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RESEARCH ARTICLE

STUDY OF OVARIAN MORPHOLOGY IN HIGH FRUCTOSE FED MICE

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ABSTRACT

Objective: The objective of our present study is to assess the histological changes in the ovary of mice fed refined and unrefined high sugar diet

Methods: Thirty six Female albino mice (15-25g) of age 21 days were randomly divided into six groups (6 animals in each group). The mice are fed unrefined high sugar diet (Palm jaggery diet**)**, refined high sugar diet (High fructose diet), normal control diet tap water ad libitum. Animals were maintained in the respective diet for 60 and 90 days. The animals were monitored closely and weighed every day from 21 day age onwards. At the end of the experimental period (i.e. 60th day, and 90th day) the animals were sacrificed. The body weight (before sacrifice the animal) and weight of the ovaries were measured. The ovaries were fixed in 10 % buffered formalin and stained with haematoxylin and eosin. The histoarchitecture of ovary was studied.

Results: the ovary of mice fed high fructose diet shows degenerating cystic follicles, many preantral follicles, and reduced number of corpora lutea. There were no significant changes in histoarchitecture of mice fed palm jaggery diet and normal control diet.

Conclusion: This study concludes that chronic exposure to high fructose diet (HFD) in female mice induces a reproductive phenotype resembling features observed in women with PCOS

Key wards: female albino mice, refined high sugar diet, unrefined high sugar diet, corpora lutea.

INTRODUCTION:

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 5-10% of reproductive age women (1, 2). Approximately one in 15 women experiences PCOS (3), and an enlarged ovary is observed on ultrasound in 22% of women (4) during their reproductive years. This syndrome is a heterogeneous disorder characterized by chronic ovulatory dysfunction and hyperandrogenism (5) and, consequently, infertility (6, 7). Although the mechanism of anovulation remains uncertain, it is known that genetic and environmental factors play a role in the origin and development of this disorder (8-10).

Polycystic ovaries, the presence of multiple (> 10) cysts in an ovary (11) is caused by the arrest of follicle development at an immature stage. PCOS is named in reference to this morphological change. As the development of these follicles is arrested well before the point of dominant follicle selection, and therefore positive estrogen feedback to the hypothalamus and pituitary axis is lacking, the LH surge is absent in PCOS patients (12-14). Consequently, ovulation and menstrual cycles are interrupted (oligovulation and oligoamenorrhea, respectively). PCOS is associated with features of the metabolic syndrome. Consequently, studies involving women with PCOS are often confounded by coexisting obesity, insulin resistance and other features of the metabolic syndrome. The cellular and molecular mechanisms of insulin resistance in PCOS have not yet been elucidated, but they are considered to be distinct from those of other diseases associated with insulin resistance (15). Insulin resistance is considered the most important pathophysiological factor in PCOS (16-18). It affects 70% of PCOS women (19-21).

The etiology of PCOS is unclear. The clinical and metabolic changes characteristic of polycystic ovary syndrome are mainly related to hyperandrogenism and insulin resistance with compensatory hyperinsulinemia. The Compensatory hyperinsulinemia is important in the development of metabolic abnormalities and also contributes to the high androgen levels observed in women with PCOS (22–27). hyperandrogenism and

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polycystic ovaries (assessed by ultrasound and chronic anovulation) are major characteristics in PCOS patients.

The main driving forces for the increased prevalence of insulin resistance are modern Westernized diets and patterns of eating associated with the dramatic rises in obesity. Insulin resistance is often linked to the macronutrient content in the diet. In the past, diets high in saturated fats have been shown to induce weight gain, insulin resistance, and hyperlipidemia in humans and animals (28-31). Recent research suggests that a high intake of refined carbohydrates may also increase the risk of insulin resistance (32-35). In addition, diets specifically high in fructose have been shown to contribute to a metabolic disturbance in animal models resulting in weight gain, hyperlipidemia (36), and hypertension (37). Studies have shown that high dosage of fructose induces hyperinsulinemia, hypertriglyceridemia, impaird glucose tolerance in rats (38, 39).

Some studies have shown that insulin resistance is one of the important reasons for reproductive problems. We hypothesizes that unrefined sugar (palm jaggery) diet is a good alternate to refined high sugar diet (high fructose diet). Studies have shown that Hyperinsulinemia affects granulosa cells in small follicles and theca cells (40). Palm jaggery is reported to be more beneficial than fructose diet. Although the use of palm jaggery is known from the ancient past, however the scientific literatures regarding its health benefits particularly in relation to reproductive system are considerably limited. In this study we studied the histology of mice ovary in response to refined sugar diet, unrefined sugar diet and normal control diet.

MATERIALS AND METHODS:

This study was conducted in the Division of Physiology, Rajah Muthiah Medical College, (RMMC), Annamalai University, Chidambaram, Tamil Nadu, India.

EXPERIMENTAL ANIMALS:

Female albino mice (Wistar strain) of three weeks old, weighing approximately 15-25g were selected. The animals were maintained in the Central Animal House, Rajah Muthiah Medical College, Annamalai University. They were housed in an animal room under controlled conditions on a 12 hour light/dark cycle at $25^0 \pm 2^0$C. The animals were provided with control diet, experimental diet and water ad libitum. The experiment were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee (IAEC), Annamalai University (Approved number: 160/1999/CPCSEA/1072).

EXPERIMENTAL GROUPS:

The animals were divided into six groups. Each group consists of six animals.

Group 1 Control diet (normal diet) - 60 days: Animals fed normal control diet for 60 days.

Group 2 Control diet (normal diet) - 90 days: Animals fed normal control diet for 90 days.

Group 3 unrefined high sugar diet – 60 days: Animals fed Palm jaggery diet for 60 days.

Group 4 unrefined high sugar diet – 90 days: Animals fed Palm jaggery diet for 90 days.

Group 5 refined high sugar diet – 60 days: Animals fed High fructose diet for 60 days.

Group 6 refined high sugar diet – 90 days: Animals fed High fructose diet for 90 days

EXPERIMENTAL DIETS:

Diets (41) were formulated based on American institute of nutrition 93G (AIN-93G) (and modified for our study) to meet recommended nutrients levels for mice as showed in table 1. Fructose, casein, vitamin mix and mineral mix was purchased from SDFCL, Mumbai, NICE CHEMICALS Pvt, Ltd, Kerala, India. All other food ingredients were purchased from local market, Chidambaram. Diets were prepared fresh daily.

SAMPLE COLLECTION:

At the end of the experimental period, the animals were placed supine on the dissecting board following dislocation of the spine at the cervical region. With a pair of forceps and scissors, the lower abdominal region was cut open. This incision was extended upwards into the upper abdominal region and subsequently into the thoracic region, to expose the contents of the abdomen and the thorax. The Ovaries and uterus were removed and separated from surrounding tissue. The weight of the dissected ovaries and uterus was measured and stored in 10 % formalin (fixation) for histological examination.

HISTOLOGICAL PROCEDURE:

After fixation, the piece of ovary was dehydrated by bathing it successfully in graded mixture of ethanol and water (70-100%). The ethanol was then replaced with a solvent miscible with the embedding medium (xylene). As the tissues were infiltrated with xylene, they became transparent (clearing). Once the tissue has been impregnated by xylene it was placed in melted paraffin in an oven maintained at 58º-60°C (embedding). The heat caused the solvent to evaporate and the spaces within the tissues became filled with paraffin. The tissue together with its impregnating paraffin hardened after it had been taken out of the oven. The hard block containing the tissue was then taken to the microtome (Rotatory microtome). Thin sections (5µm), were cut

using a Rotatory microtome (LEICA RM2125RTS). The sections were then floated on water and transferred to a glass slide and stained with heamatoxylin and eosin. The slides were viewed under light microscope with high power magnification. Light photograph of histological slides of ovaries were taken by a Nikon camera attached to light microscope.

STATISTICAL ANALYSIS:

Statistical analysis was performed with SPSS (version 17.0). Values are expressed as mean ± SD. The student's't' test was used to compare mean values. A value of P <0.05 was considered statistically significant.

RESULTS:

All control group mice ovaries show normal histology and contained fresh corpora lutea, (figure 1) indicative of recent ovulations. And also shows large number of developing follicles in cortex region (figure 2). We observed compact stromal tissue between developing follicle and we also observed antral follicle with antral cavity, oocyte with normal healthy morphology surrounded by granulosa cells, corona radiata, cumulus oophorus and compact theca cell layer. Medullar region in the center of ovary shows normal vessels network.

The weight of ovary of mice fed high fructose diet for 90 days duration (HFD 90) was increased significantly than compare to control mice (P < 0.05) and palm jaggery fed mice (PJD 60, PJD 90 day duration). Ovaries of mice fed high fructose diet for 60 days duration (HFD 60) tended to have an increased weight, although this failed to reach significance. No change in weight of palm jaggery fed mice ovary. The size of the ovary was also increased in high fructose fed mice (HFD 90 day duration). It shows very less number of corpora lutea, Graafian follicle and also shows Atretic follicle, few numbers of cystic degenerating follicles (figure 3), and these cyst like follicles were larger in size and contains fluid filled antrum. We observed these changes only in HFD 90 day's group mice. This was never observed in control group, high fructose diet 60 days duration, palm jaggery group mice. We noted macrophages in the antral fluid. We also observed enlarged vessels network and dispersed theca cell layer (figure 4) in both group (HFD 90, HFD 60 days duration). Some of the HFD 90 ovaries show thin theca cell layer which is normal in control and both groups (PJD 60, PJD 90 day duration) of Palm jaggery fed mice. Mice belongs to this palm jaggery diet shows normal ovarian changes as that of control mice (figure 5, 6).

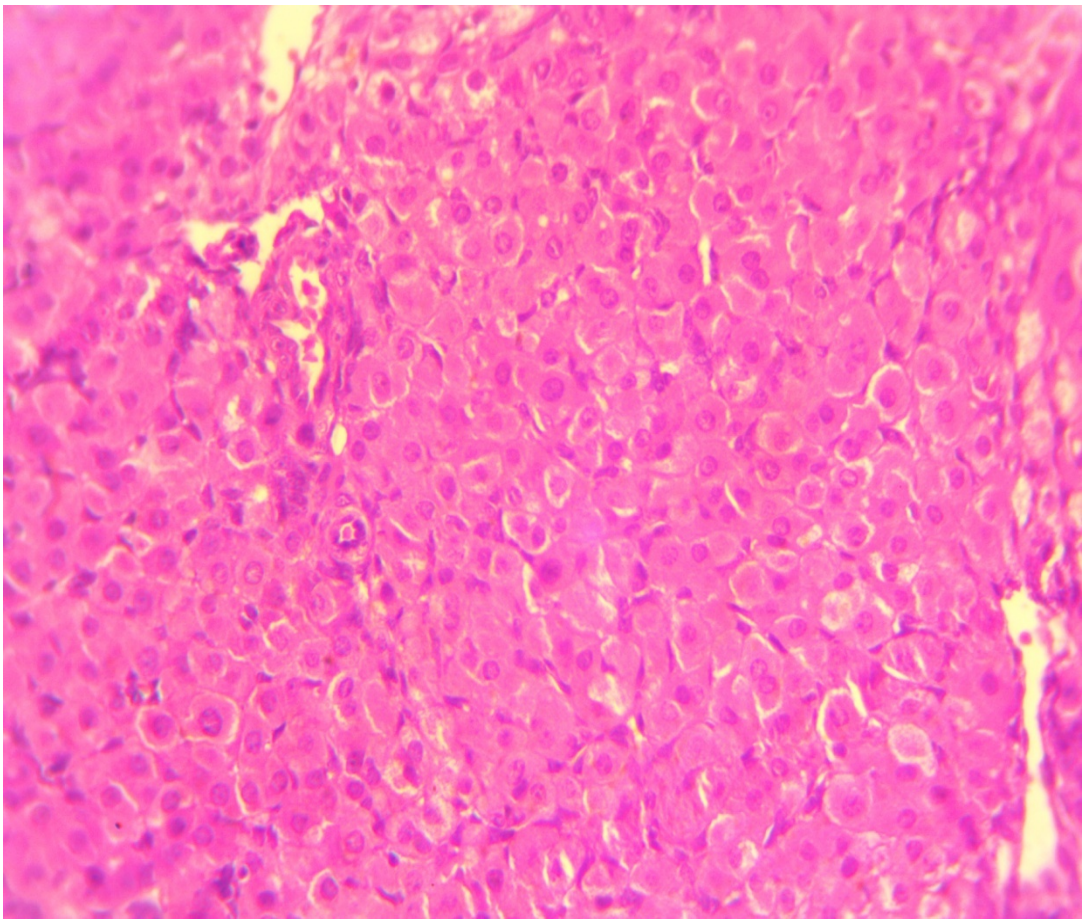

Figure 1: (H & E × 400) Light micrograph of ovarian section of mice fed normal control diet for 90 days duration showing numerous luteal cells.

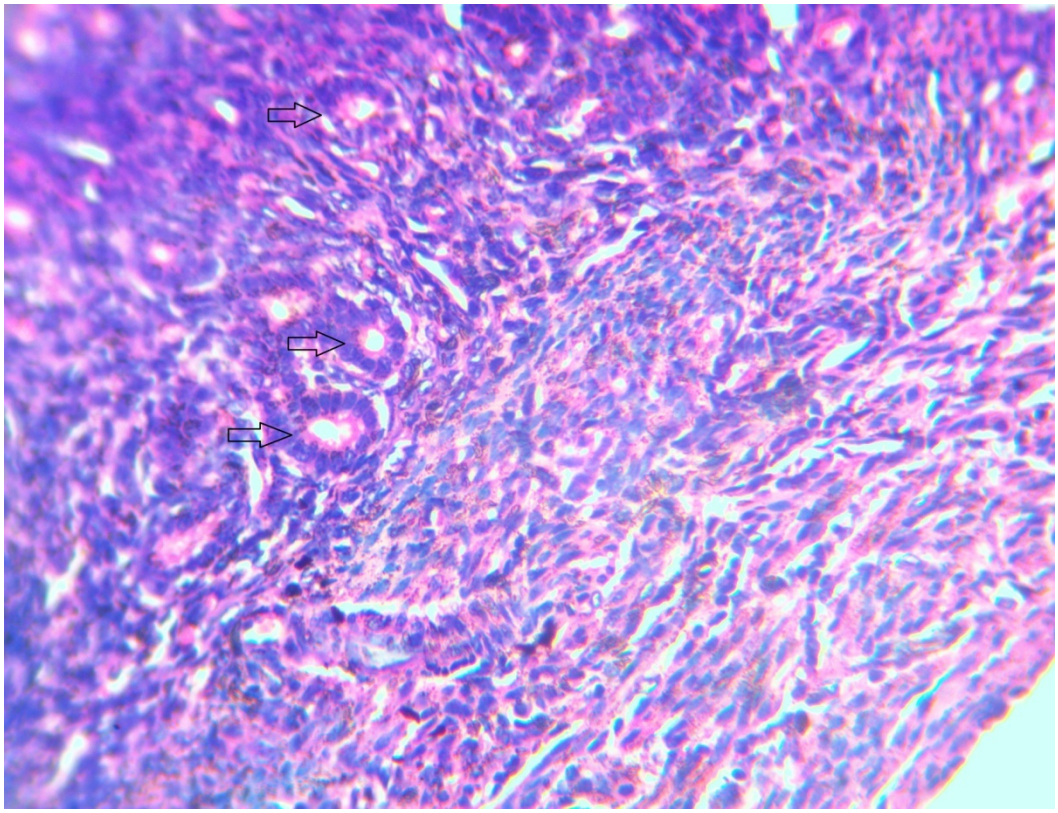

Figure 2: (H & E × 400) Light micrograph of ovarian section of mice fed normal control diet for 60 days duration showing normal developing follicles (Arrow).

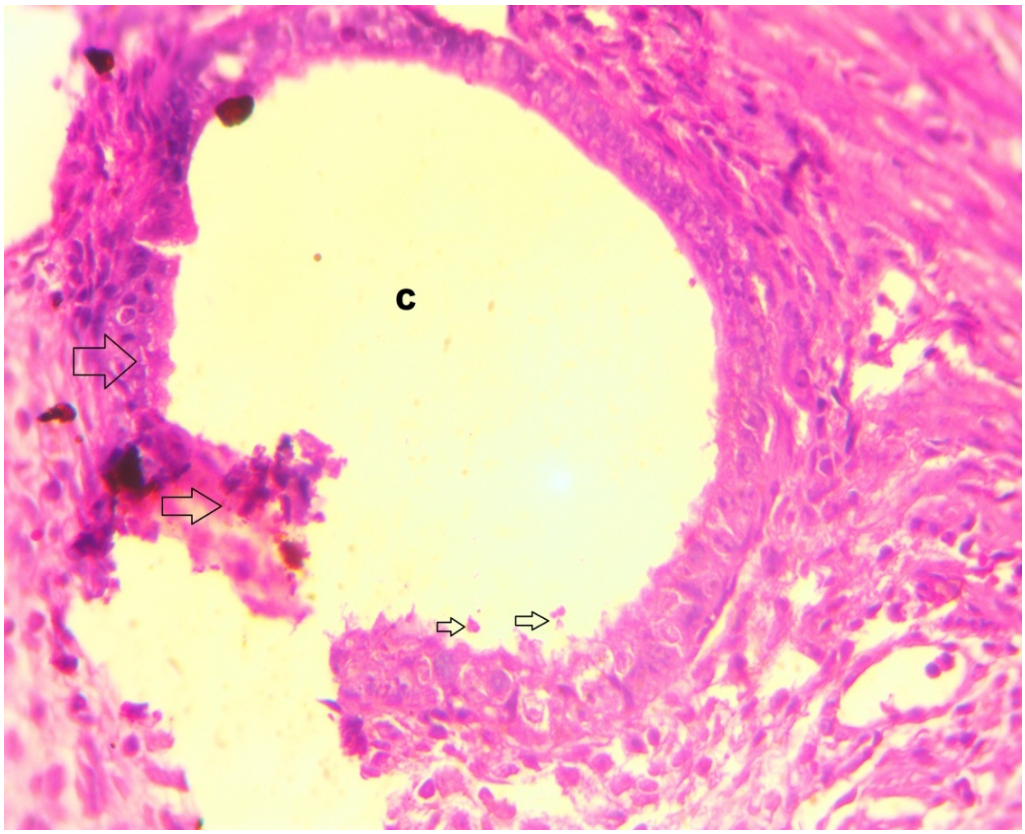

Figure 3: (H & E × 400) Light micrograph of ovarian section of mice fed high fructose diet for 90 days duration showing dispersed theca layer (Big Arrow), macrophages (small arrow), and Cystic degenerating follicle (C).

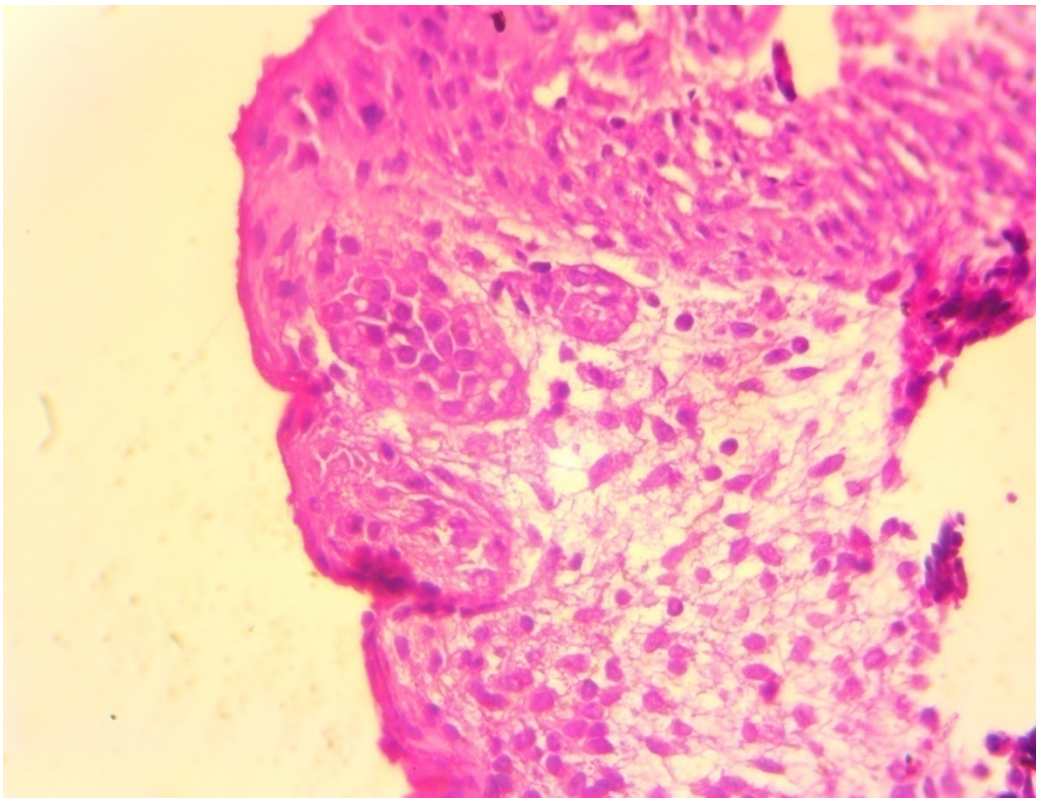

Figure 4: (H & E × 400) Light micrograph of ovarian section of mice fed high fructose diet for 60 days duration showing the presence of atretic follicle.

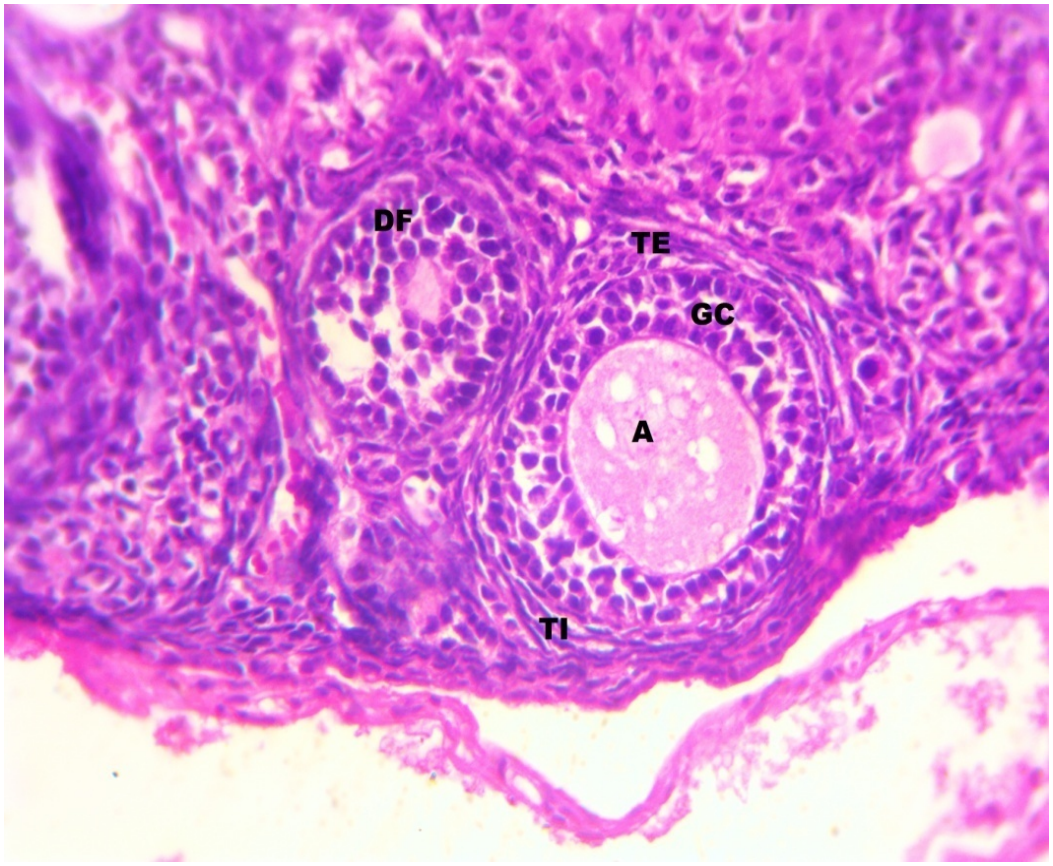

Figure 5: (H & E × 400) Light micrograph of ovarian section of mice fed palm jaggery diet for 90 days duration showing the presence of Developing follicle (DF), antral follicle (A), Granulosa cell (GC), Theca externa (TE), Theca Interna (TI).

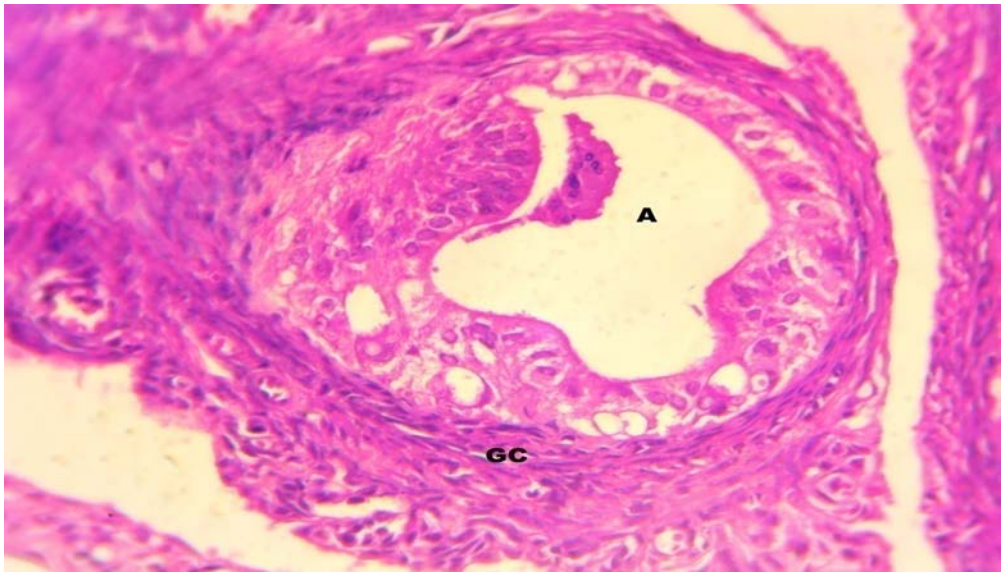

Figure 6: (H & E × 400) Light micrograph of ovarian section of mice fed palm jaggery diet for 60 days duration showing the presence of Granulosa cell (GC), antral follicle (A).

Table: 1 Composition of diets (g/100g)

Ingredients	**HFD**	**PJD**	**CONT**
Corn starch	-	-	60
High fructose	60	-	-
Palm jaggery	-	60	-
Casein(fat free)	20	20	20
Methionine	0.7	0.7	0.7
Groundnut oil	5	5	5
Unrefined sesame oil	-	-	-
Refined sesame oil	-	-	-
Wheat bran	10.6	10.6	10.6
Salt mixture♣	3.5	3.5	3.5
Vitamin mixture*	0.2	0.2	0.2

HFD - High fructose diet
PJD - Palm jaggery diet
CONT - Control diet
Un.S - Unrefined sesame oil diet
Re.S - Refined sesame oil diet

♣The composition of mineral mix (g/kg) $MgSO_4$. $7H_2O$-30.5; NaCl -65.2; KCl - 105.7; KH_2PO_4-200.2; $MgCO_3$ - 3.65; $Mg(OH)_2$. $3H_2O$ - 38.8; $FeC_6H_5O_7.5H_2O$ - 40.0; $CaCO_3$-512.4; KI-0.8; NaF-09.$CuSO_4.5H_2O$-1.4; $MnSO_4$-0.4, and $CONH_3$-0.05.

*One kilogram of vitamin mix contained thiamine mononitrate, 3g; riboflavin, 3g; Pyridoxine HCl, 3.5g; nicotinamide, 15g;d-calcium pantothenate, 8g; folic acid, 1g; d- biotin, 0.1g; cyanocobalamin, 5 mg; Vitamin A acetate, 0.6g; α-tocopherol acetate, 25g, and choline chloride, 10g.

Table 2: Body Weight and weight of the Ovary in Control and Experimental Groups.

Groups	Body Weight(g)	Weight of the Ovary(g)
Group 1	24.33±1.21	0.01±0.00
Group 2	31.00±1.78	0.01±0.00
Group 3	25.83±2.32	0.01±0.00
Group 4	29.67±2.16	0.01±0.00
Group 5	28.17±2.04*	0.02±0.00*
Group 6	34.17±2.48*	0.03±0.00*

Data are means ± SD. * P < 0.05 compared with the control group

DISCUSSION:

In the present study mice fed high fructose diet for 90 days group shows many degenerating follicles, few cystic follicles. And also shows more number of atretic follicles and less number of corpora lutea (42- 45) which was never observed in control group, palm jaggery diet group and high fructose diet 60 days duration group mice. High fructose diet group mice show characteristic features of polycystic ovary syndrome, particularly HFD 90 day's duration group mice. The wall of cyst like degenerating follicles shows thick layer of theca cells (theca interna) (45). Furthermore, our results show that the mice fed high fructose diet had an increased body weight (table 2), and their ovaries also enlarged than palm jaggery diet, control group mice ovaries. E. Leonie A.F et al (57) reported that DHT (*dihydrotestosterone)*-treated mice, ovarian weights tended to be increased. Androgens are synthesised in theca cells and then transported to the granulosa cells where P450 aromatase converts the androgens to estrone and E2 (Estradiol) (46).An increase in theca cells in HFD 90 group mice therefore suggests greater androgen production and a decrease in granulosa cells suggests conversion of androgen to estrogen is impaired. Therefore, the antral follicle morphology in these animals supports the expectation that their androgen production might be increased (47). It is in agreement with previous studies showing that androgen treatment in rats resulted in a pronounced thickening of the theca cell layer (17, 24). The fluid in the cystic degenerating follicle shows macrophages. Dying apoptotic cells in degenerating follicles can secrete soluble factors that recruit macrophages (48) which appear in the follicular fluid during degeneration or atresia. The macrophages can then cross and disrupt the basement membrane separating the vascular granulosa membrane from the theca cell layer (45).

The weight of ovaries was significantly increased in HFD 90 day duration group mice than compare to control group. HFD 60 day duration group also shows increased weight of ovaries but it was not significant statistically (45, 49). Furthermore, our results show that the mice fed high fructose diet (HFD 90 Days) had an increased body weight, and their ovaries also enlarged than palm jaggery diet, control group mice ovaries (42). Which is in accordance with earlier findings (50, 51, 45, 52, 53). The increased weight of ovary was due to presence of many preantral follicles. It indicates the follicles were arrested in that stage. This is a well-known morphological feature of women with infertility associated with polycystic ovaries. (54,55). In the present study the HFD 90, 60 day mice ovary shows less corpora lutea than compare to palm jaggery (PJD), control group mice. It indicates disturbances in ovulation and decreased frequency of estrous cyclicity (56, 52, 53). This is one of the important characteristic features of poly cystic ovary syndrome (PCOS).

In the present study, it was observed that the ovarian histology shows structural disparity in mature follicle, and the number of mature graffian follicles and corpus lutea were significantly reduced in HFD group mice. As graffian follicle is indicative of active folliculogenesis, we suggest it might be arrested in preantral stage during development of follicles. Our study indicates excess amount of fructose might have affects follicular development through alterations in hormonal balance via insulin resistance that lead to ovarian dysfunctions.

CONCLUSION:

This study concludes that chronic exposure to high fructose diet (HFD) in female mice induces a reproductive phenotype resembling features observed in women with PCOS, such as irregular cycle or acyclicity and large degenerating follicles with a cyst-like structure. In our study, mice fed high fructose diet (HFD) might have interfered with ovarian function through insulin resistance. It needs further analysis to clarify that HFD mice is a

suitable animal model to assess pathogenesis of polycystic ovary syndrome (PCOS).

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