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Research Article

Alloxan-induced diabetes and insulin resistant effects on ovulation and phases of estrous cycle in virgin female Sprague-Dawley rats

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ABSTRACT

Backgroumd: Sexual disorders have been extensively studied in diabetic men while possible changes in the sexual function of diabetic women have only recently received attention. Mammals other than primates do not menstruate, thus their sexual cycle is termed "Estrous cycle". The period of 'heat' (estrus), is the time of ovulation and corresponds to the only time which the sexual interest of the female mammal is aroused. The goal of this study was to assess the effects of alloxan-induced diabetes and insulin-resistant on hormonal pattern in diabetic female rats **Methods:** Virgin female Sprague-Dawley rats aged 6 weeks, weighing 90 – 100 g were randomly divided into 3 groups. Group 1 = Control group; fed on normal rat chow. Group 2 = Alloxan-diabetic group; at the 4th week received a single dose IV injection of alloxan monohydrate 40 mg/kg BW into the lateral tail vein. Group 3 = Insulin resistant group; fed ad libitum on a special diet containing 25% fructose mixed with 75% normal rat chow (w/w). Cervical dislocation was carried out on the animals in the three groups on the morning of specific phases of the estrous cycle and the LH, FSH, Estradiol, and Progesterone levels were determined using Enzyme-linked immunosorbent assay (ELISA) methods. On the morning of estrus phase, the oviducts were excised, viewed under the microscope and any ova found was counted. **Results:** The results of this study showed that both Alloxan-diabetes and insulin resistant diabetes distort the estrous cycle pattern, reduce significantly the "fertile period" and the ova released by acting on the pituitary-ovarian axis reducing the gonadotropins and the ovarian hormones. **Conclusion:** These data open a new field to study hormonal profile in diabetic female rats during each phase of the estrous cycle and also to investigate the hypothalamo-pituitary-ovarian axis in insulin-resistant diabetic female rats.in light of recent preferences for the use of natural medicines.

INTRODUCTION

Mammals other than primates do not menstruate, thus their sexual cycle is termed "Estrous cycle". This name derives its origin from the period of 'heat' (estrus), which is the time of ovulation and corresponds to the only time which the sexual interest of the female mammal is aroused (Freeman, 1988).

Cycling female rats have an estrous cycle of approximately 4 – 5 days in length, and this short cycle length makes the rat an ideal animal for investigating changes that occur during the reproductive cycle (Spornitz et al, 1999; Marcondes et al, 2001). A normal female rat has a cycle with 4 phases namely proestrus, estrus, metestrus and diestrus. These phases are characterized based on the proportion of 3 cell types (epithelial cells, cornified cells, and leukocytes) observed in the vaginal smear.

Diabetes mellitus has been shown to interfere with reproductive function in both laboratory animals and in man (Valdes et al, 1990, La Vignera et al, 2012, Jain and Jangir, 2014). Arikawe et al, (2006) reported that diabetes mellitus impair distinct phases of spermatogenesis thus affecting semen parameters in male rats. Agbaje et al, (2007) also reported that diabetes mellitus in man is associated with increase sperm nuclear and mitochondrial DNA damage, impairing reproduction.

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Although sexual disorders have been extensively studied in diabetic men, possible changes in the sexual function of diabetic women have only recently received attention. The prevalence of sexual dysfunction in diabetic men approaches 50%, whereas in diabetic women it seems to be slightly lower.

In their study, Arikawe et al, (2008) showed that diabetes mellitus alters corpus luteum functions and predisposes to polycystic ovarian syndrome in female rats. Also recently Spadotto et al, (2012) showed that chronic hyperglycemia during gestation and lactation affected the reproductive functions of female offspring.

Unlike the male rats, the effects of diabetes mellitus on female reproductive function have been inconclusive. According to Valdes et al, (1990); there is the need to establish if the reported abnormalities associated with diabetes mellitus in female rats are at the hypothalamic, pituitary and/or ovarian level.

Thus, this study is aimed at assessing the effects of alloxan diabetes and insulin resistance on ovulation, estrous cycle and the pituitary gonadotropins and ovarian hormones during the different phases of the estrous cycle in virgin female Sprague-Dawley rats.

MATERIALS AND METHODS

Seventy-two virgin female Sprague-Dawley rats aged 6 weeks, weighing 90 – 100 g were obtained from the Laboratory Animal Department. The animals were housed in clear polypropylene cages (43 cm x 30 cm x 15 cm) with laboratory-grade pine shavings as bedding. Animals were kept under standard conditions of temperature 27ºC – 30ºC, air exchange rate was 10 room-volumes per hour, and were also maintained under standard colony photoperiodic conditions with a 12-hour light/12-hour dark cycle (lights on at 0600 – 1800 hour) so that midnight (24 hour) was the midpoint of the dark cycle. All animals had unrestricted (ad libitum) access to water and were randomly divided into 3 groups of 24 rats per group (6 rats each for the 4 phases of the cycle). Group 1 served as Control group and was fed with normal rat chow.

Group 2 served as Alloxan-diabetic group; fed on normal rat chow and at the 4th week received a single intravenous injection of Alloxan monohydrate, 40 mg/kg body weight (Iranloye et al, 2013) into the lateral tail vein. Hyperglycaemia was confirmed 72 hours later using Dextrostix Test Strips (Bayer Corporation, U.K.) following the glucose oxidase method (Hugget and Nixon, 1957).

Group 3 served as Insulin resistant diabetic group and was fed ad libitum on a special diet containing 25% fructose mixed with 75% normal rat chow (w/w) from the 4th weeks (Arikawe and Olatunji-Bello, 2004) and continued till the 10th week. Hyperglycaemia was confirmed at the 10th week using Dextrostix Test Strips (Bayer Corporation, U. K.) following the glucose oxidase method (Hugget and Nixon, 1957). All animals had free access to drinking water throughout the duration of the study and were also weighed weekly throughout the duration of the experiment. The procedures were performed in accordance with guidelines of the College Ethical Committee on the use of laboratory animals for research.

Estrous cycle analysis

The estrous cycles were monitored by colpocytological examination (vaginal smears) daily for 14 consecutive days (two weeks). Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5 – 10 µl, UK). Filter tips containing 10 µl 0.9% saline were discarded after the vaginal secretions had been transferred to clean slides (Marcondes, et al, 2002). Colpocytological examination time was set at 0900 hours. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany) at 10x and 25x magnifications. Only female rats showing two consecutive estrous cycles of the same length were used (Iranloye and Bolarinwa, 2007)

Fasting Blood Glucose and Hormone Assay

Cervical dislocation was carried out on the animals in the three groups on the morning of specific phases of the estrous cycle and fasting blood glucose concentration was determined using one touch ultra-test strips (Lifescan Inc. Milipitas, USA) (Iranloye et al, 2013).

Blood was quickly collected by cardiac puncture into plain sample bottles, allowed to clot and centrifuged at 3,000 rpm for 15 minutes to get clear serum samples, which were subsequently kept frozen (-20ºC) until measurement of the different hormones (LH, FSH, Estradiol, and Progesterone) using Enzyme-linked immunosorbent assay (ELISA) methods procured from the Diagnostic Automation, Inc.

Ovulation assessment

On the morning of estrus phase after separation from the ovary, the fimbriated part of the ovary was dissected out from the rats and placed on the microscopic slide. The fatty tissues attached to the oviducts were gently removed leaving only the oviducts strand, then the oviducts were excised (using a surgical blade) and viewed under the microscope at magnification of X100 for the presence of ova (oocytes). Any ova found was counted (Iranloye and Bolarinwa, 2007).

Statistical analysis

All data are presented as Mean ± SEM. The data was analyzed using One-way ANOVA followed by Student

Table 1: Body weight (gm) in Control, Alloxan-induced diabetic and Insulin resistant groups

Weeks	Control	Alloxan-induced diabetic	Insulin resistant
Week 0	90.0 ± 1.1	95.0 ± 1.3	95.0 ± 1.2
Week 2	94.8 ± 1.9	104.5 ± 1.6	109.0 ± 1.3
Week 4	105.2 ± 1.6	107.7 ± 1.5	115.8 ± 1.0*$^{\alpha}$
Week 6	112.8 ± 1.5	90.8 ± 1.9*	109.8 ± 1.0*$^{\alpha}$
Week 8	123.3 ± 1.5	80.5 ± 1.7*	103.8 ± 1.1*$^{\alpha}$
Week 10	134.0 ± 1.4	67.7 ± 2.1*	95.2 ± 0.9*$^{\alpha}$

Data are expressed as mean ± SEM *P < 0.05 Vs. Control; $^{\alpha}$P < 0.05 Alloxan-diabetic Vs. insulin resistance

Table 2: Fasting blood glucose concentrations (FBG) in Control, Alloxan- induce diabetic and Insulin resistant groups

FBG (mg/dl)	Control	Alloxan- induced diabetic	Insulin resistant
Proestrus	94.4 ± 6.0	280.8 ± 9.8*	259.2 ± 9.6*$^{\alpha}$
Estrus	96.8 ± 3.3	252.6 ± 24.8*	201.6 ± 8.8*$^{\alpha}$
Metestrus	95.1 ± 5.2	263.6 ± 13.2*	252.1 ± 6.1*
Diestrus	84.8 ± 6.2	259.2 ± 8.3*	270.2 ± 7.1*

Data are expressed as mean ± SEM *P < 0.001 Vs. Control $^{\alpha}$P < 0.05 Alloxan- induced diabetic Vs. insulin--resistance

Table 3: Duration of Estrous cycle, Frequency of Estrous cycle phase, Number of ova released, and Number of atretic follicles in Control, Alloxan-induced diabetic, and Insulin resistant groups

Parameters	Control	Alloxan-induced diabetic	Insulin resistant
Estrous cycle duration (days)	4.0 ± 0.3	4.9 ± 0.3	4.4 ± 0.2
Frequency of proestrus (days)	4.8 ± 0.3	6.2 ± 0.7	5.8 ± 0.3
Frequency of estrus (days)	3.8 ± 0.2	1.4 ± 0.4†	1.8 ± 0.4†
Frequency of Metestrus (days)	1.6 ± 0.3	2.6 ± 0.3	2.2 ± 0.4
Frequency of Diestrus (days)	5.9 ± 0.3	6.8 ± 0.5	6.6 ± 0.5
Number of ova	5.4 ± 0.9	0.6 ± 0.2*	1.6 ± 0.2*
Number of atretic follicles	0.4 ± 0.1	6.0 ± 0.5*	3.2 ± 0.4*$^{\alpha}$

Data are expressed as mean ± SEM *P < 0.001 Vs Control; †P < 0.05 Vs Control; $^{\alpha}$P < 0.05 Alloxan-diabetic Vs insulin--resistance

-Newman-Keuls post-hoc test. Level of statistical significance was taken at P < 0.05.

RESULTS

There was no significant difference in body weight amongst the three groups at the beginning of the experiment utill the 4th week. The body weight began to decline significantly (P < 0.05) in both experimental groups when compared with the control group from the 6th week (112.8 ± 1.5gm for control rats, 90.8 ± 1.9 gm for Alloxan-diabetic rats and 109.8 ± 1.0gm for Insulin resistant rats) till the end of experiment ((134.0 ± 1.4 for control rats, 67.7 ± 2.1gm gm for Alloxan-diabetic rats and 95.2 ± 0.9 for Insulin resistant rats) Table 1.

The fasting blood glucose (FBG) concentration (mg/dl) in all the phases of estrous cycle (proestrus, estrus, metestrus and diestrus phases) was significantly higher (P < 0.001) in the diabetic experimental groups compared. The FBG was significantly lower (P < 0.05) in insulin resistant group when compared to the Alloxan diabetic group during the proestrus and estrus phases (Table 2).

There was no significant difference in estrous cycle duration amongst the three groups but the frequency of estrus phase was significantly reduced (P < 0.05) in both experimental groups compared to the control group (Table 3).

The number of ova released during ovulation was significantly higher (P < 0.001) in the Control rats (5.4 ± 0.9) compared to Alloxan-diabetic rats (0.6 ± 0.2) and Insulin resistant rats (1.6 ± 0.2). However, number of atretic follicles was significantly lower (P < 0.05) in

the Control rats (0.4 ± 0.1) compared to Alloxan-diabetic rats (6.0 ± 0.5) and Insulin resistant rats (3.2 ± 0.4). It was also significantly lower (P < 0.05) in the Insulin resistant rats compared to Alloxan-diabetic rats (Table 3).

Serum FSH significantly decreased (p<0.05) during the estrus and metestrus phases in Alloxan diabetic rats (15.3 ± 0.1; 14.8 ± 0.2 mIU/ml) and insulin resistant rats (15.3 ± 0.1; 15.0 ± 0.1 mIU/ml) compared to the control rats (20.4 ± 0.3; 17.6 ± 0.2). There was no significant difference in FSH concentrations at proestrus and diestrus amongst the three groups (Figure 1).

LH concentration is significantly reduced (p<0.05) during the estrus and metestrus phases in Alloxan diabetic rats (15.9 ± 0.1; 15.0 ± 0.2 mIU/ml) and insulin resistant rats (16.1 ± 0.1; 15.2 ± 0.2 mIU/ml) compared to the control rats (18.0 ± 0.2; 17.2 ± 0.1). There was no significant difference in LH concentrations at proestrus and diestrus compared to the control (Figure 1).

Estradiol concentration is significantly lower (P < 0.05) in all the experimental groups compared to the control in all the phases (estrus, metestrus, diestrus and proestrus). Serum estradiol is significantly higher (P < 0.05) in insulin resistant rats during estrus (55.8 ± 0.2pg/ml), metestrus (56.9±0.2pg/ml) and proestrus (56.0± 0.3 (pg/ml) compared with the Alloxan diabetic rats during estrus (40.5 ± 0.2 pg/ml), metestrus (48.8 ± 0.2 pg/m)) and proestrus (39.2 ± 0.2 pg/ml) Figure 1.

Progesterone concentration is significantly reduced (P < 0.05) during the estrus, metestrus, and proestrus phases in Alloxan diabetic and insulin resistant rats compared to the control. No significant difference in progesterone concentration during diestrus phase in Alloxan diabetic group (30.9 ± 0.1) and insulin resistant group (31.2 ± 0.5) compared to the control (32.0 ± 0.3ng/ml) Figure 1.

DISCUSSION

There was no significant difference in body weight amongst the three groups till the 4th week, after which it began to significantly decline (P < 0.05) in both experimental groups compared to the control group till the end of experiment (Table 1). The body weight pattern in this study is consistent with our earlier observations in both male and female rats (Arikawe et al, 2008; Arikawe et al, 2006) that body weight in insulin resistant diabetic rats begins to decline from the 4th week. The result on body weight is also in line with the view of Catena et al, (2003), who reported that fructose feeding has no significant effect on body weight of virgin female rats in the first two weeks of feeding.

Our result on fasting blood glucose concentration (Table 2) is in line with previous reports on female rats (Sheweita et al, (2002); Thirunavukkarasu et al, (2003),

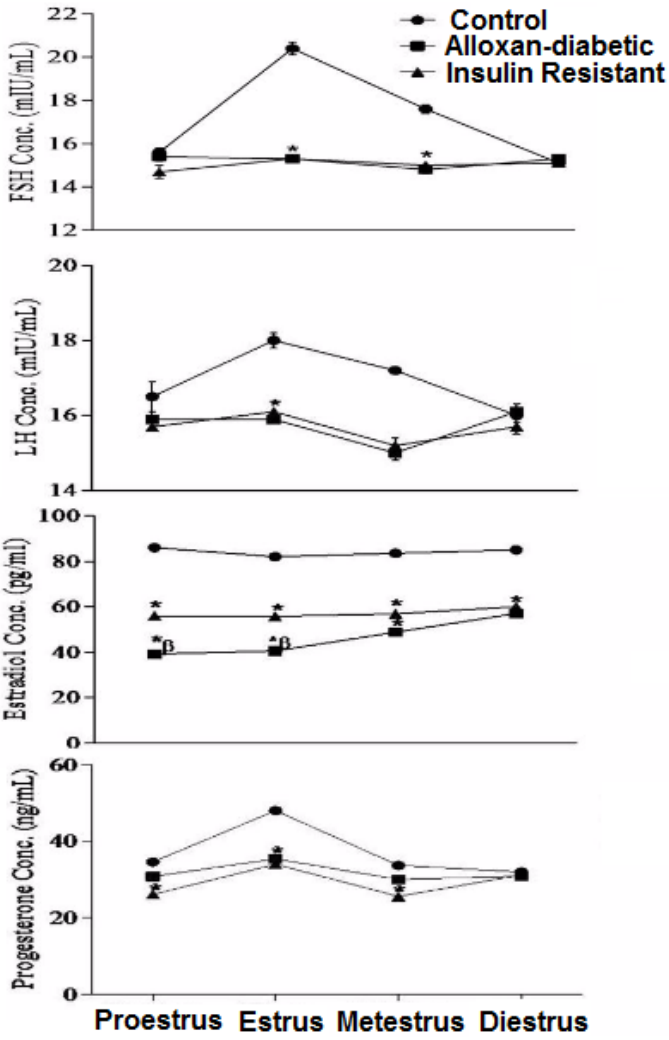

Fig.1: Hormone profile of Alloxan-induced diabetic and insulin resistant rats across the phases of the estrous cycle. *shows significant difference compared with control at P<0.05, β shows significant difference compared with insulin resistant at P<0.05.

Arikawe et al, (2012). Though the duration of the estrous cycle was not significantly different amongst the 3 groups, the phases were altered in the experimental groups with a significantly reduced duration of estrous phase thus reducing the period of "heat" which is the time of ovulation. Impairment of fertility due to impaired spermatogenesis was reported earlier in male rats by Arikawe et al, (2006). According Ding et al, (2015) diabetes may influence epigenetic modification during sperm spermatogenesis.

The experimental groups also had a significantly reduced ($P < 0.05$) number of ova when compared with the control group. This also is consistent with the view of Kovacs et al, (2003); Jawerbaum et al, (1996) and also correlates with the results on reduced LH and FSH concentrations. On the other hand, atretic follicles were significantly higher ($P < 0.05$) in the experimental groups compared to the control group. This is line with the reports of Cox et al, (1994) and Foreman et al, (1993). Tobias et al, 2015 had reported that subfertility due to ovulation disorders is strongly related to a higher risk of diabetes mellitus.

The results on hormonal concentrations during specific phase of the cycle are quite interesting. Normally pituitary gonadotrophin secretion drives follicular maturation and estradiol secretion such that LH and FSH levels begin to increase just after diestrus. FSH stimulates development of the zona granulosa and triggers expression of LH receptors by granulosa cells. LH initiates the synthesis and secretion of androstenedione which is utilised by granulosa cells as substrates in the synthesis of estradiol.

The increase in estradiol during proestrus (Smith et al, 1975) morning initiates several characteristic morphological changes in the uterus and vagina. It also suppresses release of LHRH by the hypothalamus, as well as directly inhibiting pituitary secretion of both LH and FSH. This is the negative feedback control of estradiol on pituitary LH and FSH. Estradiol levels rise during the morning, peak around midday and then fall during the afternoon of proestrus until estrus and gradually rise again with fluctuations during metestrus and diestrus (Smith et al, 1975).

Once estradiol peak is reached its inhibition of LHRH and gonadotrophin secretion ceases. At this point, estradiol starts promoting both hypothalamic LHRH release and anterior pituitary responsiveness to LHRH. This positive feedback modulation of hypothalamic-pituitary function results in a preovulatory LHRH surge and corresponding LH surge. Results on estradiol concentration of the control rats in each phase of estrous cycle follow this normal pattern described above, but was significantly reduced in experimental groups. This is in line with a previous report by Chabrolle et al, (2008).

The LH surge which closely follows the estradiol peak, occurs during the afternoon of proestrus and triggers ovulation later (Smith et al, 1975) at estrus. FSH levels peak twice in the rat; the first (preovulatory) peak is LHRH-dependent and occurs in concert with the LH peak. This is followed by a second (postovulatory) rise in FSH that occurs at the time of ovulation or shortly after. LH and FSH levels then gradually decline during metestrus and diestrus phases of the estrous cycle. The results on LH and FSH concentrations in each phase of estrous cycle in this study are in line with this pattern. There was no significant difference in LH and FSH concentrations at proestrus and diestrus phases of the estrous cycle in the three groups. On the other hand, LH and FSH concentrations were significantly lower ($P < 0.05$) at estrus and metestrus phases of the estrous cycle in the experimental rats. This is in line with previous reports (Ballester et al, 2007, Kovacs et al, 2003, Babichev et al, 1994, Steger, et al, 1993) that diabetic rats have decreased LH and FSH levels.

Progesterone levels start to increase during proestrus and peak during ovulation. Like oestrogen, progesterone feedback control of hypothalamic-pituitary function may be negative or positive, depending on the stage in the estrous cycle. Following ovulation, progesterone synergises with oestrogen to inhibit gonadotrophin secretion. Conversely, rising progesterone levels during proestrus trigger hypothalamic LHRH secretion, stimulating gonadotrophs in the anterior pituitary and reinforcing the preovulatory LH surge.

Our results on progesterone concentrations in each phase of the estrous cycle follow this pattern described above. , Progesterone levels was significantly reduced ($P < 0.05$) during the proestrus, estrus and metestrus phases of the estrous cycle in the experimental groups This is in line with a previous report Chabrolle *et al.*, (2008).

CONCLUSION

The results of this study showed that both Alloxan-diabetes and insulin resistant diabetes distort the estrous cycle pattern, reducereduce significantly the "fertile period" by acting on the pituitary-ovarian axis reducing the gonadotropins and the ovarian hormones.

These data open a new field to study hormonal profile in diabetic female rats during each phase of the estrous cycle and also to investigate the hypothalamo-pituitary-ovarian axis in insulin resistant diabetic female rats.

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