Original Research Article

Effect Of Obesity On Selected Reproductive Parameters In Female Sprague Dawley Rat (*Rattus Norvegicus)* Model.

ABSTRACT

Aims: To determine changes in frequency of occurrence and durations of estrous cycle stages and measure serum levels of cortisol and estradiol in Sprague Dawley rats.
Study design: Laboratory Experimental research Design.
Place and Duration of Study: Department of Veterinary Anatomy and Physiology, Chiromo Campus, University of Nairobi Kenya, March to June 2017.
Methodology: Obesity was induced through a High Energy Diet (HED) after which frequency of occurrence and durations of estrous cycles stages, serum estradiol 17b and cortisol hormone levels were analyzed. Twenty four, three-month-old sexually mature female Sprague Dawley rats grouped into replicates of six rats were fed either on HED (n=12) or a control diet (n=12) for seven weeks after which 12 obese rats and 12 controls were evaluated for estrous cycles durations and frequency of occurrence through vaginal smears. Six rats from control and obese groups then underwent cervical dislocation followed by collection of blood through cardiac puncture. This was followed by analysis of serum cortisol and estradiol 17b hormone levels using ELISA technique, Mean values of estrous cycle stages' frequencies of occurrence, serum levels of cortisol and estradiol were subjected to Student t-test to evaluate any significant differences at *P=.05*.
Results: Obese rats had disrupted and extended estrous cycle stages, elevated serum cortisol (5.12±1.45) and estradiol (214±17.28) levels. Student t-test analysis indicated significant differences between means of frequencies of occurrence of proestrus (t=-2.66, *P=.02*) estrus (t=5.13, *P=.00*) and diestrus (t=-2.45, *P=.02*) stages as well as serum levels of cortisol (-2.87, *P=.04*) and estradiol 17b (t=5.37, *P=.00*). There was an inverse correlation between concentrations of cortisol and estradiol in blood sera of obese rats:-r =0.64.
Conclusion: Obesity leads to an inverse relationship between estradiol and cortisol resulting to disruption in the rat's estrous cycles.

Keywords: [Estrous cycles, Obesity, High Energy Diet, Hormone Levels, ELISA,Cortisol,Estradiol }

1. INTRODUCTION

Excess body fat affects most of human populace: both men and women, young and old. Studies by[1], has shown that obesity predisposes a victim to a number of complications ranging from social, psychological to demographic whereby the females are affected more than males. According to[2}, increased consumption of high calorie diet over a prolonged period of time can elevate risk factors for severe health problems associated with obesity such as non-insulin diabetes, hypertension, coronary heart disease and depression. Obesity not only leads to increased health risks [3,4], but also compromises reproductive performance in women. Studies have demonstrated that obesity may lead to severe and long-term fertility complications in females due to considerable ovulatory problems and oligomenorrhea [5].Harmful implications of obesity continues to be a major research concern as indicated by significant studies in many animal models with induced obesity showing that overweight and obesity negatively impacts on reproductive function in females [6,7]. Although a number of obesity related studies have been done, the mechanisms by which obesity affects reproductive function are not fully understood.

Excess body fat leads to several complications in most reproductive age women. Although a number of individuals are affected by obesity, it does not mean that every other overweight or obese woman is negatively affected by having altered reproductive fitness. However a number of obese individuals have a compromised reproductive function. In women of reproductive age, obesity plays a considerable role in reproductive disorders such as anovulation, difficulties in assisted reproduction, miscarriage, menstrual disorders, and infertility [8]. Research on animal and human subjects has shown that obesity during pregnancy can also increase the risk of offspring overweight [9, 10]. Thus, if obesity is not addressed in women of reproductive age, its effects will trickle down to the female offspring and continue to affect future generations.

It is possible that obesity affects reproduction negatively by interfering with several reproductive parameters, including acting as a reproductive stressor to the female. Past studies in rats exposed to stressful conditions have shown disrupted levels of hormone cortisol [11]. Indeed limited obesity related studies have looked into obesity as a reproductive stressor [12]. This study aimed at finding out the effect of obesity as a stressor to female reproduction and its possible mode of action by determining changes in the frequency and durations of different estrous cycle stages and measuring serum hormone levels of cortisol and estradiol in Sprague Dawley rats fed on a control and a High Energy Diet.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

Sprague Dawley rats used in this experiment were caged in pairs in plastic bottomed cages with wire meshed tops (45 cm × 20 cm × 15 cm) on a 0.75 m raised surface in the laboratory animal house. Pine wood shavings were used as bedding and were replaced daily. The rats were kept in light controlled quarters at a 12 hour light–dark cycle (lights on: 07:00–19:00 h and lights off: 19:00–07:00 h). All experimental procedures were conducted during the light cycle. Average room temperature was kept at 21 ± 3℃. Effects of a HED on selected reproductive parameters: frequency of occurrence, durations of estrous cycle stages and hormone levels of estradiol and cortisol were tested in the laboratory using two sets of 12 rats (6 replicates) for the experimental and control sets respectively. Obesity was induced using a HED comprising of 23.54% protein, 20.2% animal fat, 5% fat, 20% polysaccharide, 20.3% simple sugars, 5% fiber, 5% mineral mix, 1% vitamin mix[13] , and filtered clean tap water. This was fed ad *libitum* on 6 replicate pairs of rats against a similar set fed on a normal laboratory chow (control diet). All rats were weighed using a weighing balance Shimadzu model TX423L to the nearest 0.01g and their nasal anal lengths measured to the nearest 0.1cm using a measuring board daily respectively. BMI was calculated by dividing the rat's weight (g) by the square of the nasal-anal length (cm^2) [14]
Rats with BMIs of greater than or equal to 0.68 g cm^{-2} were regarded obese. Weights and BMIs of both groups were monitored weekly. The effect of obesity on durations and frequency of occurrence of estrous cycle stages were studied using microscopic examination of the cytological features of the vaginal samples according to [15] at x40 using an Olympus light microscope model CX22LEDRFS1. Hormone levels of estradiol and cortisol were estimated using Enzyme Linked Immuno-Sorbent Assay (ELISA) technique according to Sigma Aldrich and the concentrations of both hormones determined by an ELISA Microplate reader Bio-Tek model ELx800. Mean values of estrous cycle stage durations, frequencies of occurrence and serum levels of cortisol and estradiol were subjected to Student t-test to evaluate whether there were any significant differences between those of experimental and control rats at P=.05.

3.0 RESULTS AND DISCUSSION

3.1 Obesity Induction.

Changes in BMIs of both controls and experimental sets of rats are as illustrated in Table 1.BMIS show that the rats fed on HED in experimental sets had their mean BMI's greater than 0.681, confirming that they had attained obesity.

Table 1: The Mean Body Mass Indices (BMIs) of control and experimental rats.

Group	Set	BMI Range (g/cm^2)	Mean BMI(g/cm^2)	S.D	S.E
Control Rats	1	0.53-0.67	0.62	0.03	0.005
	2	0.55-0.68	0.62	0.03	0.005
Experimental Rats	1	0.67-0.79	0.74	0.33	0.005
	2	0.68-0.84	0.73	0.03	0.033

Key: S.D=Standard Deviation S.E=Standard Error

The HED diet fed to the experimental sets of rats induced obesity after a period of 7 weeks. On the other hand, the normal diet used on the control set did not induce obesity.

3.2 Frequency of Occurrence of Estrous Cycle Stages

Frequency of occurrence of individual cycle stages refers to the number of times a stage was observed in 50 days. The cycle stages namely, proestrus and diestrus occurred over prolonged periods while estrus was shortened in the experimental rats as compared to the controls (Table 2). The diestrus stage in the experimental rats had the highest mean frequency of occurrence while that estrus stage in the experimental rats had the lowest mean frequency of occurrence.

Table 2: Frequency of occurrence for the estrous cycle stages for control and experimental rats.

CYCLE STAGE	GROUP	N	RANGE	MEAN	S.D	S.E
Proestrus	Control	12	10.00-13.00	11.08	0.64	0.26
	Exptal	12	8.00-22.00	14.50	4.36	1.26
Estrus	Control	12	11.00-14.00	12.08	0.79	0.23
	Exptal	12	5.00-11.00	7.25	3.16	0.91
Metestrus	Control	12	10.00-14.00	11.92	1.31	0.38
	Exptal	12	3.00-18.00	10.17	3.69	1.06
Diestrus	Control	12	13.00-18.00	14.92	1.56	0.45
	Exptal	12	13.00-25.00	18.08	4.19	1.21

Results of the single tailed Student t-test for comparison of means of frequency of occurrence between the control and experimental rats (Table 3) indicated that there were significant differences for the means of proestrus, estrus and diestrus stages. There was no significant difference between the means of metestrus for both controls and experimental rats.

Table 3: Single tailed student t-test on the effect obesity on the frequency of occurrence of estrous cycle stages (*P*=.05)

Stage	t value	P Value	Comments	Null Hypothesis
Proestrus	-2.66	.02	Significant	Rejected
Estrus	5.13	.00	Significant	Rejected
Metestrus	1.55	.14	Not Significant	Accepted
Diestrus	-2.45	.02	Significant	Rejected

3.3 Levels of the hormones cortisol and estradiol in serum samples.

Cortisol and estradiol in the blood sera of experimental and control rats (Table 4) indicated that experimental rats had higher values than control rats. The mean cortisol levels in experimental rats had higher variability than those of the controls. Similarly; the mean of the serum estradiol levels of experimental rats was higher than that of the control rats.

Table 4: Cortisol and estradiol hormone levels in experimental and control rats fed on HED and normal diets respectively.

Hormone	GROUP	N	Range pmol/L^{-1}	MEAN pmol/L^{-1}	S.D	S.E
Cortisol	Control	12	0.00-3.10	0.73	1.27	0.52
	Exptal	12	2.9-10.7	5.12	3.23	1.45
Estradiol	Control	12	98-143	114	15.22	0.23
	Exptal	12	139-248	214	42.32	17.28

Single tailed student t-test for comparing the means of cortisol and estradiol levels of experimental and control rats (Table 5) indicated that there were significant differences.

Table 5: Single tailed Student t test for serum cortisol and estradiol hormone levels (***P*=.05**).

Serum hormone	t value	P Value	Comments	Null Hypothesis
Estradiol	5.37	.00	Significant	Rejected
Cortisol	-2.87	.04	Significant	Rejected

An attempt was made to establish whether there was any relationship between cortisol and estradiol levels in the blood sera of experimental rats (Fig.1) Cortisol levels were related to those of estradiol by the regression model: Estradiol = -4.407 Cortisol + 256.71; R=0.64.The model indicates an inverse relationship, that is, as the level of estradiol increases in the blood sera of rats, that of cortisol decreases.

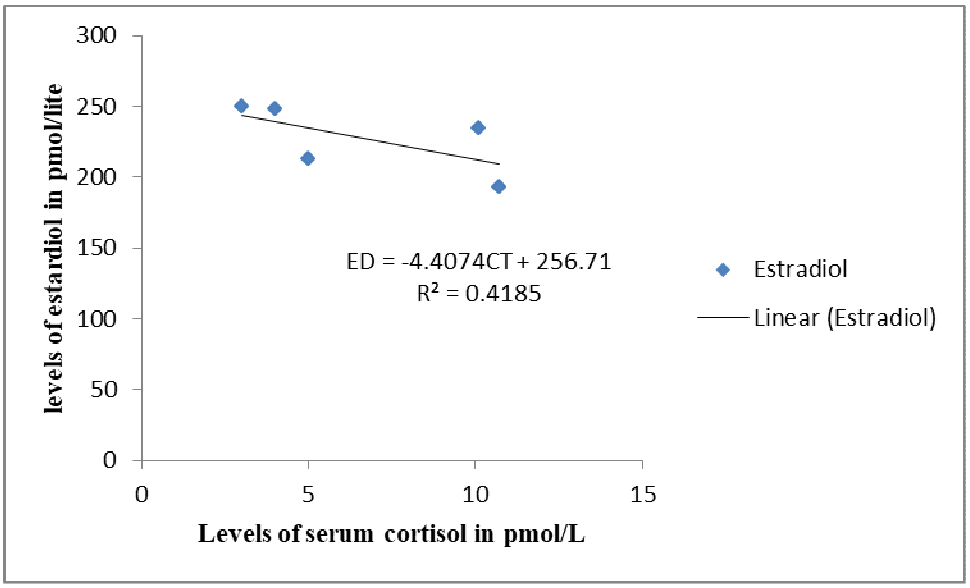

Figure 1: Blood sera levels of estradiol and cortisol in experimental Sprague Dawley rats.

3.4 Estrous Cycle durations.

The durations of different estrous cycle stages for the experimental rats are represented in Fig.2. Disruption of the cycles was due to skipping and repeating of stages contrary to the normal sequence of proestrus, estrus, diestrus and metestrus consecutively. Estrous cycle durations in obese rats were disrupted and prolonged beyond the normal length of 4-6 days. Most of the rats in the experimental group had stages namely proestrus and diestrus repeating themselves longer than the expected durations. For instance, in rat number one (Fig 2), the first stage on day one was diestrus; the second day metestrus instead of proestrus, third day was metestrus again. This trend of skipping is also observed in other experimental rats.

EXPERIMENTAL RATS						
DAY	**1**	**2**	**3**	**4**	**5**	**6**
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						
11.						
12.						
13.						
14.						
15.						

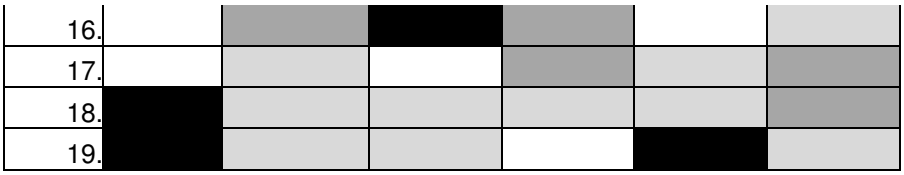

Fig.2: Lengths of estrous cycle for the obese experimental rats in a period of 19 days.

Key:

Proestrus Estrus Metestrus Diestrus

It was observed further that the proestrus, diestrus and metestrus stages extended for more than one day. For instance, rat one had proestrus stage being prolonged up to four days: between days 13-16 while metestrus in rat 5 extended up to 5 days as from day 11 to day 14(Fig. 2).Similarly, diestrus stage in rat 4 protracted for 4 days: from day 2 to day 5.Unlike proestrus, metestrus and diestrus stages that extended for longer days than normal, estrus was shorter and did not extended beyond three days (Fig 2), it occurred for periods of 1 and two days. Prolonging and of cycle stages by the obese diet caused lengthened cycles.

The durations of different estrous cycle stages for the control rats are represented in Fig. 3.Unlike in the experimental rats where there was disruption and lengthening of cycle stages, rats in the control experiment maintained the normal pattern of the estrous stage which took place in 4-6 days.

CONTROL RATS						
DAY	**1**	**2**	**3**	**4**	**5**	**6**
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						
11.						
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13.						

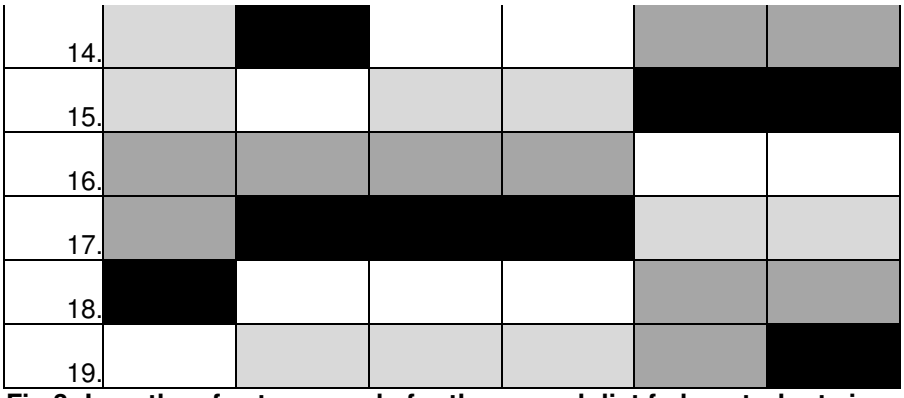

Fig.3: Lengths of estrous cycle for the normal diet fed control rats in a period of 19 days.

It was further observed that in the control rats (Fig 3) only the diestrus stage extended to periods of up to three days, this was not abnormal because in literature, diestrus normally takes up to 57 hours while proestrus estrus and metestrus stages averagely take one day in normal rats. (Westwood, 2008)

Discussion

The HED used in this study induced obesity in rats.Similary; human obesity is also as a result of consumption of high calorie diets that exceeds normal body requirements. It is presumed that with the rising dominance of sedentary lifestyles and dietary alterations, obesity is developing within the human population. Increased obesity is leading to hostile health effects, including female reproductive disorders[2] Statistics from a number of studies show an inverse relationship between BMI and female reproductive fitness as exemplified by the decline in conception rates, pregnancies rates and reproductive cycling ,[16,17]. However, the mechanism through which fertility is affected remains unclear[18]

In an effort to realize more understanding about excess fat accumulation in the adipose tissue, a number of animal models such as the Ossabaw mini-pigs, hamsters and rodents have been used. Studies by [19,7,20] show that the rodent models of feed-induced obesity has provided best equivalents in the same, to human obesity. The HED used in this study was largely constituted of carbohydrates (20% polysaccharide, 20.3% simple sugars) and fats (20.2% animal fat, 5% fats) which often result in the deposition of excess adipose tissue in the body. This is as a result of the development of fat cells known as adipocytes. These types of cells are specifically adapted for the storage of excess fat which often result in obesity. In as much as obesity is known to cause harmful effect to the body, fat storage in these cells helps to avoid harmful metabolic consequences of excess cellular lipid deposition in organs like liver, muscle, and heart. (21-23].

Studies by [24, 25], show that both the fat and non-fat cells produce and secrete various factors including peptides and steroid hormones which impact the local systemic physiology. In this study, elevated levels of the hormones cortisol and estradiol in the blood sera of Sprague Dawley rats were as a consequence of feeding the rats with a high energy diet. The resultant adipose tissue, serves as an endocrine organ by either storing or releasing preformed steroid hormones [21].Since the normal functioning of the reproductive axis depends on appropriate energy balance, the endocrine function of the adipose tissue coupled with that of the HPA and HPG axes, cause reproductive disruptions, such as disrupted and extended estrous cycles as well as unusual cortisol and estradiol hormone levels. A similar study by [26], showed elevated but lower levels of estradiol in obese female rats than in our study. Thus, the only concurrence with this study is that obesity is related to elevated estradiol levels in rats.

The study also observed that other than being disrupted, the estrous cycle stages of experimental rats extended longer than those of the control rats. This observation is similar to the studies by [27] whereby the obese female Ossabaw mini pigs demonstrated extended and disrupted estrous cycles. [26], made similar obsevations.However, her findings demonstrated that all the cycle stages were not different from

the controls except diestrus stage that had a higher frequency of occurrence. Comparably, in this study the frequencies of occurrence of proestrus, estrus and diestrus were significantly different from those of the controls. The other observed difference between this study and the former is the reduction in frequency of occurrence of the estrus stage in our experimental rats. Since the estrous cycle is driven by pituitary gonadotropins and ovarian steroid hormones, a disruption of the hormonal balance particularly estradiol interferes with normal estrous cycling. As demonstrated by [27], a cycle comes to an end and another one starts once the estradiol levels drop in order to trigger the hypothalamus to release GnRH which elicits the next cycle. Therefore continued high levels of estradiol may lengthen the duration of estrous cycle stages as observed in this study. Despite this, high estradiol levels did not affect the metestrus stage as also observed by [26].In this study the estradiol levels were slightly lower in the metestrus stage than in other cycle stages but still remained at higher levels in experimentals compared to the controls. This slight drop in the estradiol level should be the reason as to why there was no effect on the metestrus stage.
[28], reports that extended and prolonged estrous cycles in obese rats approximates to the prolonged and sometimes disrupted menstrual cycles commonly observed in obese human females. The estrous cycle in rats follows the same pattern as that of human except that in rats, the duration and frequencies are different and the sloughed off endometrium is reabsorbed hence no menses. For successful functioning of the reproductive system, there has to be well balanced energy requirements for the body in order to maintain proper reproduction as mediated by the HPA and HPG axes.

4. CONCLUSION

HED causes obesity leading to a disruption in the rats 'estrous cycle by extending the duration and increasing the frequency of occurrence of proestrus, estrus and diestrus stages. The diet also lowers the frequency of occurrence of the estrus stage. Since the normal functioning of the reproductive axis depends on appropriate energy balance, the metabolic and endocrine function of the excess adipose tissue coupled with that of the HPA and HPG axes, cause reproductive disruptions, such as disrupted and extended estrous cycles as well as unusual cortisol and estradiol hormone levels. Furthermore the diet leads to an inverse relationship between estradiol and cortisol

ETHICAL APPROVAL

All authors hereby declare that the study protocol was approved by the National Commission for Science; Technology & Innovation (NACOSTI) review committee who issued the Research Permit number NACOSTI/P/16/50358/11300. The study was conducted in accordance with the internationally accepted doctrines for laboratory animal use and care as outlined by Voipio, Baneux, de Segura, Hau&Wolfensohn, (2008).

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