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Endocrine, Metabolic and Ovarian Features of Human PCO Repeat in Sprague Dawley Rats- An Experimental Study

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Abstract

Polycystic Ovary Syndrome (PCOS) affects 9-18 % of women in the reproductive age group, and is manifested as hyperandrogenism and infertility. However, the etiology of PCOS is still not clear, so knowledge from experimental animal models may perhaps enhance the knowledge regarding the mechanisms of establishment and advancement of PCOS. The present study was undertaken to validate the role of high fructose intake and the consequent alterations in the hormone levels as the causative factors of polycystic ovaries in females. Healthy, young colony-bred female albino rats, Sprague Dawley breed, weighing 80-85 g, were divided into three groups. Rats in group I served as placebo control. Group II rats received fructose, 10 g/10 mL/kg body weight (bw) per day through oral gavage. Group III rats received *ip* injection of insulin at 0.5 IU/kg bw/day. The treatment lasted 90 days. A significant (p<0.05) increase was detected in FSH, LH, insulin, testosterone and estradiol levels in fructose- and insulin-treated animals compared to the control. The levels of blood glucose, protein, cholesterol and triglyceride were significantly (p<0.05) increased in the treated groups. Cytoplasmic vacuolation, altered hepatic sinusoids, hepatic necrosis, etc., were observed in liver sections of treated rats. The histoarchitecture of ovary of treated animals presented fluid-filled cysts without granulosa cells and follicles. In the treated animals, histolpathological examination of fallopian tubal segments revealed infiltration of inflammatory cells with harshly crowded epithelial cells. Thus, fructose and insulin treatments reiterate certain endocrine, metabolic and ovarian characteristics of human polycystic ovary in therat.

Keywords: Estradiol, Histoarchitecture, Polycystic Ovarian Syndrome, Testosterone

1. Introduction

Polycystic Ovary Syndrome (PCOS) is one of the most common causes of female infertility, affecting 5 %-10 % of women[1], causing infertility in consequence of anovulation, distinctive polycystic ovaries and hyperandrogenism, accompanied by metabolic abnormalities including obesity, insulin resistance, hyperinsulinism[2], increased risk of type 2 diabetes[3] and cardiovascular disease. A unifying notion for PCOS has yet to become known from the endocrine-metabolic changes associated with the disorder. In spite of its popularity and health impact, the etiology of PCOS remains obscure. Etiological assumptions for the sources of PCOS encompass hormonal imbalance, epigenetic changes in fetal life, genetic abnormalities, lifestyle, and environmental factors[4].

Women diagnosed with PCOS usually have greater craving, depend on more energy-dense high Glycemic

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Index (GI) foods and saturated fat, have inadequate fiber intake and have reduced scores for PCOS-related quality of life, while their total energy intake, physical activity and resting metabolic rate are identical to normal subjects[5]. There are imminent chances that the quality of diet would cause metabolic and endocrine irregularities of PCOS, while there has been very limited research on this matter[6,7]. There is indeed a complicated association between various nutritional elements and the endocrine status. It is recognized that diet plays a significant role in the control of metabolism of sex steroids and LH release. Thus, a woman's diet may eventually influence her fertility, especially ovulation.

The utilization of sugar-sweetened diets has increased in most of the developing countries due to growing rates of urbanization and globalization[8]. Growth of income and technological advancements have contributed to a radical change to the Western dietary lifestyle i.e., diets rich in simple sugars are progressively replacing traditional diets which are high in complex carbohydrates and fiber[9]. The production of High Fructose Corn Syrup (HFCS) in the 1970s mainly gave rise to the enhanced utilization of sweeteners, as a result of the easiness with which HFCS was solublized in processed foods. Fructose is useful because it is at any rate 1.5 times sweeter than sucrose and low-priced to produce; therefore, it has been used extensively in foods during the preparation of tinned fruits, jams, jellies, paste candies, cakes powder for beverages, and soft drinks.

The promptness with which too much dietary fructose consumption wields antagonistic metabolic consequences is noticeable. It has been reported that diet containing high-fructose triggers hypertriglyceridemia, hyperinsulinemia, and insulin resistance, disrupts glucose tolerance and increases body weight[10,11]. Therefore, the present study aimed at demonstrating experimentally the effect of high fructose and elevated levels of insulin on the endocrine profile and the development of polycystic appearing ovaries in Sprague Dawleyrats.

2. Materials and Methods

2.1 Experimental Protocol

The animal experiments were conducted at Pushpagiri Research Centre, Thiruvalla. This study was conducted as per the guidelines of Institutional Animal Ethics Committee (IAEC) and the approval number is IAEC/PIMS & RC/2018/7. In this study, the technique of randomization and blinding was not used. The experiment was carried out on healthy, young, colony-bred female Sprague Dawley albino rats weaned at 7-8 weeks of age. These animals weighed 80 g-85 g each. They were reared in the Department's animal house at a temperature of 22±2 ^{0} C and exposed to 10-12 hr of day light. Different experimental groups of the animals were caged separately and an average of 4 animals per cage was maintained. Rats in the control as well as experimental groups were given free access to standard chow and water *ad libitum*. All the animals were housed in polyethylene cages and provided with water in plastic bottles.

The experimental animals were divided into 3 groups, each consisting of 12 rats. Group I was maintained as control. The experimental groups consisted of fructose-treated (group II) and insulin-treated animals (group III). Fructose and insulin were used as test substances. Fructose was dissolved in water to make the dosage at 10 g/10 mL/kg body weight and was administered through an oral gavage. The insulin treatment group of animals received an intraperitoneal injection of insulin at a dosage of 0.5 IU/kg bw/day. The treatment was continued for 90 days.

After 90 days of treatment, the rats were anaesthetized with ether and blood was collected by cardiac puncture. Blood was kept for 2-4 hrs after which serum was separated by centrifugation and stored at -20 ^{0}C for hormonal assay of FSH, LH, insulin, testosterone and estradiol. Chemiluminescence Enzyme Immune Assay (CLIA) technique was carried out for the hormonal assays[12].

The metabolic parameters including glucose, protein, cholesterol and triglyceride levels were also determined. The blood glucose level was determined using a glucometer (One Touch Horizon) with the help of test strips. Here glucose in the blood samples mixes with special chemicals (glucose oxidase) on the test strip and a small electric current is produced. This current is measured by the one touch horizon meter and displayed as the blood glucose[13]. The cholesterol level was determined by ferric chloride method[14]. The level of protein was detected using Lowry method[15]. GPO-POD method was used for the estimation of triglycerides[16].

After blood collection, the animals were autopsied for histopathological examination of vital organs and reproductive organs. Tissues of liver, ovary and fallopian

tube were excised out, blotted free of blood and fixed in alcoholic Bouin's fixative. The tissues were processed for histological examination adopting the standard methods. The paraffin-embedded samples were cut at a thickness of 6 microns using Yorco Rotary Microtome. Sections were processed for staining in hematoxylin and eosin, mounted in DPX mountant. The stained slides were examined in Olympus S761 (Model SZ2-ILST) research microscope, and photomicrographs were obtained using Lumenera-Infinity 1 (Model N9032789) camera.

2.2 Statistics

The statistical Package for Social Sciences (SPSS 16.0 version for windows) was used for all data analysis. A minimum of six replicates were taken for each parameter. Differences in the hormonal and metabolic parameters of treated and control groups were analyzed by one way ANOVA. The Tukey test was used for multiple comparisons. Post hoc analysis was done, wherever required, to single out pairs of observations having significantly different levels of observations. Data was expressed as mean ± S.E. Statistical significance was accepted at $p<05$.

3. Results

3.1 Hormone Profile

The mean value of LH was 1.05 mIU/mL in fructose-treated and 1.15 mIU/mL in insulin-treated animals. The mean LH levels were significantly ($p<0.05$) increased in the treated rats as compared to control. The mean value of FSH was 0.5 mIU/mL in fructose-treated and 0.6 mIU/mL in insulin-treated animals. The FSH levels were significantly ($p<0.05$) increased in the treated rats as compared to control. Likewise, LH:FSH ratio was also significantly ($p<0.05$) increased in the treated rats. Moreover, insulin, testosterone and estradiol levels showed significant increase in relation to control (Table 1)

3.2 Metabolic Profile

The mean blood glucose level observed in fructose-treated (153.50) and in insulin-treated (156.33 mg/dL) animals showed statistically significant ($p<0.05$) increase in comparison to control (Figure 1). Similarly, the mean protein level of treated animals also significantly increased ($p<0.05$) as compared to control (Figure 2) Moreover, mean cholesterol and mean triglyceride levels were found to be significantly ($p<0.05$) increased after insulin and fructose treatment (Figures 3,4).

3.3 Histology

Sections of liver of control animals showed parenchyma composed of congested central veins with hepatocytes radiating outwards from the central veins to the portal tract. The portal tract show hepatic artery, portal venule and bile ductules along with sparse inflammatory infiltrate composed predominantly of lymphocytes along with plasma cells. However, in fructose- and insulin-treated animals, cytoplasmic vacuolation, alteration in the radial organization of the sinusoids and hepatic necrosis were seen (Figure 5A, 5B and 5C).

Histological observation of ovary of control animals showed ovarian cortical parenchyma with multiple variably sized follicles, predominantly cystic Graafian follicles. Corpus luteum with central edematous soft tissue was also seen. The medullary region showed sheets of mature adipocytes and intact vessels. In fructose-treated and insulin treated animals, medullary region with sheets of mature adipocytes and congested vessels and reduced number of corpus lutea were observed.

Table 1. The hormone profile of control and treated female albino rats

Parameter	Control	Fructose-treated	Insulin-treated
LH (mIU/mL)	0.05 ± 0.007	*1.05 ± 0.14	*1.15 ± 0.19
FSH (mIU/mL)	0.1 ± 0.02	*0.5 ± 0.11	*0.6 ± 0.13
LH:FSH Ratio	0.6 ± 0.12	*2.4 ± 0.39	*1.7 ± 0.20
Insulin (uIU/mL)	4.2 ± 0.13	*12.31 ± 2.74	*11.44 ± 1.03
Testosterone (ng/dL)	41.31 ± 1.35	*56.15 ± 1.11	*68.01 ± 1.52
Estradiol (pg/mL)	29.41 ± 1.40	*58.5± 2.12	*57.6 ± 1.25

*significant ($p<0.05$)

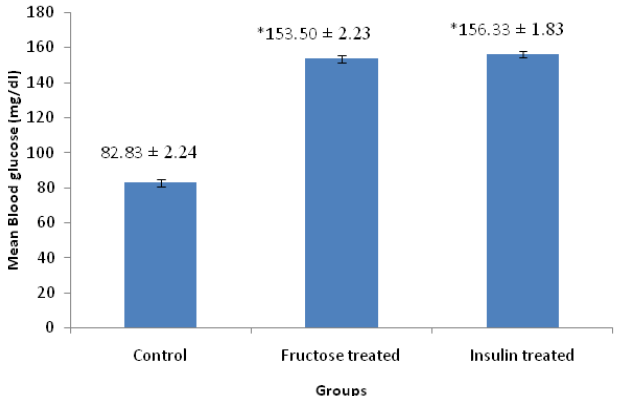

Figure 1. The mean blood glucose (mg/dL) of control and treated female albino rats.
*significant (p<0.05)

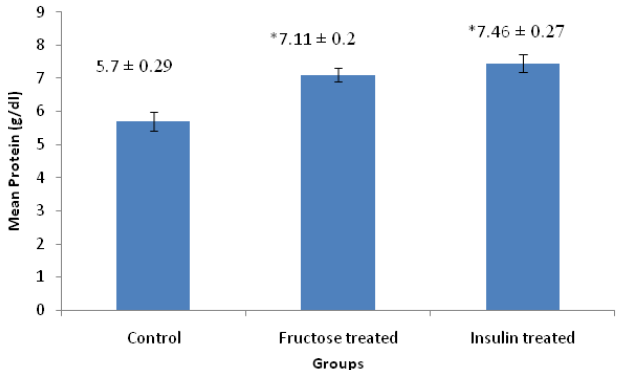

Figure 2. The mean protein (g/dL) of control and treated female albino rats.
*significant (p<0.05

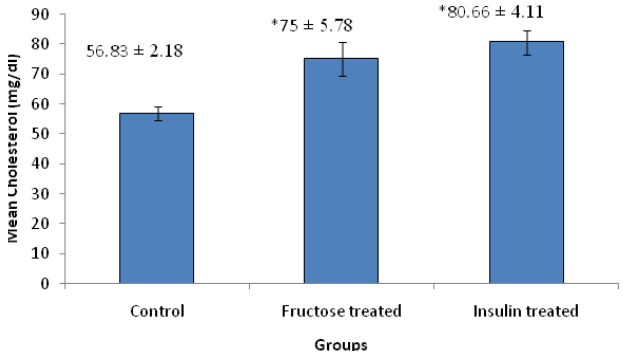

Figure 3. The mean cholesterol (mg/dL) of control and treated female albino rats.
*significant (p<0.05

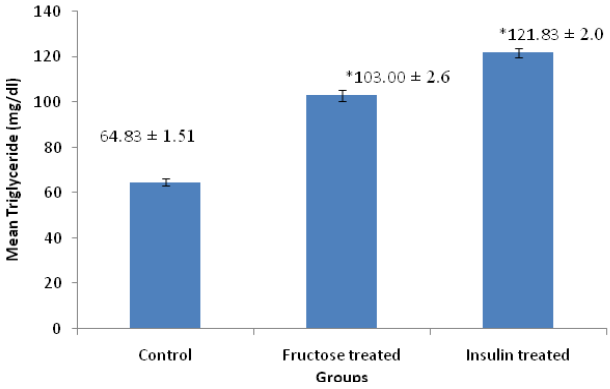

Figure 4. The mean triglyceride (mg/dl) of control and treated female albino rats.
*significant (p<0.05)

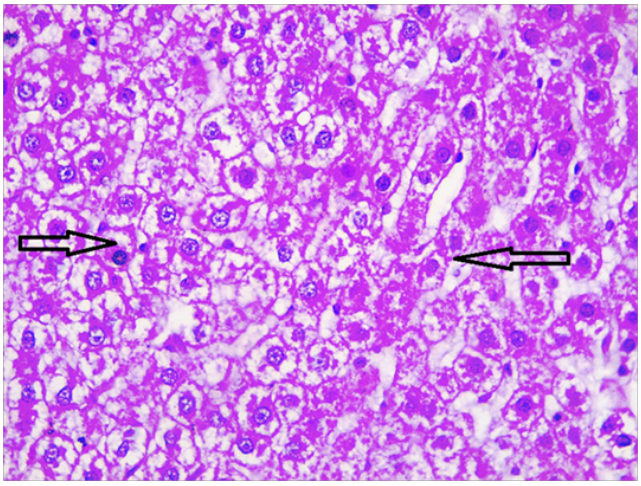

Figure 5A. Histology of a control liver showing normally arranged hepatocytes.

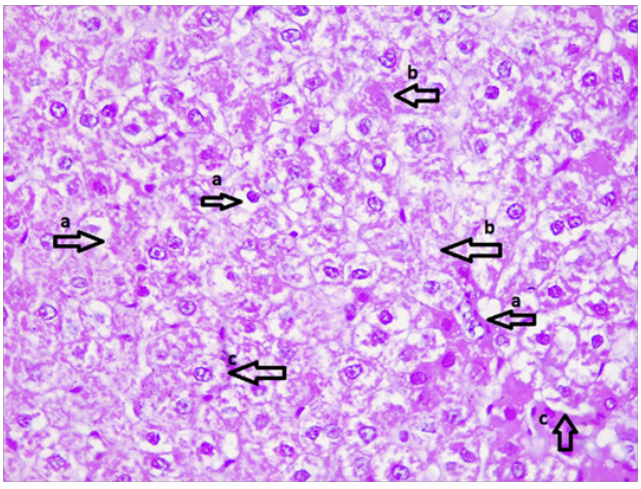

Figure 5B. Histology of liver of a fructose-treated animal showing **(a)** Cytoplasmic vacuolation, **(b)** disorganized sinusoids and **(c)** hepatic necrosis.

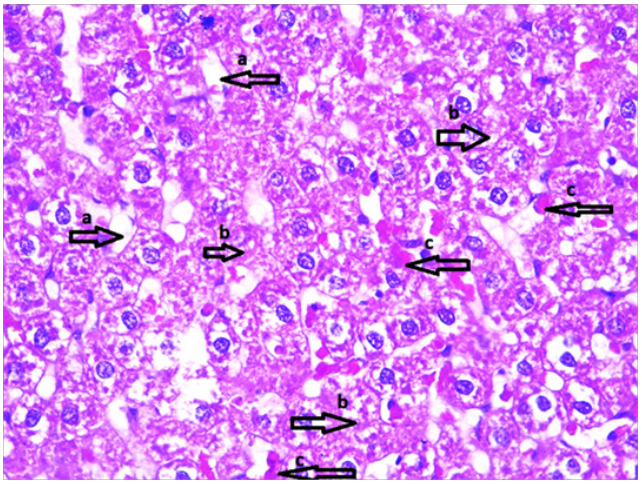

Figure 5C. Histology of liver of an insulin-treated animal showing **(a)** cytoplasmic vacuolation, **(b)** disorganized sinusoids and **(c)** hepatic necrosis..

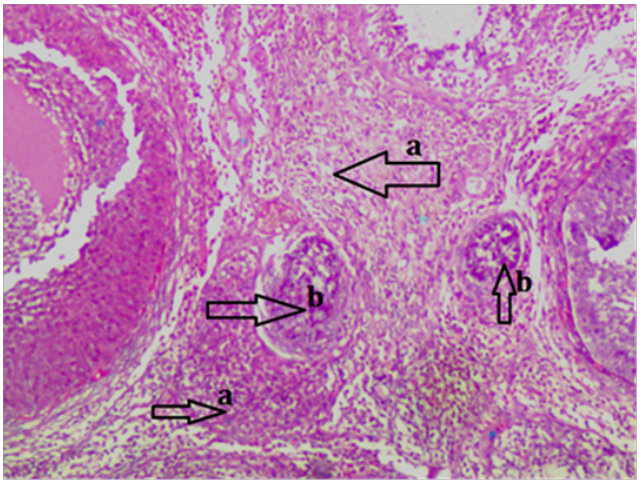

Figure 6A. Histology of ovary of control animal showing **(a)** corpusluteum and **(b)** antral follicle.

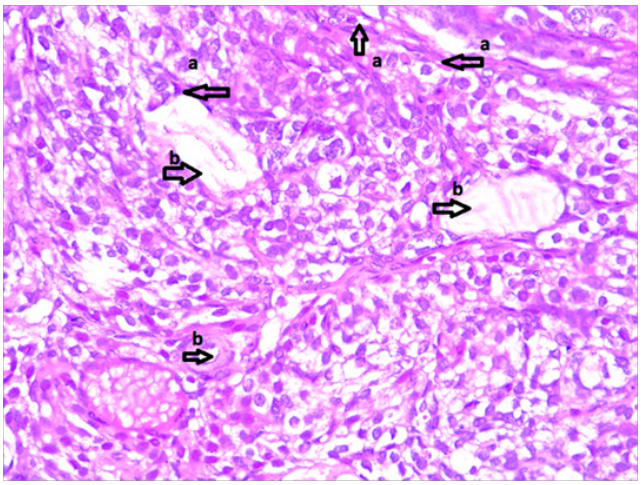

Figure 6B. Histology of ovary of fructose-treated animal showing **(a)** atretic follicle and **(b)** cyst-like regions.

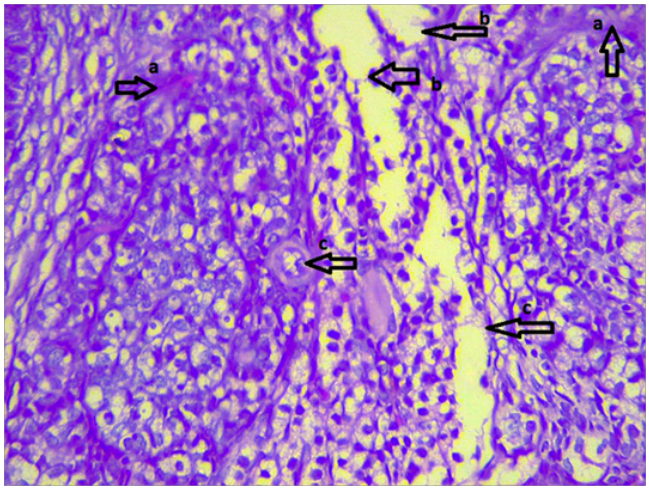

Figure 6C. Histology of ovary of insulin-treated animal **(a)** atretic follicle, **(b)** cyst-like regions and **(c)** stromal

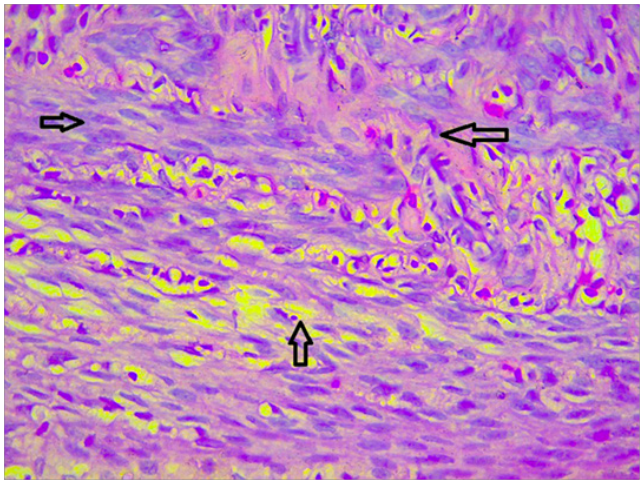

Figure 7A. Histology of fallopian tube of control animal-Normal tubal parenchyma.

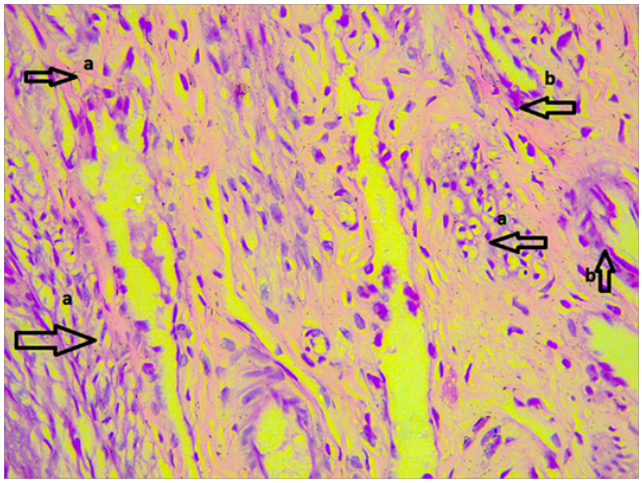

Figure 7B. Histology of fallopian tube of fructose-treated animal showing **(a)** congested epithelial cells and (b)cellular hypertrophy.

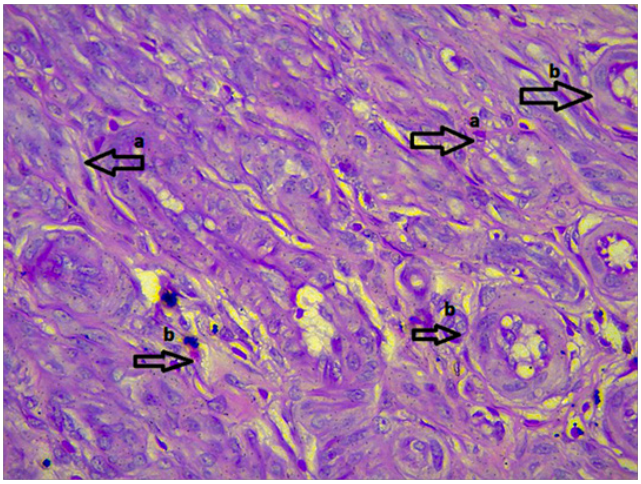

Figure 7C. Histology of fallopian tube of insulin-treated animal showing **(a)** congested epithelial cells and (b)cellular hypertrophy.

Moreover, too much atretic follicles, stromal hypertrophy, thin layer of granulosa cells and hyperplasia of theca cells were observed (Figure 6A, 6B and 6C)

The histoarchitecture of both fallopian tubal segments of control animals presented normal tubal parenchyma with the lumen composed of variable tubular structures lined by ciliated low columnar epithelium with the overlying lamina propria and congested vessels, muscularis propria and adventitia. In fructose- and insulin-treated animals, both fallopian tubal segments showed penetration of inflammatory cells with harshly crowded epithelial cells, hyperplasia of the tubular epithelial cells, cellular hypertrophy, degenerative and atrophic changes (Figure 7A, 7B and 7C).

4. Discussion

The present study aimed at validating the role of high intake of dietary carbohydrate (fructose) and the resultant alterations in the hormonal levels as the causative factors for human PCOS in the rat model. Data assembled in clinical experiments reveal strong evidence to propose that the added sugar and particularly added fructose supplied from high-fructose corn syrup cause a grave and increasing public health problem, to the extent of outbreak of type 2 diabetes[17,18]. At present, fructose is utilized as a taste-enhancer to prepare more delicious and appealing food. It is merely a transitional molecule in glucose metabolism and there is no biological requirement for dietary fructose. The normal level of fructose (~0.01 mmol/L) in peripheral blood is extremely small in comparison to glucose (~5.5 mmol/L)[19]. Curiously, a little amount of fructose creates a reduced glycemic response to proxy sucrose and starch in the diet in diabetic patients[20]. Moreover, ingestion of fructose in the recent times is too much on the higher side due to intake of artificially sweetened beverages and food. We thus carried out this study to evaluate the effect of high fructose and elevated levels of insulin on the endocrine and metabolic profile and the development of polycystic-like ovaries in Sprague Dawley rats.

LH and FSH levels were increased in treated rats in response to fructose administration. Also, endogenous insulin levels were stimulated by fructose. In *in vitro* studies researchers have established that insulin also has receptors in the hypothalamus and the pituitary, in which it enhances the secretion of FSH and LH under basal conditions and after GnRH stimulation[21,22]. In insulin-treated animals also LH and FSH levels were increased. In *vitro* data, reported based on cell culture models, revealed that co-incubation of insulin and FSH with bovine oocytes stimulates up-regulation of LH receptors on granulosa cells of antral follicles. It promotes arrest of follicular growth, obstructs aromatase activity, and generates ovarian hyperandrogenism[22]. In the present study testosterone levels were elevated in treated animals in line with these reports.

Serum estradiol levels detected in treated animals are in line with previous studies[23]. Testosterone levels were obviously increased in fructose- and insulin-treated animals compared to control rats. In keeping with the present study, increased testosterone levels were reported in pregnant rats after chronic fructose consumption[24]. Another study revealed that initiation of letrozole treatment during puberty or adulthood in female mice caused reproductive characteristics of PCOS, comprising elevated testosterone levels and anovulation[25]. In another letrozole study, considerably reduced estrogen level (27 %) and elevated testosterone level (87 %) was observed in treated group compared to control[26].

Hormonal imbalances were not detected in sucrose treated animals of the present study. It has been earlier established that insulin plays a direct role via its own receptor in PCO theca cells to enhance androgen synthesis[27]. Although PCOS women may be in an insulin-resistant state, the ovary keeps on responding to insulin signaling for the secretion of androgens[28]. Consequently, the increase of testosterone levels could

occur by means of insulin-induced cyst development, whereby hyperplasia of the cal cells in the walls of these cysts may stimulate steroidogenic activity[29]. Similarly, insulin levels were also increased in treated groups like testosterone levels. Researchers reported that abnormal fructose feeding causes insulin resistance. It was established that nine week high-fructose feeding triggers glucose intolerance and insulin resistance in humans[30]. Moreover, in another study, investigators selected a group of young workers whose food supplemented with sugar-sweetened beverages offering either an additional 40 g (~9 % total energy consumption) or 80 g of fructose (~14 % of total energy consumption), compared to 80 g of glucose or sucrose, for three weeks[31]. Hepatic insulin resistance was only obvious in the excessive fructose-fed group. Furthermore, researchers provided an isocaloric weight sustaining diet to eight healthy men for nine days in which they consumed either complex carbohydrate or fructose (25 % of energy requirements)[32]. Abnormal fructose feeding caused elevation in lipogenesis and damaged insulin-mediated inhibition of hepatic glucose production. Consequently, when fructose consumption is enough to enhance lipogenesis hepatic insulin resistance also builds up.

Cholesterol levels were increased in rodents after sucrose and fructose administration and insulin treatment. According to researchers, considerable inflow of fructose into the liver produces deposition of triglycerides and cholesterol due to its lipogenic features, which resulted in reduced insulin sensitivity, insulin resistance and glucose intolerance[33,34]. Consistent with these reports, blood glucose and insulin levels showed an increasing trend in fructose and insulin treated animals. Different from glucose, fructose circumvents phosphofructokinase and instead forms fructose-1-phosphate, which enters glycolysis and can be utilized directly for the synthesis of triglycerides. As a result, excess lipids are generated. Similarly, triglyceride levels were increased in sucrose-, fructose- and insulin-treated rats. Rodent models validate the mechanisms in which fructose consumption, usually >40 % kcal, has enhanced adiposity, augmented levels of cholesterol and TGs, and imbalance in glucose tolerance[33]. Moreover, fructose consumption elevates portal vein intensity of bacterial endotoxin, a suggested stimulus of metabolic irregularity[35]. It also has been revealed that most of the detrimental outcomes of high-carbohydrate diets may be partially due to the metabolic conversion of glucose in these diets into fructose[36]. Furthermore, metabolism of fructose to fructose-1-phosphate decreases intracellular ATP levels, consequent to which AMP deaminase is triggered catalyzing the breakdown of AMP to inosine monophosphate and finally, uric acid[37]. Uric acid may act as a potent antioxidant, but excess uric acid may cause oxidative stress and cellular dysfunction[38,39]. In another study, 10 % fructose for 21 days produced glucose intolerance in rats showing resemblance to the present study[40]. It has been established that metabolic syndrome could be produced in Wistar rats by feeding 10 % fructose solution for 8 weeks[41]. As revealed in the present study, researchers have reported that plasma glucose, insulin and triglyceride concentrations were elevated after the intake of 10 % and 20 % HFCS-containing solutions for a period of 12 weeks[42]. The utilization of 10 % fructose-containing feeding triggers increase in plasma leptin level and abdominal adipose tissue in male rats in comparison to the control group[43].

High-fructose or high-sucrose diets are obviously accompanied by IR increase and disrupted glucose homeostasis in rodents[44]. When rats consumed a diet in which sucrose replaced starch produced numerous changes in glucose and lipid metabolism over time. Between 2 and 5 weeks, fasting hyperinsulinemia was produced, demonstrating whole-body insulin resistance. In rats treated 66 % fructose for 3 weeks the insulin receptor mRNA and the number of insulin receptors on skeletal muscle were remarkably reduced as compared to rats fed a standard diet. Moreover, it was observed that after fructose feeding for 28 days, there was no variation in the concentrations of insulin receptor, but its auto-phosphorylation, a required mechanism for its action, was declined to 72 % in the liver[45]. The impact of a high-fructose diet validates its glycemic effect, which was obvious in oral-glucose-tolerance tests carried out in rats after they were treated 20 % fructose for 18 wk. Compared with control rats, blood glucose concentrations were significantly higher in the fructose-fed rats at 30 wk, and the return to baseline was incomplete during the response tests[46]. The fructose constituent of added sugar is further harmful than glucose, and methods for fructose's contributions to *de novo* lipogenesis, insulin resistance, lipid dysregulation, and obesity are largely assessed[47]. Fructose is carried into cells through GLUT5, a particular fructose-carrier very much expressed along the brush border of the small intestine, and GLUT2, a transporter for both glucose and fructose expressed in the liver, small intestine (near the basolateral membrane)

and pancreas. The majority of ingested fructose passes via the portal circulation to the liver where it is rapidly cleared. Insufficient amount of this escapes the liver first-pass, controlling top marginal fructose levels to elevated micromolar or low millmolar concentrations[48,49]. Intracellularly, during fructose metabolism, the triose phosphates resulting from "fructolysis" become substrates for gluconeogenesis, lipogenesis, or cellular respiration. Augmented usage of fructose-derived substrate for lipogenesis (both fatty-acid synthesis and fatty-acid esterification into triglyceride) may largely account for the increase in plasma triglyceride concentrations after short-term, high fructose exposure. However, continued fructose exposure could activate signalling events to additional enhancement in lipogenesis. Severe fructose ingestion promotes lipid synthesis by means ofthe fluctuation of fructose carbons into lipogenic precursors. Prolonged fructose exposure could enhance enterocytes' potential to soak up fructose by upregulating GLUT5, and may also augment enterocyte lipid synthesis[50].

It was reported that liver specimens of 12 h (HFCS access during dark period and 12 h tap water) and 24 h (24 h HFCS only access) animals revealed that the numbers of fat-containing vacuoles augmented in comparison to control samples. Liver histology ensured the adverse effect of HFCS consumption on these tissues[51]. The present study also confirmed the effect of fructose on liver tissues in terms of cytoplasmic vacuolation and hepatic necrosis. Fructose consumption in concentrations of 15 %[33] results in hepatic steatosis since fructose causes an enhanced *de novo* lipogenesis in liver [52] resulting in lipid accumulation in hepatocytes.

The histoarchitecture of fallopian tube of fructose- and insulin-treated animals supported the earlier research conducted in female Wistar rats to investigate the effect of letrozole in the development of PCOS. The histological examination of the female reproductive organ (fallopian tube) of letrozole- and/or fructose-treated rats revealed permeation of inflammatory cells with severely clogged epithelial cells and also hyperplasia of the tubular epithelial cells[53].

Histological examination of ovary of fructose- and insulin-treated animal showed fluid filled cysts without granulosa cells and follicles. Researchers had reported that histopathology of ovaries of fructose- and letrozole-treated animals revealed considerable resemblance to human PCOS. In agreement with the present study, the authors showed reduced corpus luteum in comparison to vehicle control group and showed abnormalities in ovulation and declined frequency of estrouscyclicity. They also reported a large number of subcapsular cysts lined with a thin layer of granulosa cells and hyperplasia of theca interna cells. Their observations revealed the occurrence of active levels of FSH and elevated LH[54] as observed in the present study. According to their studies, the number of Graafian follicles were also remarkably decreased in treated group as observed in the present study. Since Graafian follicle is an indication of active folliculogenesis, its development could be paused in preantral stage during follicular development. Moreover, another letrozole study reported that abnormal secretion of sex hormones produced structural changes in ovary. The number of cystic follicles was enhanced and cystic wall became highly thickened represented by reduced or diminished granulosa cell layer and congealed theca cell layer in letrozole-treated rats. Further changes detected were thin layer of granulosa cells, hyperplasia of theca cells and atresia of follicles. During atresia, ovum is damaged and granulosa cells undergo apoptosis, which are changed by fibrous material[26].

The large amount of fructose may have influenced it through disturbances in hormonal balance *via* insulin resistance which resulted in ovarian dysfunctions. Such hormonal imbalances were established in our study. The present study also shows resemblance to the ovarian characteristics of the another study where letrozole treatment of adult female mice caused ovaries with reduced Corpora Lutea (CL), Cystic Follicles (CF) and Hemorrhagic Cysts (HC) in comparison to placebo-treated mice[25].

It could be concluded that fructose- and insulin-treatments influence the endocrine status and ovarian functions of female albino rat, in the context of experimentally induced PCOS.

5. Acknowledgment

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6. References

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