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EFFECTS OF MAJOR DIETARY CONSTITUENTS ON ESTROUS CYCLE BY NON INVASIVE METHOD IN ALBINO MICE

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ABSTRACT

The aim of our present study is to assess the estrous cycle of mice fed refined high sugar diet (fructose), unrefined high sugar diet (palm jagerry), unrefined oil diet (unrefined sesame oil), refined oil (refined sesame oil) diet. Thirty Female albino mice of age, 21 days were randomly divided into five groups. The mice are fed unrefined high sugar diet, refined high sugar diet, unrefined oil diet, refined oil diet taps water ad libitum. The stage of the cyclist was determined by daily observation of vaginal smear. It was observed that mice fed high fructose diet shows significant decrease in the number of estrous cycle with concomitant significant increase in the duration of diestrus phase. Alterations in estrous cycle were also observed in metestrus and diestrus phases of unrefined and refined oil diet group mice. The present study concludes that mice fed refined high sugar diet exhibit irregular cycle.

KEYWORDS: Refined high sugar diet, unrefined high sugar diet, unrefined sesame oil diet, refined sesame oil diet.

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INTRODUCTION

The short reproductive cycle length observed in rodents, called the estrous cycle, makes them an ideal animal model for investigation of changes that occur during the reproductive cycle. And is characterized as proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II) [1, 2]**.** Short cycle length makes rodents an ideal animal model for investigating changes occurring during the reproductive cycle and historically rats have been the chosen model. Rats display, most of time, regular cycles; they are easy to manipulate; and the cycle is not disrupted easily even with the routine stress in the animal facility. However, as use of mice lines continues to increase, an understanding of the mouse estrous cycle is critical for investigators. There are few published studies involving estrous cycle in mice. The stages of the estrous cycle are not as visually discernible as in rats, and handling mice requires more caution due to their aggressive behavior. This study will be a useful one to investigate the estrous cycle by noninvasive method particularly for those who are doing research in estrous cycle in longer duration. The ovulation occurs from the beginning of proestrus to the end of estrus [3, 4]. From the onset of sexual maturity, up to the age of 12 months, the mean cycle length in the female rat and mice is 4 days [1, 2, 5] and this short cycle length makes the rats and mice an ideal animal for investigation of changes occurring during the reproductive cycle [6,7]. Assessing reproductive status in rodents is useful not only in the study of reproductive dysfunction, but is also required for the production of new mouse models of disease for further investigations. Daily assessment of the relative ratio of nucleated epithelial cells, cornified squamous epithelial cells, and leukocytes present in vaginal smears can be used to identify estrous stages. The degree of invasiveness, however, employed in collecting these samples can alter reproductive status and elicit an inflammatory response that can confound cytological assessment of smears. In this study, we followed Mclean's [8] method of non invasive techniques to assess the estrous cycle changes in mice, Thereby avoiding the chance of artificially induced cornification of the vaginal epithelium. It is a well known and tragic fact that in the formulation of the great majority of today's refined foods, the primary considerations of the manufacturer are taste, mouth feel, appearance, shelf life and profit. Sadly, all important considerations, these being the nutritional value of the product, and the effect that the product will have upon the health of the consumer, are not always given the attention that they deserve. Eating refined foods, particularly refined sugar were practically none existent in the past. Today it is included in almost everything people eat in prepackaged foods and in restaurants – soups, sauces, muffins, breads, cookies, spreads, jellies, chips, and etc. Refined sugar are included in 90 % in processed convenience foods. White sugar is a refined sugar. It is called simple sugar, or sucrose for scientific names. Half of the sucrose content is fructose. Fructose is also called levulose and fruit sugar. It is present in fruits and honey and is responsible for their sweet taste. It is a sweetest tasting carbohydrate, found in many fruits and vegetables However, the major source of fructose worldwide is sucrose, or table sugar, which is derived from sugar cane and sugar beets. In addition to sucrose, the other major source of fructose is high fructose corn syrup (HFCS).HFCS is often a major ingredient in soft drinks, pastries, desserts, and various processed foods. [9, 10]. HFCS consists of fructose and glucose mixed in a variety of concentrations, but most commonly as55% fructose and 45% glucose. [11, 12.] Fructose consumption has increased dramatically over the past several decades [11], and with the incidence of metabolic syndrome. The metabolic syndrome is a constellation of pathologies including obesity, insulin resistance, dyslipidemia and hypertension [13]. Fructose has been shown to be involved in the progression of metabolic syndrome, through dysregulation of many molecular signal factors [14, 15]. In the past, dietary intake of fructose was used to be 16-20 grams per day, mainly from fresh fruits and vegetables. But in the last three decades, increased consumption of industrialized foods such as

soft drinks, fruit juices, bakery products, canned fruits, jams, jellies and cookies, containing added sugars (sucrose, high fructose corn syrup, honey, molasses, and other syrups) has resulted in a significant increase in fructose intakes of 85-100 grams per day [16,17]. Insulin resistance, a feature of metabolic syndrome is found to be the result of high intake of dietary fructose. Studies have shown that insulin resistance is one of the important reasons for reproductive problems, particularly menstrual disturbances such as problems in ovulation. In india palm jaggery has been used as a healthy sweetener, It is a relatively unrefined sugar as no chemicals or bleaches are added during the process. While manufacturing processes in sugar utilize chemicals such as sulfur dioxide, lime and other bleaching agents, but jaggery is prepared in a natural way, without removing the minerals. Jaggery is known to have various medicinal properties and other health benefits. Sesame oil is important cooking oil in south India. Virgin vegetable oil (unrefined sesame oil) is reported to be more beneficial than refined vegetable oil (refined sesame oil). Although the use of oil is known from the ancient past, however the scientific literatures regarding its health benefits are considerably limited. In this study, we assessed the changes of estrous cycle in response to refined sugar diet, unrefined sugar diet and refined oil diet, unrefined oil diet.

METHODOLOGY

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. The animals were kept in polypropylene cages, 2 per cage, with relative humidity (55%) in a 12 hour light/dark cycle at $25^0 \pm 2^0$C. They received a normal control diet, experimental diet (table 1) and water ad libitum. The experiment carried out as per the guidelines of the 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.

EXPERIMENTAL DIET

Unrefined Sesame oil was purchased from a sesame oil extraction unit in Chidambaram. Where sesame oil is extracted manually by ghani method [18, 19]. Refined sesame oil, Palm jaggery, wheat bran, corn starch, groundnut oil, groundnut, Bengal gram was purchased from local market, Chidambaram. Fructose, casein, vitamin mix, mineral mix was purchased from SDFCL, Mumbai, NICE CHEMICALS Pvt, Ltd, Kerala, India. Diets [20] were formulated based on American institute of nutrition 93G (AIN-93G) to meet recommended nutrient levels in mice as showed in table 1. Diets were prepared fresh daily.

Table 1
Composition of diets (g/100g)

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

EXPERIMENTAL GROUPS

The animals were divided into five groups
Group 1: Normal control diet: Animals fed normal control diet for 90 days.
Group 2: Unrefined high sugar diet: Animals fed Palm jaggery diet for 90 days.
Group 3: Refined high sugar diet: Animals fed High fructose diet for 90 days.
Group 4: Unrefined oil diet: Animals fed unrefined sesame oil diet for 90 days.
Group 5: Refined oil diet: Animals fed refined sesame oil diet for 90 days.

DETERMINATION OF ESTROUS CYCLE

The phases of estrous cycle were determined by daily examination of vaginal smear as described by Marcondes et al [21]. Slight modification of staining the slides with 0.5% aqueous methylene blue solution. Every morning between 8:00 and 9:00a.m. (With a light: dark cycle of 12:12 and lights on at 06:00 GMT), vaginal smear cytology was performed for determination of the mouse estrous cycle. Latex bulb is placed on the end of a sterile 200 µl tip and drawn up approximately 100 µl of saline using the gradations on the tip as a volume guideline. Mouse was lifted out of her cage and places her on the cage hopper (lid). The end of the saline filled tip is placed at the opening of the vaginal canal and the bulb is gently depressed to expel a quarter to half of the

volume of water (~25-50 µl) at the opening of the vaginal canal. The liquid is spontaneously aspirated into the canal without insertion of tip into the vagina of a mouse. After this, the pressure exerted on the bulb was released slowly. The fluid is withdrawn back into the tip. This step is repeated 4-5 times using the same tip, bulb, and fluid to obtain a sufficient number of cells in a single sample. Slides are pre-labelled with the study number, animal numbers and date, using a permanent marker. A different glass slide was used for each animal. The fluid was placed on a glass slide, colored with 0.5% aqueous methylene blue solution, covered with a cover slip and examined with a light microscope. Photomicrographs are taken at time of analysis to document cytology. For cytology definitions a classification guide was used [21].

Figure1

Photograph shows the process of vaginal lavage collection from female mice

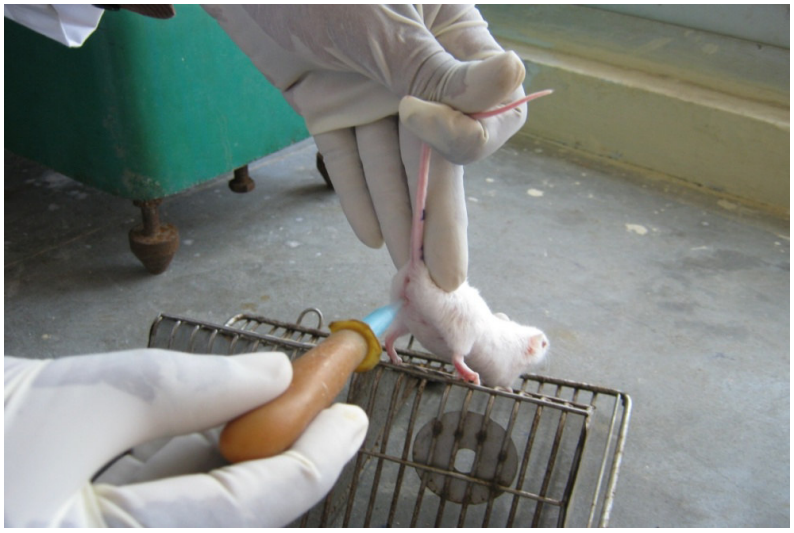


Figure 2 ***Plate A***

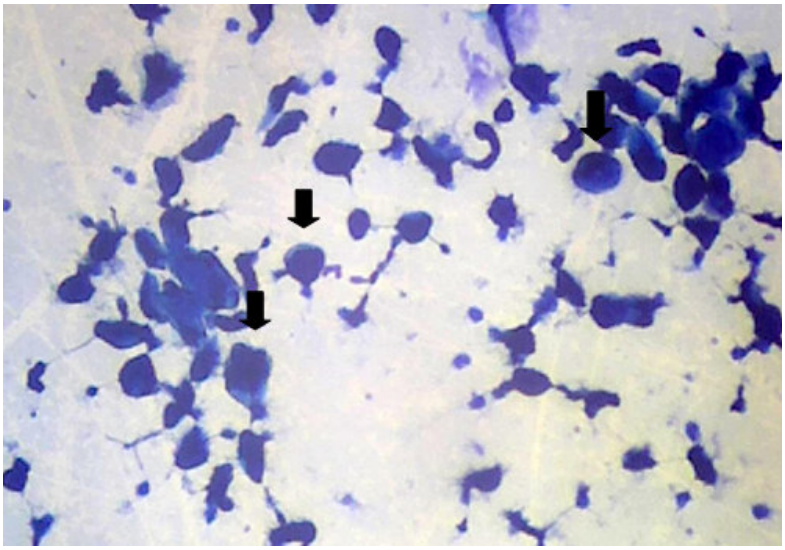


Plate B

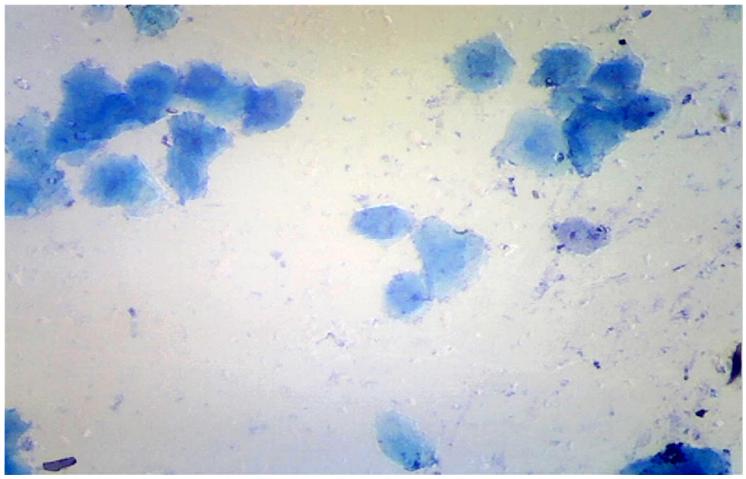


Plate C

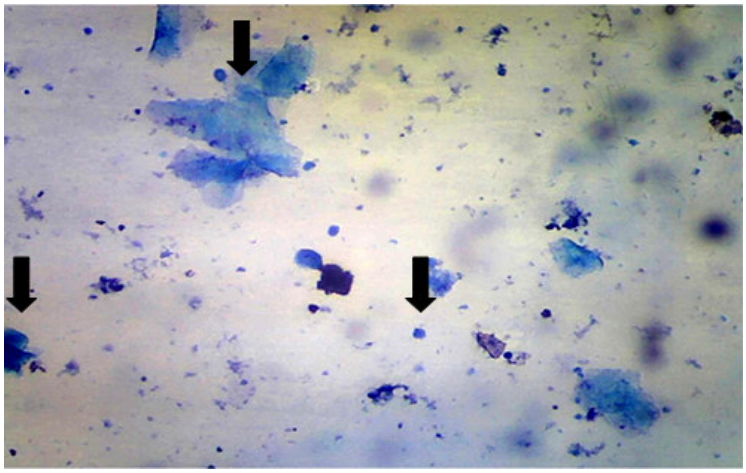


Plate D

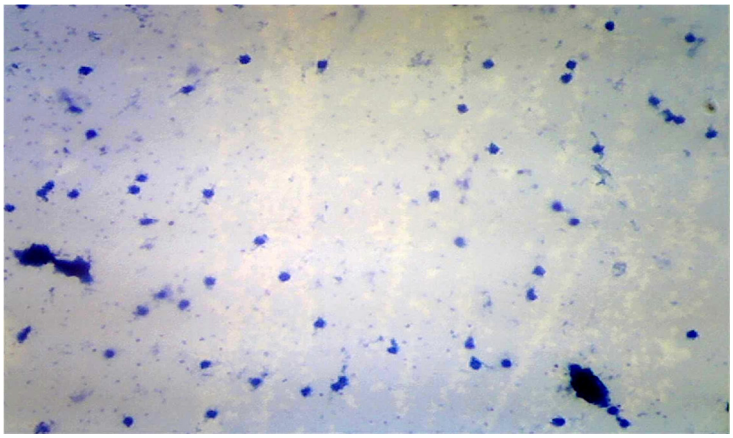


Figure 2 ***Plate A: Photomicrograph of Methylene blue stained vaginal smear from female mice at proestrus phase of the cycle,showing predominance of round and nucleated cells (arrowed). Plate B: Photomicrograph of Methylene stained vaginal smear from female mice at estrous phase of the cycle, showing predominance of irregular cornified cells. Plate C: Photomicrograph of Methylene stained vaginal smear from female mice at metestrous phase of the cycle, showing nucleated, cornified and leucocyte cells (arrowed). Plate D: Photomicrograph of Methylene stained vaginal smear from female mice at diestrous phase of the cycle, showing predominance of leucocyte cells.***

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS (version 17.0). Results are expressed as mean ± SD and the statistical analysis of data was done using the student's't' test. Probability level less of 0.05 was considered statistically significant.

RESULTS

The control mice showed regular estrous cycles and normal duration of each phases of the estrous cycle. Estrous cycle was affected by showing significant (P<0.05) decrease in the number of estrous cycles (figure: 4) in high fructose fed mice (HFD) with concomitant significant increase in the duration of diestrus phase (P<0.01)(figure: 3).It indicates that it was arrested in this stage for long days. It also shows significantly decrease in the duration of estrus phase (P<0.01), Metestrus phase (P<0.01) in high fructose fed mice. The mean duration of proestrus was increased in palm jaggery group (PJD) mice, but it was not statistically significant, But estrus phase (P<0.05), Metestrus phase (P<0.05) was decreased significantly in this group. No significant changes were seen in the diestrus phase of palm jaggery fed mice. Mice fed unrefined sesame oil diet (UNS) did not show significant changes in proestrus and estrus phases of estrous cycle. But Metestrus phase was significantly decreased (P<0.01) and diestrus phase was significantly increased (P<0.01) in UNS group. Mice fed refined sesame oil (RES) diet shows decreased phases of estrus (P<0.01), metestrus (P<0.01) significantly. Moderate changes were seen in diestrus phase of unrefined sesame oil diet. The diestrus phase was significantly increased in RES group (P<0.01). We observed that the total number of cycle in mice fed high fructose diet group (P<0.01), palm jaggery diet group (P<0.01), refined sesame oil diet group (P<0.01) was decreased significantly But total number of estrous cycle during experiment period was less than compare to palm jaggery diet group, refined oil group mice. No significant changes were observed in unrefined sesame oil diet group. We also observed the results of total number of cycle in first 30 days and last 30 days of experimental duration. In this type of observation only high fructose diet group mice shows significant changes. The number of cycles was decreased in first 30 days of this group (P<0.01), no significant changes were observed in other groups. During last 30 days of experiment period, mice fed a high fructose diet was decreased significantly (P<0.01). The number of cycles was reduced significantly in refined sesame oil diet group (P<0.01), but actually it shows normal cycle. No significant changes were observed in the unrefined oil diet group.

DISCUSSION

An estrous cycle is a rhythmic reproductive cycle occurring in sexually mature female mammals which depend upon the periodic release of gonadotropic releasing hormones, gonadotropins and sex hormones [22, 23] and gives a fair index of ovarian and uterine function. The estrus cycle reflects the function of gonadal glands in female mammals [24].Estrus cycle includes 4 phases. It starts with proestrus phase, which lasts approximately 17~21 h in the rat and mice. In this phase, estrogen level starts to increase and the follicles are starting to grow. It is followed by estrus phase that lasts 9~15 h, characterized by a peak level of estrogen. The estrus phase corresponds to the pre-ovulatory and ovulatory phases. In the pre-ovulatory and ovulatory phases, the shedding cells are mainly the epithelial cells and thus this is also known as the epithelial phase. In the post-estrus phase, the estrogen level starts to decrease and this phase lasts approximately 10~14 h, at which time the corpus luteum forms and progesterone is secreted. In the diestrus phase, estrogen reaches a minimal level and this phase usually lasts 60~70 h. The post-estrus and diestrus phases are characterized by an increase in the number of leukocytes, and thus are also known as the leukocyte phase. Normally the length of the estrous cycle is 4 or 5 days. Our results confirmed that the mean duration of the estrous cycle of control mice was 5 days (Figure: 4).Similar observation has also been made by Radhika*et al*. [25]. Observation of estrous cycle changes in unrefined high sugar diet (palm jaggery diet) and unrefined oil diet

(unrefined sesame oil diet) mice were normal. These groups show regular cycle. But the number of metestrus phase was decreased slightly in all experimental diet groups than compare to control group mice. Prolonged diestrus phase was observed in refined sesame oil group mice. It needs further investigations in refined sesame oil diet group mice. In the present study, altered estrus cycle was observed in high fructose fed mice. There was a significant decrease in the total number of estrous cycles in high fructose fed mice (Figure: 4). No major changes were observed between first 30 and last 30 days of the experimental period. The number of cycles was slightly decreased in refined oil group in last 30 days diet period than compare to control group. It has also been observed that mice fed high fructose diet shows prolonged diestrus phase ($P<0.01$) and decreased numbers of estrus phase than compare to the control group mice. These changes may be due to hormonal imbalance. Insulin resistance, a feature of metabolic syndrome found to be the result of high intake of fructose rich diet [26]. It may play the major role in reproductive dysfunction such as irregular cycles or anovulation. But in our study anovulation does not occur in high fructose diet group mice. But the high fructose fed mice shows irregular cycle and the duration of diestrus phase was prolonged. Many Studies have shown the link between high fructose diet and insulin resistance [27, 28].The association of insulin resistance and reproductive abnormalities with clinical hyperandrogenism in a woman was first demonstrated by Achard and Thiers in the diabetes of a bearded woman" [29]. Our study exhibit reproductive dysfunction in mice through an altered estrous cycle in response to high fructose diet. The oil diet group mice shows regular cycle, but changes have been observed in refined sesame oil group mice. After the complete assessment in our study, we consider that unrefined diet group was better than refined diet group.

Figure 3

Mean ± SD of the Percentage of mice at different phases of estrous cycle in different diet groups

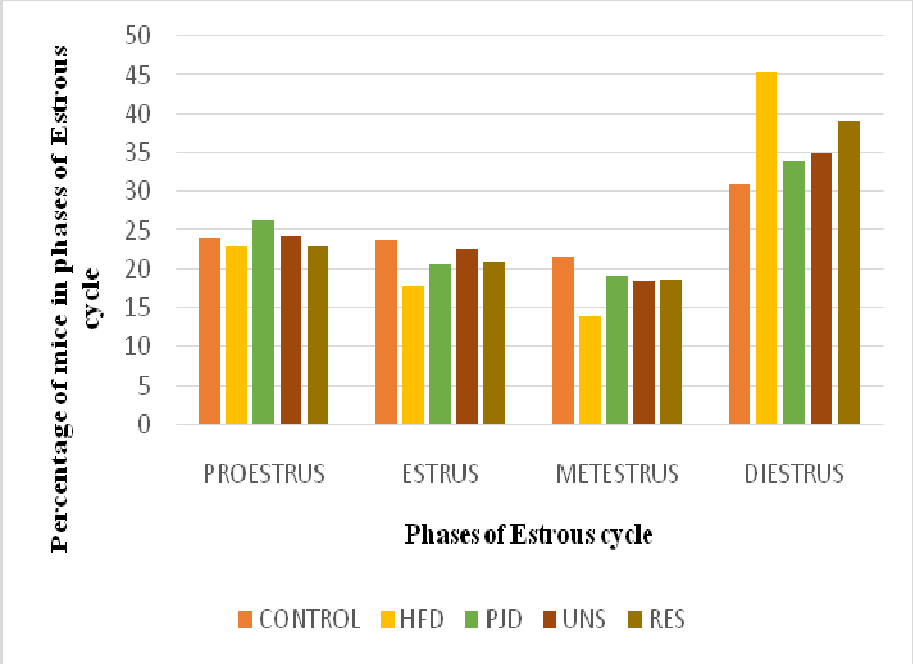
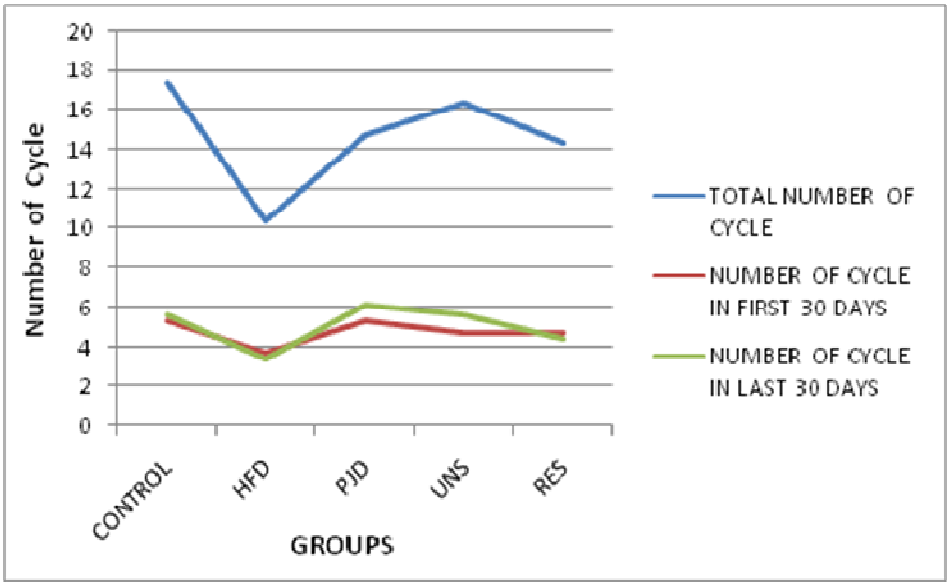


Figure 4
Number of estrous cycle



CONCLUSION

In the present study vaginal smears indicated that the estrous cycle of mice fed a high fructose diet were lengthened and irregular. Our study suggests that intake of palm jaggery will be a good alternative to fructose to maintain the normal menstrual cycle. It needs further explanations in the view of hormonal, histopathological and molecular level assessment to understand the ovarian pathology in response to these different diet ingredients.

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