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THE EFFECT OF LEPTIN ON THE ONSET OF PUBERTY IN NORMAL PREBUPERTAL FEMALE ALBINO RATS

By

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ABSTRACT

Background: Many studies have focused on leptin, the product of the LEP (ob) gene, searching for a possible link between energy balance and reproduction. **Objective:** Investigating the effect of daily leptin injection on prepubertal female rats and the possible mechanisms by which leptin can affect reproductive axis.

Materials and method: This study was applied on eighty female albino rats weaned after 21 days. They were divided into two equal groups, group I (*GI*, control) was injected with saline 5uL/g and group II (*GII*) injected with leptin in a dose of 5µg/g. The injection was daily, intraperitoneally (i.p.), during the light cycle from 21 days age till one day before sacrification. The animals were assessed daily for body weight and vaginal opening (VO). At 28, 32, 38 and 42 days of age, 10 rats from each group were anesthetized and dissected. Blood was collected from the heart of each dissected animal for E_2 and LH assay. The right ovaries were dissected and fixed in Bouin's solution and stained with Hx & E for histological examination.

Results: Daily i.p. leptin injection (5ug/g) resulted in a statistically significant decrease in body weight, a statistically significant earlier occurrence of VO, a statistically significant increase in serum estradiol (E_2) levels, and early appearance of LH surge in *GII* as compared with *GI*. Also, there was a statistically significant increase in the mean numbers of primary, growing and mature follicles and corpora lutea with earlier occurrence of ovulation in *GII* when compared with *GI*.

Conclusion: Daily i.p. injection of leptin 5ug/g BW to prepubertal female albino rats led to statistically significant decreased body weight and earlier onset of puberty and its manifestation in treated group. Leptin may act on the reproductive axis both centrally and peripherally. Leptin could be a possible link between the body fat and timing of puberty.

INTRODUCTION

Puberty is the result of a complex series of biological processes including activation of the gonadotrophic axis, with increased serum levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH), and the achievement of reproductive capacity. Gonadotrophin-releasing hormone (GnRH), released from the hypothalamus, plays an important role which finally dictate the pulsatile release of LH and FSH from pituitary gonadotrophs (**Navarro et al., 2004**). Although the GnRH is subjected to be influenced by a number of factors, it is unclear whether puberty results from the presence of an activator or the withdrawal of an inhibitor of GnRH neuronal activity (**Terasawa and Fernandez, 2001**).

The dependency of puberty on tissue mass reveals the value of physiological signals from metabolic regulatory tissues to the reproductive axis (**Martin et al.,**

2008). For adipose tissue, the primary signal is leptin (**Rosales et al., 2014**). White adipose tissue (WAT) is the main site of leptin synthesis, but it is now evident that the hormone is also produced in other tissues as brown adipose tissue (**Moinat et al., 1995**), placenta, ovary (**Spicer and Francisco, 1997**) and rat and mouse pituitary glands (**Jin et al., 2000**). Leptin decreases appetite and increases energy expenditure (**Garcia et al., 2015**), stimulates growth hormone secretion (**Allensworth-James et al., 2015**), has antidiabetic effect **(Rachel et al., 2014)**, and plays a role in pregnancy (**Margetic et al., 2002**) and lactation (**Vernon et al., 2002**).

Leptin plays an essential role as a neuroendocrine integrator linking the magnitude of body fat stores to different neuroendocrine axes, including the reproductive system. **(Cecilia et al., 2013).** Whether leptin can affect the onset of puberty or not is a subject of controversy. Some researchers postulated that leptin can regulate the hypothalamic-pituitary-gonadal axis and affect time of onset of puberty (Miguel and Manuel, 2013). Others reported that puberty in female mice is not associated with increase in either body fat or leptin **(Bronson, 2001).** So, this study aimed to clarify the physiological effect of leptin on the onset of puberty in female prepubertal female albino rats, and discuss the possible mechanisms by which leptin engages in the regulation of different elements of HPG axis.

MATERIALS AND METHODS

This randomized control trial study was carried out in Physiology Department, Faculty of Medicine, Sohag University. Eighty prepubertal female albino rats with average body weight of 50-60 g, weaned after 21 days age were used. Obese and underweight pubs were excluded. The animals were obtained from the animal house at Assiut Faculty of Medicine, Assiut University.

The rats were housed in groups of four in metal shoe box cages (20 x 32 x 20 cm) at room temperature and normal light / dark cycles. The rats were fed a standard diet of commercial rat chow and tap water.

The rats were divided into two equal groups: group I (control, *GI*) was injected with saline 5µL/g body weight and group II (*GII*) was injected with leptin, in a dose of 5µg/g body weight. The injection was intraperitoneally (i.p) daily during the light cycle, from 21 days age till one day before sacrification. The body weight of each rat was assessed daily and recorded. The rats were examined daily for the occurrence of vaginal opening (VO).

Ten rats from each group were scarified and dissected after being anesthetized with ethanol at ages of 28, 32, 38 & 42 days successively. Blood samples were collected from the rats' heart, serum harvested and stored at -22°C till the time of hormonal assay. Right ovary was taken from each rat, fixed in Bouin's, processed for paraffin blocks and stained with hematoxylin-eosin (Hx & E) stain for histological examination.

Recombinant leptin from rat, expressed in Escherichia coli, was obtained from Sigma-Aldorich, supplied as a lyophilized powder from 0.2µm filtered solution of PBS, pH 7.4, with no carrier protein. The

contents of the vial was reconstituted by adding 1ml sterile tris HCL, pH 8.0, to prepare a working stock solution of 1 mg/ml. After reconstruction, the vial was stored at 2-8°C according to the product instructions.

Mouse/rat estradiol ELISA from Calbiotech, Inc (CBI) estradiol (E2) ELISA Kit was used for the quantitative determination of Estradiol (E2) concentration in mouse/rat serum and plasma. Rat LH ELISA Kit (S-type) from Biovendor research and diagnostic products were manufactured by Shibayagi Co., Ltd. was a sandwich ELISA system for quantitative measurement of rat LH. The kits were used according to the product instructions.

Data were compared between two groups using unpaired t- test. Data were expressed as mean ±SD. A two-tailed $P < 0.05$ was considered significant in all the statistical tests. Statistical analysis was performed by SPSS version 17.

RESULTS

Effect of leptin hormone on the body weight: There was a statistically significant increase in rats' weight with time in group I starting at initial weight of 58.5±1.7 g at age of 21 days reaching 124±2.5 g at age of 42 days, and also in group II starting at initial weight of 58.6±1.7 g at 21 days reaching at age 42 days to 118.4±1.6 g. On comparing weights of group II with those of group I, there was statistically significant decrease in body weight in rats of group II (71.2±2.5, 83.6±2.7, 104.8±1.3, 118.4±1.6 g at ages of 28, 32, 38 and 42 days respectively) versus (73.8±3.2, 86.7±2.6, 107.4±1.6, 124±2.5 g at ages of 28, 32, 38 and 42 days respectively) in rats of group I (Fig. 1)

Effect of leptin on the timing of vaginal opening (VO): There was a statistically significant earlier appearance of vaginal opening (VO) in group II (mean age was 32.5±2.45 days) when compared with group I (mean age was 35±1 days, fig. 2).

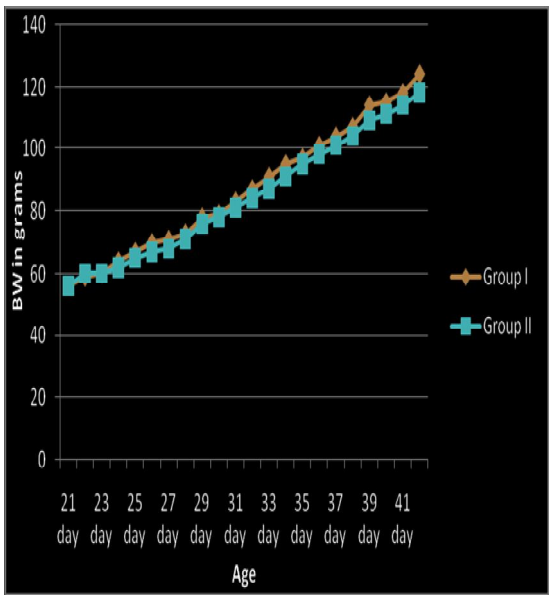

Figure (1): Mean body weight in *GI* and *GII* from initial weight measured at 21 days age after weaning till the day of sacrification.

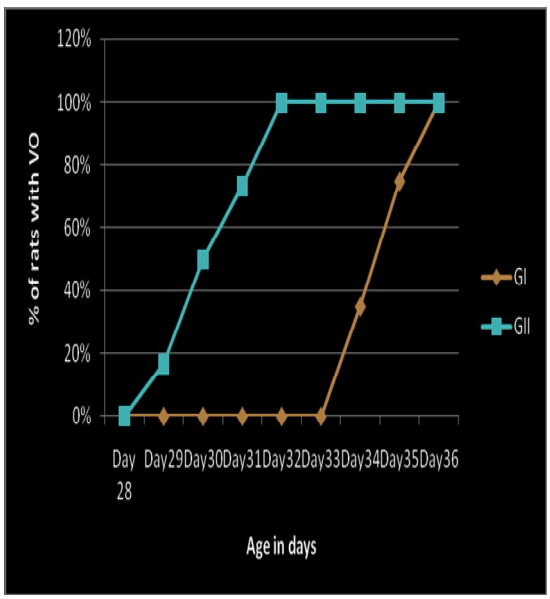

Figure (2): Percentage of rats in *GI & GII* that had vaginal opening in relation to age in days.

Effect of leptin on serum estradiol levels (E_2): There were statistically significant higher levels of serum E_2 at ages of 28, 32, 38, and 42 days in GII (14.94±1.633, 19.58±0.939, 17.23±0.686, 17.88±0.588 pg/ml respectively) versus GI (6.8±0.789, 12.64±1.787, 11.96±0.814, and 12.35±0.793 pg/ml respectively, Fig. 3)

Effect of leptin on serum LH levels: LH level at day 28 in group II was (0.039± 0.005 mlU/ml). This level was statistically significantly higher when compared with LH level in rats of *GI* at the same age (< 0.01mlU/ml), and then LH levels decreased at the following days in *GII*. On the other hand, LH level first increased at age 32 day in *GI* (0.022± 0.002 ml U/ ml), then decreased to (0.012± 0.001 mlU/ml) at ages 38 & 42 days. (Fig. 4).

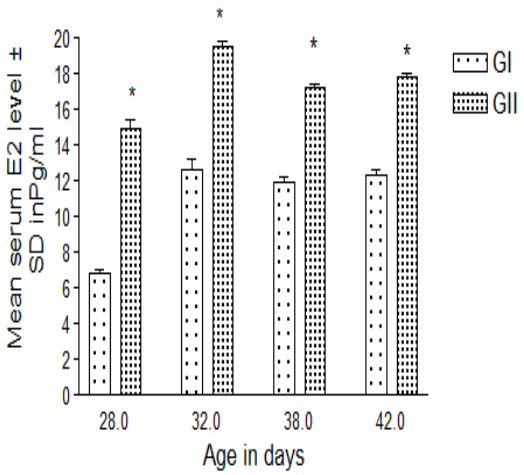

Figure (3): Mean serum estradiol (E_2) levels in Pg/ml ± SD in *GI* & *GII* at ages of 28, 32, 38 and 42 days, * statistically significant difference

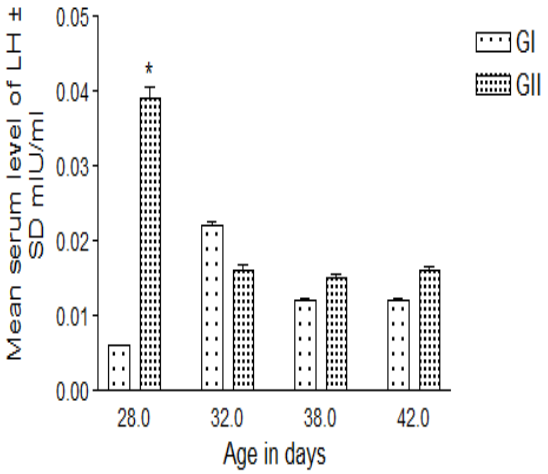

Figure (4): Mean serum LH levels in mIU/ml ± SD in *GI* & *G*II at ages of 28, 32, 38 and 42 days, ***** statistically significant difference

Effect of leptin on histological structure of the ovary: As compared with control group, leptin injection resulted in a statistically significant increase in the mean numbers of primary, growing and mature follicles, and corpora lutea in group II with earlier occurrence of ovulation in rats of group II (Table 1and Fig. 5-12). The change in mean number of growing follicles at day 32 was statistically non significant in both groups.

Table (1): Mean numbers ± SD of primary, growing and mature follicles, and corpora lutea in *GI & GII* at ages of 28, 32, 38 and 42 days.

Group \ Age		Day28	Day32	Day38	Day42
GI	Primary follicles	5±2.1	3±1.2	3±2	4±1.1
	Growing follicles	12±4.3	11±4.1	6.5±3	6.5±3.4
	Mature follicles	0.25±0.5	2±1.3	6±3	3±1
	Corpora lutea	0	0	0	0.25±0.5
GII	Primary follicles	6±2.2*	6±1.3*	9±3.2*	8±1.2*
	Growing follicles	12±4.1	10±2.2	15±4.2*	12±4.1*
	Mature follicles	3±1.2*	7±1.2*	7±1.1*	3±2.3
	Corpora lutea	0	0	5.5±1.5*	2.5±1.2*

*statistically significant

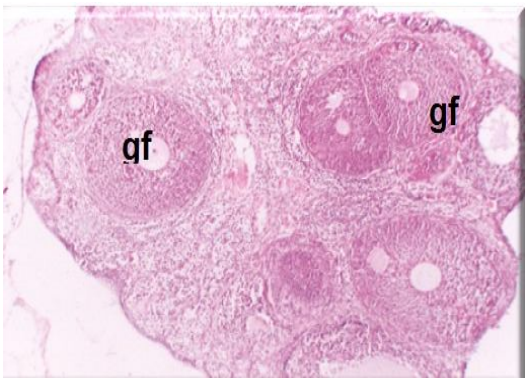

Figure (5): Photomicrograph of the ovary at 28 days age, *GI*, cross-section showing growing follicles (gf) (Hx & E × 10)

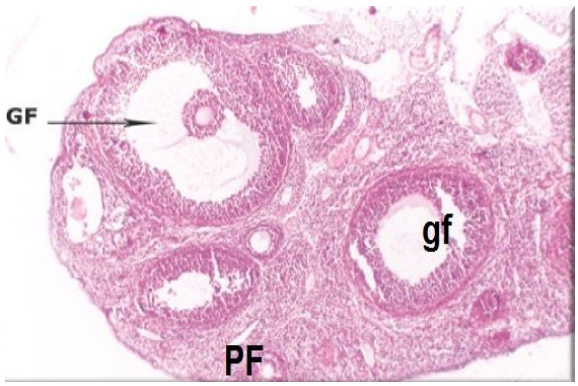

Figure (6): Photomicrograph of the ovary at 28 days age, *GII*, cross-section showing primary follicles (PF), growing follicles (gf) and mature Graafian follicle (GF) (Hx & E × 10).

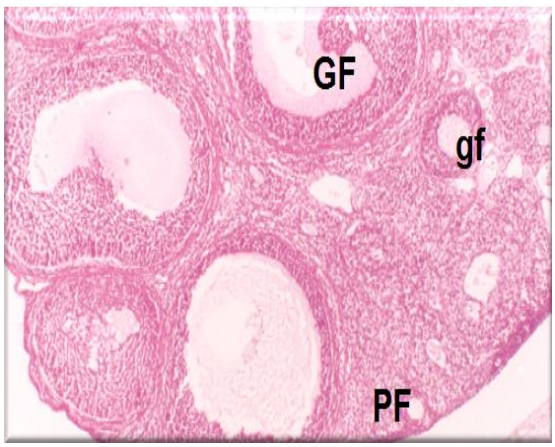

Figure (7): Photomicrograph of the ovary at 32 days age, *GI*, cross-section showing primary follicles (PF), growing follicles (gf) and mature Graafian follicle (GF) (Hx & E × 10).

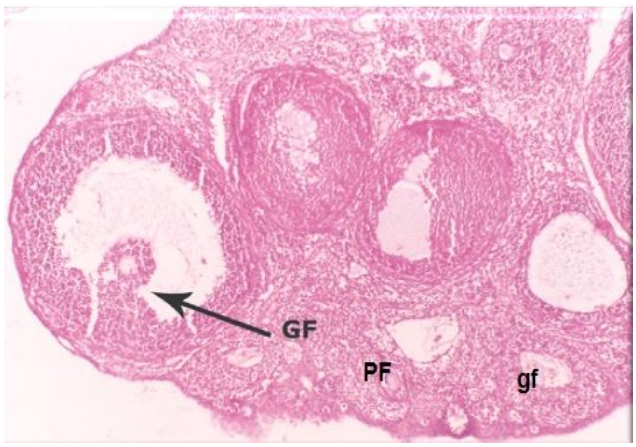

Figure (8): Photomicrograph of the ovary at 32 days age, *GII*, cross-section showing primary follicles (PF), growing follicles (gf) and mature Graafian follicle (GF) (Hx & E × 10).

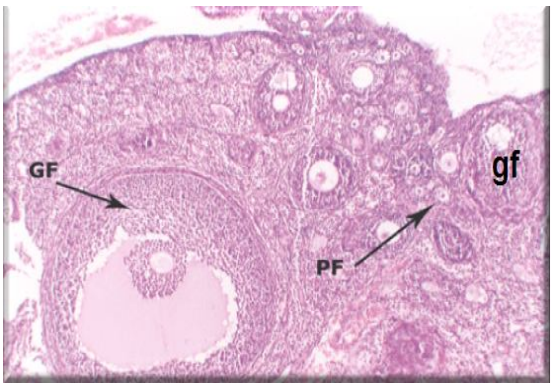

Figure (9): Photomicrograph of the ovary at 38 days age, *GI*, cross-section showing primary follicles (PF), growing follicles (gf) and mature Graafian follicle (GF) (Hx & E × 10).

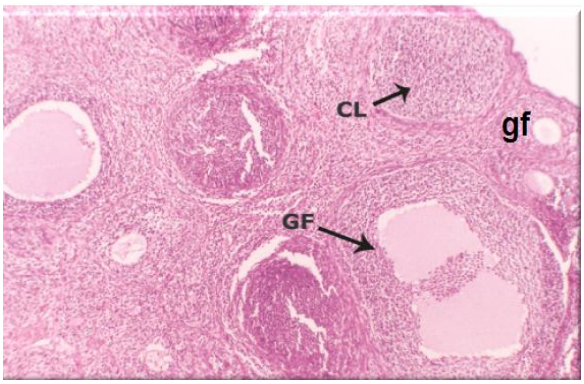

Figure (10): Photomicrograph of the ovary at 38 days age, *GII*, cross-section showing growing follicles (gf) mature Graafian follicle (GF) and corpus luteum (CL) (Hx & E × 10).

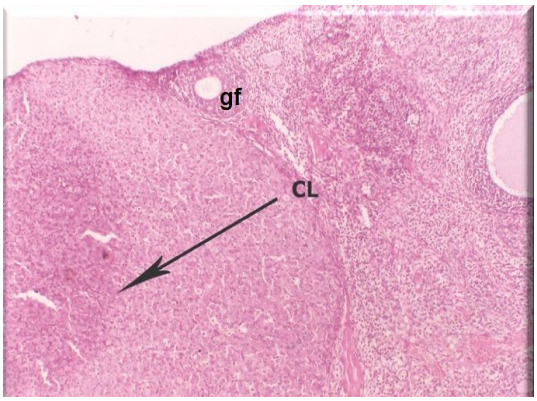

Fig (11), Photomicrograph of the ovary at 42 days age, *GI*, cross-section showing growing follicles (gf), corpus luteam (CL) (Hx & E × 10).

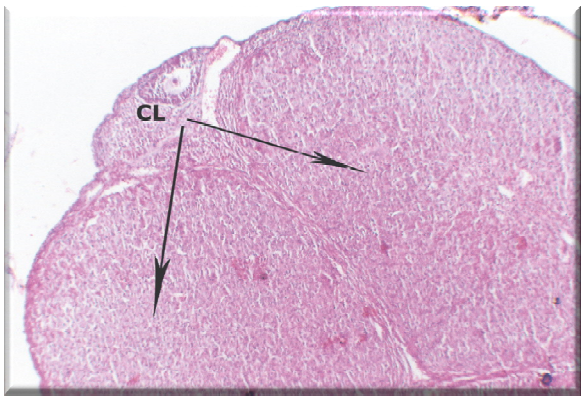

Fig (12), Photomicrograph of the ovary at 42 days age, *GII*, cross-section showing corpora lutea (CL) (Hx & E × 10).

DISCUSSION

Leptin is one of the most important adipose-derived hormones. It plays a key role in regulating energy intake and expenditure (**Brennan and Mantzoros, 2006**). An adequate energy balance is a physiological requirement must be maintained to allow reproduction. Leptin is an essential neuroendocrine integrator linking the magnitude of body fat stores to different neuroendocrine axes including the reproductive system (**Rosales et al., 2014**).

The effect of leptin on reproduction was studied at different doses (**Barkan et al., 2005** and **Ricci et al., 2006**). In this work, the effect of leptin on the onset and manifestations of puberty in normal prepubertal female albino rats was studied in a dose of 5µg/g./day. In this study, leptin was injected during the light cycle. Photoperiod can affect leptin expression through the effect of melatonin which is a photoperiod hormone. It can affect adipose tissue through sympathetic inner-vations projecting directly into fat tissue from suprachiasmatic nuclei that are rich

in melatonin receptors (**Bartness et al., 2001**).

Leptin dose used in this experiment resulted in statistically significant decrease in body weight in treated group as compared with control group. This was agreed with **Heike and Steven (2015)** who reported that leptin injection in leptin deficient cases completely correct obesity. Also **Russell et al. (2015)** reported that hypothalamic leptin gene therapy reduces body weight. However **Bronson (2001)** and **Ravussin et al. (2014)** reported that leptin-infused mice did not change the body weight. Also **Rosenbaum et al. (2005)** reported that administration of physiological or supra-physiological doses of leptin to rodents or humans has little to no effect on energy expenditure or food intake. On the other hand, leptin injected in this experiment showed a statistically significant earlier occurrence of VO in leptin treated group with mean age of 32.5±2.45 versus 35±1 days in control group. This was confirmed by the results of **Donato et al. (2011) and Cecilia et al. (2013).**

Mean plasma E_2 levels measured at 28,32,38,42 days of age, showed a statistically significant higher levels of E_2 in leptin treated rats than in control group, which explains the earlier occurrence of VO and ovulation in the leptin treated group. This was confirmed by the results of **Cheung et al. (2001). Ahima et al. (1997)** reported that leptin injection did not alter serum E_2 concentration until day 26, but increased after that. The increase in plasma E_2 levels in leptin treated group can be explained by both the central and peripheral effects of leptin. Leptin stimulates GnRH release from the hypothalamus, which in turn increase LH and FSH secretion from the pituitary (**Elias and Purohit, 2013**). Peripheral leptin receptors were found on many cells within the ovary including theca, granulosa and oocytes. Leptin targets these ovarian cells to affect steroid genesis and signal transduction (**Silveira et al. 2013**).

In this study, LH hormone was measured at ages of 28, 32, 38 and 42 days. There was a statistically significant increase in LH levels in leptin treated group (0.039 ± 0.006 mIU/ml) versus (< 0.01 mIU/ml) in control group at day 28 age. Then LH levels decreased in the following days till the age of 42 days. On the other hand, LH levels in *GI* was first increased at day 32 (0.022± 0.002 ml U/ ml) then decreased at ages of 38 and 42 days. This pattern of LH in both groups can be explained by the normal physiology of female rats. The onset of puberty in the female rat results from a cascade of events following establishment of a pulsatile luteinizing hormone (LH) release after the fourth postnatal week (approximately thirty days of age) that leads to ovarian maturation **(Andrews and Ojeda, 1981). Ponzo et al. (2001)** reported that leptin increase the plasma levels of LH at 15 and 30 days of age in female rats treated with leptin at a dose of 30µg/kg, (i.p single injection of leptin) 90 minutes before sacrification. **Donato et al. (2011)** and **Kirsz et al. (2014)** reported higher levels of LH in leptin treated mice compared with control group.

Histological examination of the ovaries in this study showed that leptin injection induced earlier and more follicular development as evidenced by

statistically significant increased mean numbers of primary, growing and mature follicles and corpora lutea in *GII* when compared with *GI*. The change in mean number of growing follicles at day 32 was statistically non significant in both groups. **Elshafie et al. (2008)** reported the occurrence of maturational changes in ovarian components from 26 days of age in leptin treated female rat with ovulation occurring at the age of 30 days. This also was agreed by **Donato et al. (2011)**, who reported earlier ovulation in leptin treated groups. **Castaneda et al. (2013)** also reported that, there was a positive relationship between the number of follicles and plasma leptin concentration in cows. **Barkan et al. (2005)** reported that treatment of normal and hypogonadal mice with leptin led to induction of follicular development and corpora lutea formation, and postulated that leptin may replace HCG as an inducer of ovulation. On the other hand, **Ricci et al. (2006)** reported that high levels of leptin inhibited ovulation in female rat aged 26-28 days. **Duggal et al. (2000)** also contradict our results and they demonstrated that excessive leptin is inhibitory to ovulation. This difference in results may be due to species or dose difference.

Considered one of the major mechanisms whereby leptin affecting the timing of puberty is via its ability to regulate the hypothalamic Kiss1 system (**Miguel and Manuel, 2013**). Also, there may be an important area for the permissive effect of leptin on timing of puberty and reproduction outside the Kiss1-enriched hypothalamic nuclei; the ventral premamillary nucleus. This nucleus was considered a connecting point for the transmission of environmental cues to the reproductive centers well before the identification of the role of kisspeptins (**Elias and Purohit, 20013**).

As additional note to the mechanisms by which leptin can affect timing of puberty, is the emergence of experimental evidences suggesting that leptin can act at levels of the gonadotropic axis other than the hypothalamus, including the pituitary and the gonads. At the pituitary level, leptin has been shown to stimulate LH and FSH secretion (**Elias & Purohit, 20013** and **Dagklis et al., 2015**). **Laurent et al. (2014)** reported that, leptin treatment activates the gonadotropic axis by inducing LH pulsatility and ovulation in nutrition-restricted animals and women with hypothalamic amenorrhea. In addition, leptin may have the ability to mimic LH in inducing follicular rupture and ovulation (**Barkan et al., 2005**). In the gonads, leptin has a stimulatory effect on ovarian cycle proteins and apoptotic peptides. So leptin control folliculogenesis and have a remodeling effect on proliferation and apoptosis of ovarian cells. Leptin stimulates steroid genesis and up regulates E2 receptors in the ovary (**Silveira et al., 2013**).

CONCLUSION

Leptin hormone injected in a dose of 5 ug/g BW in prepubertal female albino rats decreased body weight and accelerated the onset and manifestations of puberty, as evidenced by earlier onset of vaginal opening, earlier onset of ovulation, increased levels of E_2 and earlier occurrence of LH surge in the treated group. Leptin could serve as a metabolic signal between nutritional status and the reproductive axis.

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تأثير هرمون لبتن على بدء البلوغ فى إناث الجرذان البيضاء

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خلفية البحث: ركزت العديد من الدراسات فى السنوات الأخيرة على هرمون لبتن المنتج من جين السمنة، من أجل إيجاد رابط محتمل بين توازن الطاقة وعملية التكاثر داخل الجسم.

الهدف من البحث: تحرى تأثير الحقن اليومى بهرمون لبتين على إناث الجرذان فى مرحلة ما قبل البلوغ و مناقشة الآليات الممكنة التي قد يؤثر بها اللبتن علي محور التكاثر.

مواد و طرق البحث: أجريت هذه الدراسة على 80 من إناث الجرذان البيضاء بعد فطامها فى عمر 21 يوم. و قسمت الجرذان إلى مجموعتين متساويتين. حقنت المجموعة الأولى (المجموعة الضابطة) يوميا داخل البريتون بمحلول الملح بجرعة 5 ميكروليتر/ جم وزن، بينما حقنت المجموعة الثانية بهرمون لبتين تركيز 5 ميكروجرام / جم وزن. و قد وزنت الجرذان وفحصت يوميا لتحديد النضج المهبلى، و عند أعمار 28 و 32 و 38 و 42 يوما علي التوالي تم تخدير ثم تشريح 10 من الجرذان من كل مجموعة، و جمعت عينات الدم من القلب من كل جرز مشرح لقياس نسبة هرمونى الإستروجين والهرمون المصفر فى مصل الدم، وقد تم تشريح المبيض الأيمن من كل جرذ مشرح و صبغه بصبغة الهيماتوكسيلين و الإيوسين ثم فحصه هستولوجياً.

النتائج: تبين أن وزن الجرذان فى كل من المجموعتين قد ازداد مع الوقت ولكن كان هناك نقص ذو دلالة إحصائية في أوزان جرذان المجموع الثانية عند مقارنتها بأوزان جرذان المجموعة الضابطة. كما حدث النضج المهبلى بشكل أسرع فى جرذان المجموعة الثانية مقارنةً بجرذان المجموعة الضابطة.

وقد لوحظت زيادة ذات دلالة إحصائية في نسبة هرمون الإستروجين و أيضا ظهور مبكر لحدوث الإندفاع في الهرمون المصفرفى مصل الدم في المجموعة المحقونة باللبتن عند مقارنتها بنتائج المجموعة الأولي.

وعند فحص المبايض هستولوجيا وجدت زيادة ذات دلالة إحصائية فى أعداد الحويصلات الأولية والثانوية والناضجة فى جرذان المجموعة الثانية عند مقارنتها بجرذان المجموعة الأولي ، ولوحظ أن التبويض حدث مبكرا فى جرذان المجموعة الثانية مقارنة بالمجموعة الأولي.

الإستنتاج: الحقن اليومى لإناث الجرذان البيضاء بهرمون لبتن أدى إلي نقص ذو دلالة إحصائية في أوزان الجرذان كما أدي إلى الإسراع بتوقيت ومظاهر عملية البلوغ، وهذا الهرمون قد يؤثر مركزيا أو طرفيا علي محور التكاثر. وعلى هذا فهرمون لبتن هو رابط محتمل بين دهون الجسم وتوقيت البلوغ .