21 days. Rats of group III from day 6 onward were given walnut oil in 1mg/kg of body weight for 15 days as a cotreatment. Every morning at 10 am their vaginal smears were taken to observe their estrous cycle. All the rats were weighed daily. On 22th day of experiment the rats were sacrificed and their ovaries were extracted for histological investigation.

RESULT

Letrozole induced PCO rats showed atretic follicles and thin granulosa layers and remained in diestrus phase throughout the experiment. Walnut treated PCOS rats also demonstrated diestrus phase throughout the treatment with few alterations in the number and the type of cells. Weight remained constant and there was a significant decrease observed in glucose, lipid, cholesterol and testosterone of Letrozole + Walnut treated rats.

CONCLUSION

Walnut's potential for treating the symptoms and causes of PCOS was observed in this study and we concluded that with proper amount of dose, walnut oil may be considered as a significant dietary-therapeutic agent for PCOS patients.

Key words: *PCOS, Juglans regia L., Walnut, Letrozole, Phytoestrogen, Testosterone, Cholesterol.*

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INTRODUCTION

Poly Cystic Ovary Syndrome (PCOS) is a heterogenous disorder with obscured etiology, affecting as many as 6% to 21 % of females of reproductive age[42]. PCOS remains a syndrome with no single diagnostic criteria sufficient enough for clinical diagnosis. It is manifested with reproductive, psychological and metabolic features including ovulatory dysfunction[25], Polycystic ovaries[16], hyperandrogenemia[40],abnormal gonadotrophin concentrations[13], insulin resistance[11], hyperinsulinemia, androgen secreting tumors to thyroid disorders[6] are the manifestations attributed to endocrine alterations with excess androgen production either of ovarian or adrenal origin with subsequent chronic anovulation or oligo-ovulation that occurs because of arrested follicular development. Due to aberrant folliculogenesis, there is excessive thickness of ovarian stroma and pool of small follicular cysts that yields a characteristic morphology on ultrasound[36].

PCOS has been observed in girls before their reproductive years suggesting occurrence in prenatal life during ovarian development and oogenesis, proving that abnormalities during fetal development and can be considered as one of the etiological factors for this complex disorder[60]. Genes committed for the early follicular growth, for instance Transforming growth factor beta (TGFB) family including anti-Mullerian hormone (AMH) and growth differentiation factor-9 (GDF-9) and androgen exposure are said to cause earlier follicular growth which may form basis for PCOS[59]. Hyperandrogenism is considered to be the core functional disorder in both women with PCOS[52] and primates that are exposed to high androgen levels prenatally[61]. Hyperinsulinemia have proved to be a key factor in producing hyperandrogenism. In female body testosterone is found bounded to SHBG, because of the decrease in serum SHBG levels, the circulating testosterone concentration available to tissue increases[12]. Recent evidence suggests that hyperinsulinemia stimulates the activity of tissue 5a-reductase activity and act as a growth factor causing hyperandrogenism at tissue level, thus increasing the conversion of testosterone to dihydrotestosterone (DHT) which is its active form[31]. Hence any treatment that is aimed at lowering insulin levels decreases androgen levels[33]. Overweight PCOS women have more chances to be anovulatory and to have symptoms of hyperandrogenemia. Studies have reported that women suffering from PCOS have higher insulin resistance as compared to women with same age and (BMI) body mass index[19]. Other Studies have shown that administering of insulin- sensitizing drugs in PCOS patients, significantly improves many characteristic features such as, anovulation, monthly cycle and infertility[38,30].

Some researchers have hypothesized autosomal dominant transmission either linked to a single genetic defect or with polygenic pathology[24]. Work done by Rosenfield *et al*. [51] showed that gene CYP17 encodes P450c17α \was associated with PCOS[10]. CYP11a Alleles were evidently associated with both hirsutism and hyperandrogenemia in PCOS women[26].

The health benefits credited to walnut are attributed to its chemical composition. J. regia is enriched with potential neuroprotective compounds including gamma tocopherol (Vit. E), melatonin, folate, phenolic acid (ellagic acid), flavonoids and a significant amount of n-3 α-linolenic acid (ALA) which is a plant-based omega-3 fatty acid[47] and essential fatty acids[5,4]. Linoleic acid serves as a major fatty acid, followed by oleic, linolenic, palmitic, and stearic[5,53,54]. It also have high content of poly-unsaturated fatty acids (PUFA) which evidently reduces the risk of heart disease by decreasing total and LDL-cholesterol and enhancing HDL-cholesterol[15,56].

Juglan regia L. have hypoglycemic effect owing to its high concentration of phenolic compounds[3]. Its antidepressant activity is attributed to the presence of omega 3 fatty acid[50]. The husk of regia is a promising apoptotic agent and is protective against certain cancers[32]. Its omega 3 and 6 PUFAs increases proinflammatory vascular response and lowers cardio vascular risk factors in non-

hyperlipidemic individuals[28]. It is hepatoprotective[55] and shows a significant impact on nephron cell regeneration[1]. Walnut leaf extracts showed an increase in antioxidant enzymes, including catalase and superoxide dismutase. It reduces alanine aminotransferase, aspartate aminotransferase, total and plasma albumin. Walnuts are also antimicrobial and anti-inflammatory nut[20].

Owing to its nutritional profile we have therefore hypothesized that walnut oil if given to PCOS women as a dietary supplement it can improve hormonal state with amelioration of other risk factors associated with this disorder.

MATERIAL AND METHODS

Research was conducted in accordance with health research extension act of 1985 and Ethical Guidelines of Institutional ERB. Accepted standards of animal care were used. The PCOS rat model was developed with aged matched Wistar rats, weighing approximately about 150–200 g. Rats were purchased from (DUHS) Dow University of Health and Sciences, Ojha Campus. These rats were housed under controlled conditions of 12-h light/12-h dark cycle at 24-26 C temperature. They were allowed free access to diet and water. Standard diet with normal sugar level was fed throughout the experiment.

PLANT MATERIAL AND HYDRAULIC PRESS EXTRACTION

Dried Walnuts were purchased from local market located in Sadar Bazar Karachi. Seeds were ground to a size between 1 and 3 mm and then oil was extracted from them by using a pilot plant hydraulic press, for 1 kg of each nut a pressure of 60 kg cm–2 for 10 min was applied. After oil extraction, a centrifugation step was carried out to eliminate the remaining solid particles from the sample. The oil extracted was stored in dark glass bottle to avoid oxidation until use.

STUDY PROCEDURE

The rats were divided into three groups (n=6)

1. Group I: control group (no treatment given),
2. Group II: (letrozole treated group) treated with letrozole - 1 mg/body weight daily for 21 days),
3. Group III: (letrozole + walnut oil treated group) treated with letrozole + Walnut oil - 1 mg/kg and 1 ml/kg body weight daily for 21, 15 days respectively.

Group II and III Animals were treated with letrozole every morning for 21 days, to induces acyclicity in their estrus cycle and anovulation, follicular cystic ovaries[45,37,63]. Both group II and III were administered with letrozole stock (10mg in 50 ml 0.9% NaCl solution) orally via gavage. The stock was prepared freshly every 3rd day.

After the rats were confirmed as positive models via diestrus phase of estrus cycle group III was given cotreatment with walnut oil extract in concentration of 1 ml per body weight, orally-daily for 15 days.

Vaginal Smear Preparation

For 21 days ovarian cycle using vaginal cytology was observed, the stage of estrus cycle was determined microscopically by analyzing chief cell type in vaginal smears. Every morning between 10-11 a.m. smear was obtained from each rat using a cotton tipped swab, dipped in physiological saline (0.9%) and that was inserted in the vagina of the restrained rat to a depth of approximately 1.0 cm, the swab was then gently turned and rolled against vaginal wall at an angle of about 45° to the animal's body, to obtain cells that were relocated to a dry glass slide which was then heat fixed and then stained with crystal violet.

After staining slides were rinsed, covered with coverslip and then examined at 10x and 40x magnification under bright field illumination. Stages of estrous cycle were identified according to the type of cells present cornified epithelial, leukocytes, and nucleated epithelial cells[21,9].

BIOCHEMICAL ASSESSMENTS

Glucose[2], Triglycerides[23], total cholesterol[41], high-density and low-density lipoprotein[44] and testosterone[14] were estimated in Control, Letrozole treated and Letrozole + Walnut treated rats.

HISTOPATHOLOGICAL ASSESSMENT OF CONTROL, LETROZOLE TREATED AND LETROZOLE + WALNUT TREATED RAT

Ovaries were removed and fixed in Bouins fixative solution for 24 h and then transferred

to 80% ethanol. After 24 hours each ovary was trimmed in accordance with the procedure outlined by the Registry of Industrial Toxicology Animal-data[7]. the fixed tissue samples were embedded in paraffin after dehydration and cut in sections of 2–3 mm. Histology sections were then stained with hematoxylin–eosin (HE)[58].

The degree of ovarian morphological disruption from histologic section is graded via following scoring:

Score –: No noticeable alterations (0%).

Score +: Mild destruction approximately 10-20 %

Score ++: Moderate changes in the morphology (30-50 %)

Score +++: Severe evidence of destruction (>50 %)

STATISTICAL ANALYSIS

Research results are represented by mean ± standard error of mean. Statistical analysis was done via SPSS (Statistical Package for the Social Sciences) Version-16. Statistical significance and comparison between different groups was done by the application of (ANOVA) one-way analysis of variance followed by the (Least Significance Difference Post hoc multiple comparison test) called as LSD test, Statistical significance was tested minimally at $p<0.05$.

RESULTS

Rats Estrus-Cycle Phase Identification:

Estrous cycle of rats has four phases; proestrus, estrus, metestrus and diestrus. The mean cycle length is 4 days[43]. The identification of each phase is based on the proportion of three types of cells observed in the vaginal smear:

1. Estrus phase = Cornified cells: these are large, angular and irregularly shaped, non-nucleated.
2. Pro-estrus phase = epithelial cells are mostly nucleated with a granular appearance.
3. Metestrus phase = epithelial cells tend to be non-nucleated and less granular.
4. Diestrus = Leucocytes: these cells are very small and round, nuclei not evident at the low magnifications. (OECD org).

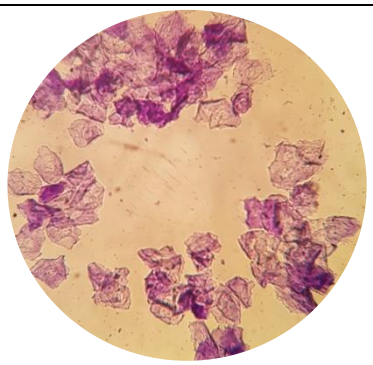
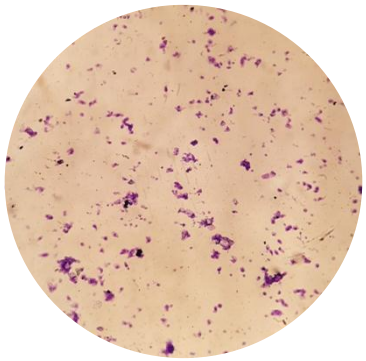
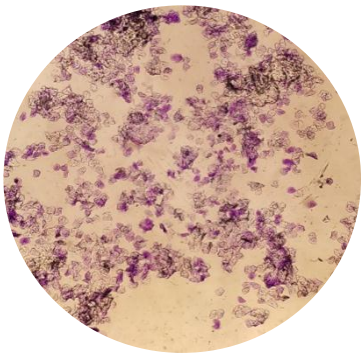
DAY: 1		
CONTROL GROUP	LETROZOLE TREATED	LETROZOLE+WALNUT TREATED
 **ESTRUS**	 **DIESTRUS**	 **ESTRUS**

Table: I

Vaginal smears of all three groups taken at 10x and 40x magnification. Images defines the phase of rats on the first day of experiment. Smears shows cornified, leukocytes and cornified respectively.

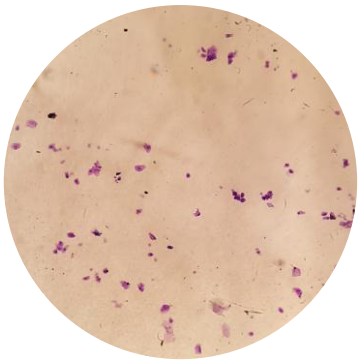
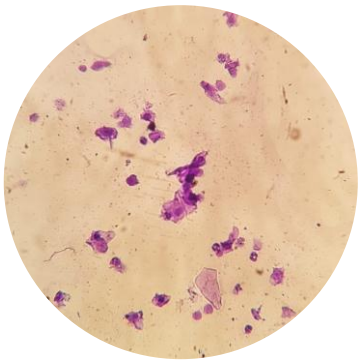
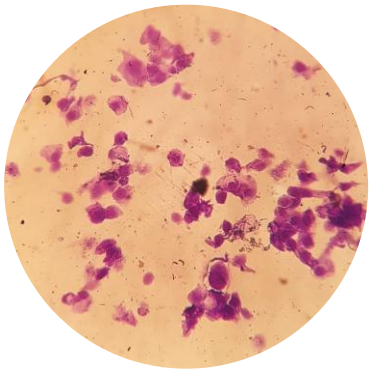
DAY: 6		
CONTROL GROUP	LETROZOLE TREATED	LETROZOLE+WALNUT TREATED
 **DIESTRUS**	 **DIESTRUS**	 **DIESTRUS**

Table: II

The slides show the vaginal smear on day 6th, the leukocyte cells of diestrus phase in these slides are indicator of PCOS in rats. Co-treatment of Juglan Regia oil started after the confirmation of PCOS

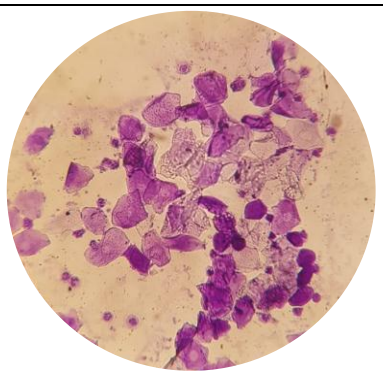
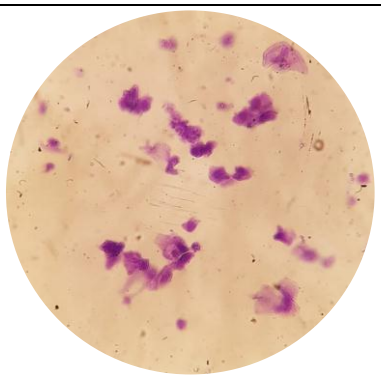
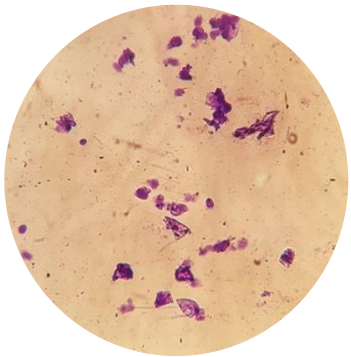
DAY: 15		
CONTROL GROUP	LETROZOLE TREATED	LETROZOLE+WALNUT TREATED
 **METESTRUS**	 **DIESTRUS**	 **DIESTRUS**

Table: III

Vaginal smears on day 15 showed no change in the estrus phase of letrozole and letrozole + Walnut treated rats. The non-nucleated cells depict metestrus phase.

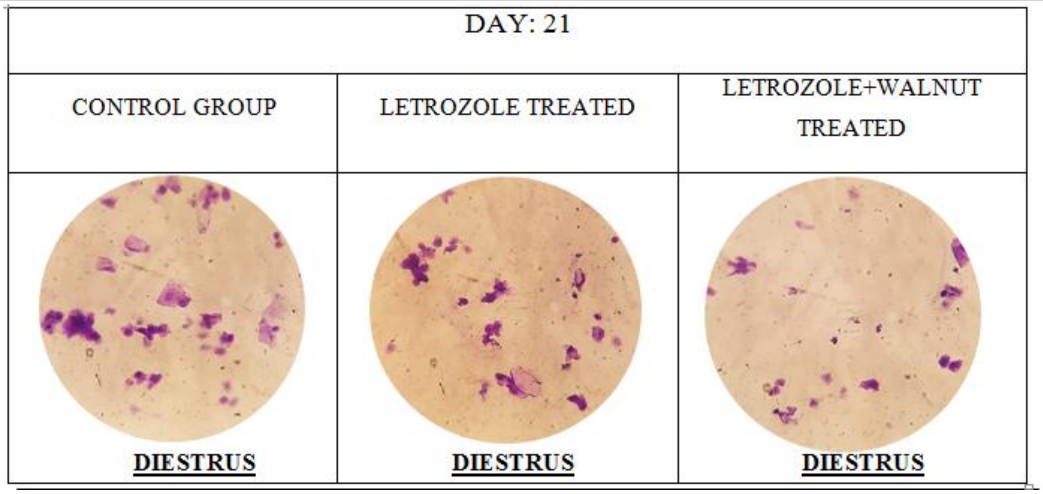
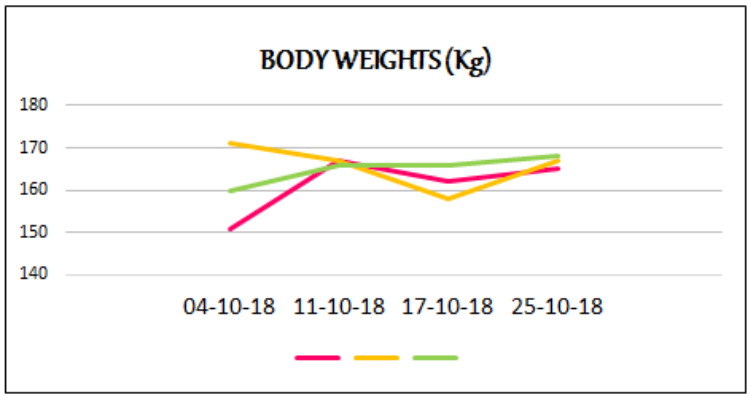


Table: IV

These slides of vaginal smear show that the rats remained in the diestrus phase.



Graph: I

Changes in body weight of Control (C), Letrozole treated (LT) and Letrozole + Walnut treated (LWT) rats.

Table VI: BIOCHEMICAL ESTIMATION OF CONTROL, LETROZOLE TREATED AND LETROZOLE + WALNUT TREATED RATS

PARAMETERS	CONTROL	LETROZOLE TREATED[1]	LETROZOLE+WALNUT TREATED[2]	LSD
TRIGLYCERIDE	147.62 ± 4.95	172.93 ± 2.12$^{\alpha}$	109.96 ± 9.21$^{\alpha}$	26.127
CHOLESTEROL	137.46 ± 2.46	197.06 ± 0.57$^{\beta}$	111.16 ± 4.85$^{\alpha}$	10.458
HDL	56.51 ± 0.67	53.88 ± 0.82^{n}	50.1 ± 0.65$^{\alpha}$	3.953
LDL	42.47 ± 2.34	114.35 ± 3.14$^{\beta}$	38.82 ± 3.96$^{\beta}$	9.56

Table V: HISTOPATHOLOGICAL ASSESSMENT

	HISTOLOGICAL FEATURES	CONTROL	LETROZOLE TREATED	LETROZOLE + WALNUT TREATED
1	HYPERPLASIA OF THECA INTERNA	+	+	+
2	CAPSULAR THICKENING	_	_	_
3	ATROPHY OF CORPORA LUTEA	_	_	_
4	HYPERTROHY OF CORPORA LUTEA	_	++	++
5	NUMBER OF FOLLICLES	32	16	28
6	FOLLICLE SIZE	1 mm	2mm	2mm
7	ATRETIC PRIMARY FOLLICLE	+	+	+
8	ATRETIC SECONDARY FOLLICLE	++	+++	+
9	ATRETIC GRAFFIAN FOLLICLE	_	_	_
10	GRANULOSA LUTEINIZATION	+	+	+
11	ENCAPSULATED GRANULOSA	_	_	_
12	NUMBER OF CYST	_	_	_

"-" No noticeable alterations, "+" Mild destruction approximately, "++" Moderate changes, "+++" Severe evidence of destruction.

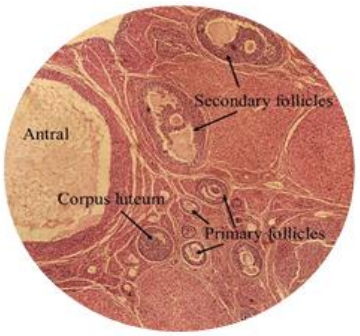


Figure: I: Control:

Secondary follicles, primary follicles and normal corpus luteum.

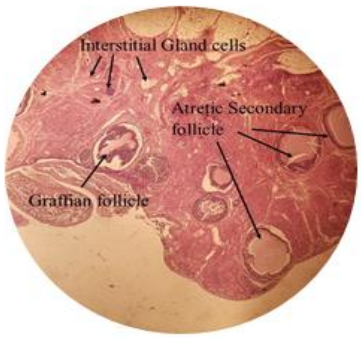


Figure: II: Letrozole Treated:

Antral follicles and few Secondary Atretic follicles with normal gland cells.

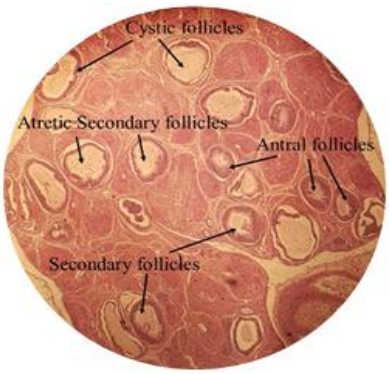
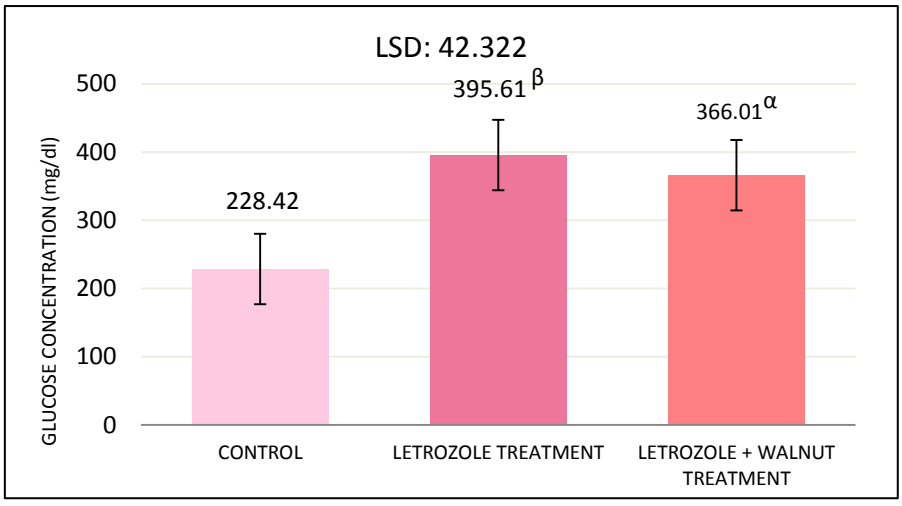


Figure: III: Letrozole + Walnut Treated:

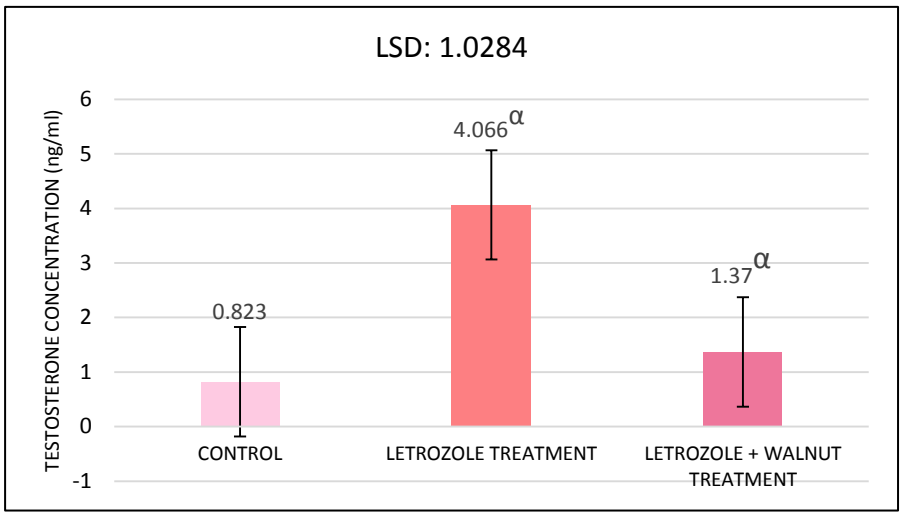
Many secondary atretic follicles with few cysts and antral follicles.

BIOCHEMICAL ESTIMATION OF CONTROL, LETROZOLE TREATED AND LETROZOLE + WALNUT TREATED RATS



Graph: II

Biochemical Assessment of Glucose in Control, Letrozole treated and letrozole + Walnut treated groups.



Graph: III

Biochemical Assessment of Testosterone in Control, Letrozole treated and letrozole + Walnut treated groups.

DISCUSSION

PCOS if not treated may foster some serious complications including infertility[35], Gestational diabetes[27], preeclampsia[39,34], miscarriage or premature birth, Nonalcoholic steatohepatitis, Metabolic syndromes that significantly increase the risk of cardiovascular disease[17], diabetes mellitus[18], Sleep apnea[22], Depression, anxiety[8], eating disorders[46] certain cancers including ovarian cancer breast cancer or uterine cancer and abnormal uterine bleeding. Obesity is a probable outcome of PCOS which can worsen complications.

To date our study was the first to be conducted on rats for the treatment of PCOS with dietary oil. For the entire period of cotreatment no microscopic change in diestrus phase of estrus cycle that is constant with PCOS model[45] was evident. The histological assessment of rat's ovaries (Figure I, II & III) however, revealed that letrozole treated group (figure III) had more secondary atretic and graffian follicles than control group (figure II). The follicles were larger with thinner granulosa layer. There was also presence of normal secondary follicles. In letrozole + walnut treated ovaries (figure III) few cystic follicles were observed; these cystic follicles are large fluid-filled cyst with thin granulosa cell layer and thick theca interna cell layer. There were also secondary follicles and many antral follicles, showing a progress towards normal histology. None of the group showed atrophy or corpus luteum and formation of corpus albicans. However, letrozole treated and Juglan treated group both had hypertrophy of corpus luteum (figure II & III).

PCOS is seen to accompany with increase in weight[29,57] and cholesterol[62]. Walnut on the other hand has proved to have PUFA which evidently reduces total and LDL-cholesterol and enhances HDL-cholesterol[15,56]. Our biochemical results fall on the positive side and have significant values proving that walnut significantly decreases LDL levels ($p<0.0001$) but there was not much increase in HDL levels however, p-value lied on the significant side being $p<0.022$ (Table VI). There was also evidence for the reduction of glucose in walnut treated rats ($p<0.027$) which was seen increased in letrozole PCOS model (Graph II). Triglyceride levels also reduced significantly ($p<0.012$) and fall back closer to those observed in control group (Table VI). Total cholesterol profile significantly decreased with the p-value ($p<0.004$) (Table VI).

The presence of Phyto-estrogenic compounds in walnut that we hypothesized were found to be effective in lowering androgen levels and enhances the female

hormonal profile. Testosterone levels of Juglan treated rats were significantly lowered when compared to those of untreated PCOS model ($p<0.010$) (Graph III). Isoflavones that are present in walnuts[49] may be the estrogen promoting factor. They act as dietary estrogens and have been considered as one of the nuts that enhances estrogen profile in females and is effective against irregular menstruation and anovulation.

CONCLUSION

Our results that walnut is infact a potent dietary supplement that can lower androgen profile and may be effective against elevated cholesterol and triglyceride levels. Similarly. it also acted as an antidiabetic agent. If taken in body calculated levels walnut can do wonders and can be concluded in light of this research as a positive treatment regime.

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