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Ghrelin-like effects induced by feed restriction and clove supplementation in female *sprague dawley* rat on estradiol and progesterone level during estrous cycle.

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Ghrelin is a 28 amino acid peptide. It is mainly secreted by gastric X/ A- like cells and stimulated by food deprivation. Serum ghrelin level was observed to increase by fasting. The potential effect of ghrelin in controlling gonadal functions has received attention recently. The Objective of this work is to study the effect of chronic food restriction and clove supplementation on estradiol and progesterone level during estrous cycle in adult female rats. The present study was carried out on 64 adult female Sprague Dawley rats equally divided into 4 groups: Group I (control group) fed on a standard ration (15 g/animal/day) Group (II) (Feed Restricted Group) received 50% of the daily standard ration (7.5g/ animal /day). Group (III) were fed on standard rat ration (15 g/animal/day) to which clove flowers powder was added at rate of (15 mg/kg/day) and Group (IV) were fed on standard rat ration (15 g/animal/day) to which clove flowers powder was added at rate of (30 mg/kg/day). Mean body weight of food restricted rats decreased during the period of the experiment, while increased in clove supplemented groups than the control group. Food restriction and clove-supplemented groups produced significant decrease in serum Estradiol and Progesterone level. Ghrelin could be one of the hormones responsible for the suppression of female reproductive axis in case of negative energy balance. Thus, ghrelin could provide a link between energy homeostasis and reproductive capacity in adult female rats.

Keywords: Ghrelin, Food Restriction, Clove, Estradiol, Progesterone, Rats.

INTRODUCTION

Ghrelin hormone, the hunger hormone, is a peptide hormone produced by gastric cell that act as a neuropeptide in the central nervous system (Dickson et al., 2011). Ghrelin increase food intake by stimulating hypothalamic neuropeptides in the brain. on the contrary, after feed consumption lower GI tract secrete gut hormone peptide YY (PYY) suppress food intake to mediate satiety (Chen, 2004). Forasmuch PYY motivate reproductive hormone release, ghrelin has been proved its inhibitory effect on reproductive function (Dhillo et al., 2007). Besides regulating appetite and energy balance, ghrelin has a significant impact on controlling the function of gonads (Burger and Berner, 2014).

Reproductive function is tightly regulated by nutritional status. Indeed, it has been well described that under nutrition can lead to sub fertility or infertility (Rance et al., 1994). Many studied suggested that feed restriction can produce suppression in pulsatile gonadotropin

secretion which reflexed on induction abnormal estrous cycles and inhibition reproductive behavior (Wade and Schneider, 1992).

Recently, several investigations elicited that ghrelin act as possible regulators of both homeostasis and reproductive function. It acts as a pleiotropic modulator of multiple endocrine and nonendocrine functions including feed intake and energy balance (Fabio et al., 2006). Data has been drawn in last few years have proved that ghrelin has an inhibitory effect on estradiol and progesterone secretion thought-out the estrus cycle (Rak and Gregoraszczuk, 2008).

Ghrelin hormone was also identified in some appetite-stimulating plants like clove (Syzygiumaromaticum) (Aydin et al., 2011).

The aim of the present work was planned to study the effect of feed restriction which may lead to ghrelin secretion and clove supplementation which was proved to induce ghrelin –like effect on estrous cycle and reproductive hormone level in female Sprague Dawley rat.

MATERIALS AND METHODS

Animals and Housing

Eighty sexually mature, healthy female Sprague Dawley rats, with an initial body weight of 130 ± 10 g, were obtained from the Animal House Colony of Abou-Rawish, Giza, Egypt. The animals were caged at the animal house of physiology department-Cairo University under standard conditions (humidity, 60-65%; temperature, 23 ± 2 ºC; automatic ventilation; photoperiod 12L: 12D and free access to water). The rats were fed on a standard granulated rat feed mixture (15 g/animal/day) containing 86% dry matter, 18% crude proteins, 2.5% fat, 5.5% fiber, vitamins and minerals. All animal procedures were performed after approval from the Ethics Committee of Cairo University- Institutional Animal Care & Use Committee (CU-IACUC) and in accordance with the recommendations for the proper care and use of laboratory animals (CU-II F5318). Before initiation of feeding manipulation, all animals were adapted for 15 days to receive their daily feed ration. Thereafter, 64 female which showed 2 successive regular 4- or 5-days estrus cycle were selected and randomly divided into 4 equal groups of 18 rats each with 3 replicate cages with 6 rats in each. The study was carried out for 4 weeks. The rats were treated as follows: The 1st group (I) was used as (control group) and fed on a standard ration (15 g/animal/day) (Sirotkin et al., 2008). This meal dosage was empirically calculated as equivalent to the standard daily feed intake of adult female rats fed ad libitum. The 2nd group (II) (feed restricted group) received 50% of the daily standard ration (7.5g/ animal /day). The 3rdgroup (III) were fed on standard rat ration (15 g/animal/day) to which clove flowers powder was added at rate of (15 mg/ kg/ day). The 4thgroup (IV) were fed on standard rat ration (15 g/animal/day) to which clove flowers powder was added at rate of (30 mg/ kg/ day). Dry Clove flowers was obtained from local market and ground in powder form to be used in this study. It was mixed with ration at 2 different doses, (15 mg/ kg/ day) and (30 mg/ kg/ day), respectively according to Mishra and Singh (2008) after conversion of mouse dose to rat dose by using Freireich et al., (1966) formula.

Body weights

Body weight was recorded once a week. To minimize the potential influence of immediate feed and/or water consumption on actual body weights, access to feed and water was prevented 12 h before weighing of animals.

Collection of samples:

Blood samples:

After the end of 2nd and 4th weeks of the experiment all rats were fasted overnight. In the morning, vaginal smears were obtained from each rat of the 4 groups and the phase of estrus cycle was determined. Vaginal smear was performed by pipette smear technique (Long and Evans, 1922). Blood samples were collected from each rat by orbital sinus puncture using heparinized capillary tubes under diethyl ether anesthesia according to Schermer (1967). Collected blood was left to clot, centrifuged for (10 min at 4ºC 3000 rpm) and sera were aspirated and stored at -20ºC until assays were carried out.

Biochemical Assay for female reproductive hormones

The levels of estrogen, progesterone in serum of rats during the different phases of estrus cycle were determinate by double antibody immunoassay using specific ELISA kits purchased from EIAab Company and performed by using Elisa analyzer according to the methods described by Manufacture instruction.

Statistical methods

The data were analyzed using version 11.0 of the computer-based statistical product and service

solutions (SPSS, 2001, Chicago, IL, USA). All the data are expressed as Mean ± standard error of mean. Analysis of the data was done using two-way Anova to detect the significant ($p < 0.05$) difference among the studied groups. A level of $p<0.05$ was defined as statistically significant ($p < 0.05$).

RESULTS

Clear changes in estrus cyclicity were recorded in feed restricted group. These changes represented by onset of prolonged diestrus at the end of the experiment. Relying on this, the ovaries of these animals showed mature follicles and necrotic corpora lutea.

Effect of Feed Restriction and Clove supplementation on body weight in (gm) of Adult Female Rats during the experiment:

The results depicted in Table (1) showed the effect of feed restriction (FR) and clove supplementation on the body weight of adult female rats. The results releveled that there was a significant ($p < 0.05$) increase in the body weight in the control (I) and clove supplemented groups III and IV throughout the experimental period. On the other hand, feed restricted group which showed a significant ($p < 0.05$) decrease in the body weight when compared with the control group, III and IV throughout the experimental period. While there was a significant ($p < 0.05$) increase administrated with both doses of clove (III and IV group) started after 2 weeks of experiment as compared to the control group.

Effect of Feed Restriction and clove supplementation on Estradiol (E2) level during estrous cycle in rat.

The results summarized in Table (2) showed the effect of FR and clove supplementation on estradiol (E_2) of the adult female rats during 15th day and 30th day of the experiment. Estradiol is significantly ($p< 0.05$) increase during proestrus phase when compared with estrus and diestrus phases in all treated groups and control group. While, feed restriction showed a significant ($p < 0.05$) decrease in estradiol level during 15th day of experiment as compared to the control, while at 30th day of experiment, there were a non-significant decrease in estradiol level when compared with the control group.

Effect of Feed Restriction and clove supplementation on Progesterone (P_4) level during estrous cycle in rat.

The results represented in Table (3) showed the effect of FR and clove supplementation on Progesterone (P_4) of the adult female rats during 15th day and 30th day of the experiment. Progesterone is significantly ($p< 0.05$) increase during proestrus phase when compared with their corresponding estrus and diestrus phases in all treated groups and control group. Feed restricted group during 15th day and 30 day showed a significant ($p < 0.05$) decrease in progesterone level in diestrus when compared with control group and clove groups.

Table (1): Mean ± SE changes in body weight in grams in control, feed restricted rats and clove supplemented groups during the experiment.

Groups / Age (wks.)	Control (Group I)	Feed Restriction (Group II)	Clove (15mg/kg/day) (Group III)	Clove (30 mg/kg/day) (Group IV)
Initial Weight	131.78± 4.9*	130.83 ±4.3*	132.23± 3.9*	133.18 ± 3.2*
Week 1	136.56 ± 5.69^{a*}	125.94 ± 3.65^{b*}	137.19 ± 4.46^{a*}	138.13 ± 4.79^{a*}
Week 2	140.31 ± 4.1^{a*}	118.44 ± 4.2^{b**}	145.19 ± 5.4^{a**}	147.75 ± 3.66^{a**}
Week 3	145.31 ± 5.45^{a**}	109.38 ± 3.44^{b***}	154.44 ± 4.44^{c***}	159.69 ± 3.77^{c***}
Week 4	152.19 ± 5.6^{a***}	97.5 ± 4.3^{b****}	165.19 ± 4.7^{c****}	171.56 ± 5.2^{c****}

- Data are represented as Mean ± SE of 18 rats/group; Significant difference from the control group in the same raw is represented by a, b, c letters at $p <0.05$.
-Significant difference between weeks in the same column is represented by asterisks at $p < 0.05$.

Table (2): Effect of Feed Restriction and clove supplementation on Estradiol (E_2) level in (pg) Mean ± SE during estrous cycle in rat.

Groups / Phases	Control (Group I)	Feed Restriction (Group II)		Clove (15 mg/kg/day) (Group III)		Clove 30 mg/kg/day (Group IV)	
		15-day	30-day	15-day	30-day	15-day	30-day
Proestrus	9.4 ± 1.47^{a*}	6.4 ± 0.61^{b*}	-	7.53 ± 0.46^{ab*}	7.26 ± 0.52^{ab*}	7.4 ± 0.35^{ab*}	7.09 ± 0.40^{ab*}
Estrus	7.18 ± 0.69^{a**}	4.0 ± 0.23^{b**}	-	5.83 ± 0.42^{ab**}	5.3 ± 0.81^{ab**}	5.7 ± 0.21^{ab**}	5.13 ± 0.69^{ab**}
Diestrus	5.4 ± 0.12^{a**}	3.67 ± 0.50^{b**}	4.63 ± 0.78^{ab}	4.6 ± 0.51^{ab**}	4.36 ± 0.53^{ab**}	4.46 ± 0.38^{ab**}	4.13 ± 0.87^{ab***}

-Data are represented as Mean ± SE; Significant difference from the control group in the same raw is represented by a, b letters at p <0.05.
-Significant difference between weeks in the same column is represented by asterisks at p < 0.05.

Table (3): Effect of Feed Restriction and clove supplementation on Progesterone (P_4) level in (ng) Mean ± SE during estrous cycle in rat.

Groups / Phases	Control (Group I)	Feed Restriction (Group II)		Clove (15 mg/kg/day) (Group III)		Clove 30 mg/kg/day (Group IV)	
		30-day	30-day	15-day	30-day	15-day	30-day
Proestrus	12.69 ± 1.37^{a*}	8.34 ± 0.44^{b*}	-	11.28± 0.96^{ab*}	10.88 ± 1.04^{ab*}	11.13± 0.98^{ab*}	10.23 ± 1.05^{ab*}
Estrus	6.37 ± 0.72^{a**}	4.26 ± 0.26^{b**}	-	5.29 ± 0.43^{b**}	4.47 ± 0.43^{b**}	5.05 ± 0.64^{b**}	4.23 ± 0.35^{b**}
Diestrus	7.97 ± 0.15^{a**}	5.56± 1.03^{b***}	5.23± 1.59^{b}	6.53 ± 0.48^{c**}	6.23 ± 0.49^{c**}	6.06 ± 0.54^{c**}	5.77 ± 0.64^{c***}

-Data are represented as Mean ± SE; Significant difference from the control group in the same raw is represented by a, b, c letters at p <0.05.
-Significant difference between weeks in the same column is represented by asterisks at p < 0.05.

Feed restriction group showed significant (p< 0.05) decrease in progesterone level during proestrus and estrus phases when compared with control groups while with non-significant decrease when compared with clove groups.

Moreover, clove supplemented groups showed a significant (p< 0.05) decrease in the level of progesterone during diestrus allover the experimental period when compared with feed restricted group and control groups.

DISCUSSION:

Feed restriction for 30 days lead to irregularity of estrus cycle and ended by a persistent diestrus. The same results were reported by Terry et al., **(**2005) who found that there was strong relationship between the feed restriction and the onset of prolonged diestrus, 60% of the animals subjected to feed restriction (7.5 gm/animal/day) showed increased incidence of the cycling dysfunction and the percentage of animals experiencing prolonged diestrus gradually increased over 2 weeks. The onset of prolonged diestrus was also responsive to feed restriction at the end of their study. Carney et al., (2004) also reported that in animals exposed to severe feed-restriction a marked reduction of LH and FSH secretion was recorded and with sharp decrease in LH plasticity that leads to rapid onset of anestrus in rats.

The present work revealed a significant ($p < 0.05$) reduction in the body weight of feed restricted group as compared with the control group. This finding match with the results of Sirotkin et al., (2008) who reported a significant ($p < 0.05$) decrease in body weight when adult male rats were subjected to 50% restriction in daily feed intake for 20 days. Rather than the FR group which showed decline in body weight, there was a significant ($p < 0.05$) increase the body weight in clove supplemented groups. These results are in consistent with the results of Wahba (2013) who noticed that the addition of clove to broilers feed improved their performance as indicated by increase in body weight, weight gain and feed intake. The author attributed this improvement to the increase in blood ghrelin concentration due to clove supplement this concurred with study performed by Aydin et al., (2011) was designed to determine whether ghrelin is present in the appetite-stimulating plant, clove, they demonstrated that a ghrelin-like substance was present at concentrations of 4070.75 pg/mg in tissues of the flower buds of clove.

Regarding to the reproductive hormones, serum estradiol and progesterone levels were significantly ($p < 0.05$) decreased in feed restricted and clove supplemented groups as compared to control group. This result may be explained on the basis that the granulosa cells act as the main site for ovarian IGF-1 synthesis, reception, and action (Duleba et al., 1997). IGF-1 can increase the activity of gonadotropins and to amplify their steroidogenic production i.e., to promote progesterone and estradiol synthesis (Balasubramanian et al., 1997). IGF-1 can also enhance StAR expression, responsible for the transfer of cholesterol into the mitochondria for steroidogenesis in granulosa-lutein cells (Devoto et al., 1995). As mentioned by (Thissen et al., 1994) IGF-1 system is extremely sensitive to metabolic alterations. So, during hyperghrelinaemia, the first observed change is an increase in IGF-binding protein-1 (IGFBP-1 ,(an inhibitor of IGF-I action) (Colin and Adda, 2015). Through these pathways, hyperghrelinaemia could lead to suppression serum level of E_2 and P_4 in adult female rats (Ahmed et al., 2012). There is also evidence in the literature that injection of ghrelin hormone inhibited the release of E2 and P4 during the estrous cycle through its restrain of LH secretion at all stages of the estrous cycle and direct inhibits aromatase activity thereby decreasing E2 secretion (Fernández-Fernández et al., 2005). In addition, ghrelin could inhibit P4 secretion through mitogen-activated protein kinase (MAPK) and protein kinase A (PKA)-dependent intracellular mechanisms, at the post-receptor level (Sirotkin and Grossmann, 2008).

CONCLUSION

Feed restriction and clove supplementation significantly increase Ghrelin level in rat. Ghrelin has negative correlation with reproductive hormones (estradiol, progesterone) that reflect on suppression the estrous cycle in rat. Feed restriction for long time persist diestrus in rat.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Morsi. A.S performed the experiments and wrote the manuscript, Hodallah. H. Ahmed, Omaima. M. Kandil, Fatma. A Wahba and E. Seifelnasr designed the experiment and reviewed the manuscript. All authors read and approved the final version.

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