Effects of Peripheral Administration of Kisspeptin on Pubertal Maturation and Serum Leptin Levels in Female Rats

Dişi Ratlarda Pubertal Olgunlaşma ve Serum Leptin Seviyeleri Üzerine Periferik Kisspeptin Uygulanmasının Etkileri

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ABSTRACT Objective: The aim of this study was to investigate the effects of exogenous kisspeptin on pubertal maturation in immature female rats. **Material and Methods:** Wistar female rats were weaned when they were 21 days old. The rats were divided into two groups. Controls (n=10) received saline only (1 ml/kg). Experimental rats (n=9) were intraperitoneally injected with daily 100 nmol kisspeptin-10 between 09.00h-10.00h a.m. starting from the day 26. Body weight and food intake were daily determined, and vaginal opening (VO) was daily monitored starting from day 26. The animals were decapitated when the first diestrus was determined by vaginal smears. Upon decapitation, serum was separated and stored at -20 °C until measurement of leptin, luteinizing hormone (LH) and estradiol. Uterus and ovaries were dissected out and weighed. **Results:** Intraperitoneal injection of 100 nmol kisspeptin-10 did not change median VO ages. There were no differences in food intake, and percentages of body weight change, between control and kisspeptin groups during the experimental period. Kisspeptin administration elicited significant ($P<0.01$) increases in uterus weight over control values. Serum leptin levels were significantly lower ($P<0.05$) in kisspeptin-treated group compared to vehicle group. Kisspeptin administration increased ($P<0.05$) serum LH and estradiol levels. **Conclusion:** Chronic peripheral administration of kisspeptin-10 does not advance puberty onset as estimated from the date of vaginal opening, but potentiates other conventional indices of maturation of reproductive axis such as elevated uterine weight and increased serum levels of LH and estradiol.

Key Words: Puberty; kisspeptin-10; leptin

ÖZET Amaç: Çalışmanın amacı ekzojen kisspeptinin immatür dişi ratlarda pubertal olgunlaşmaya etkilerini araştırmaktır. **Gereç ve Yöntemler:** Wistar dişi ratlar 21 günlükken sütten kesildiler. Ratlar iki gruba ayrıldılar. Kontrollere (n=10) yalnızca izotonik sodyum klorür verildi (1 ml/kg). Deney ratlarına (n=9) 26. günden itibaren başlayarak günlük 100 nmol kisspeptin her sabah 09.00 – 10.00 arasında intraperitoneal olarak enjekte edildi. Kilo ve gıda alımı ile vajinal açılma (VA) 26. günden itibaren günlük olarak izlendi. Vajinal smear ile ilk diestrus geliştiğinde hayvanlara dekapitasyon uygulandı. Dekapitasyon sonrası elde edilen serum leptin,luteinizan hormon (LH) ve estradiol bakılıncaya kadar ayrılarak -20 °C'de saklandı. Uterus ve overler diseke edilerek tartıldılar. **Bulgular:** İntraperitoneal 100 nmol kisspeptin-10 enjeksiyonu ortalama vajinal açılma yaşını değiştirmedi. Deney süresince gıda alımındaki faklılıklar ve vücut ağırlığı değişim yüzdesi yönünden kontrol grubu ile kisspeptin grubu arasında farklılık bulunamadı. Kisspeptin uygulaması kontrol grubuna göre anlamlı düzeyde ($p<0.01$) uterus ağırlığında artış yönünden farklılık oluşturdu. Serum leptin düzeyleri kisspeptin grubunda kontrol grubuna göre anlamlı düzeyde daha düşük bulundu ($P<0.05$). Kisspeptin uygulaması serum LH ve estradiol düzeylerini artırdı ($P<0.05$). **Sonuç:** Kronik periferik kisspeptin uygulaması vajinal açılma zamanı ile belirlenen puberteye ulaşım zamanını etkilememekle birlikte, üreme aksının uterus ağırlığında artış, LH ve estradiol seviyelerinde artış gibi konvansiyonel göstergelerini potansiyalize etmektedir.

Anahtar Kelimeler: Puberte; kisspeptin-10; leptin

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Kisspeptins, which are alternatively called as metastin since they were originally identified as products of metastasis suppressor gene KiSS-1[1], are the natural ligands for the G protein-coupled receptor 54 (GPR54).[2-4] Kisspeptins are termed kisspeptin-10, -13, -14 and-54 in accordance with their number of constituent amino acids. The decapeptide kisspeptin-10 which is shared by all the members of kisspeptin family is required for biological activity.[2] Although previous studies implicated the kisspeptins as anti-metastatic factors, recent studies have focused on their exciting role in puberty and other aspects of reproduction. GnRH neurones have been shown to express GPR54 receptor[5] through which kisspeptins activate GnRH secretion.[6] Mutations in GPR54 are associated with sexual immaturity and infertility in humans and rodents.[7-10] Kisspeptin[11] or GPR54 knockout mice[6] are found to be infertile. Kisspeptins are reported to be the most potent activators of hypothalamus-pituitarygonadal (HPG) axis to date.[12] They potently elicit GnRH release and LH secretion even at the pre-pubertal periods.[13] Central or peripheral kisspeptin administration stimulates gonadotropic axis.[14-16] Chronic central administration of KiSS-1 peptide to immature female rats was reported to induce the precococious activation of gonadotrophic axis,[15] and peripheral injection of kisspeptin was shown to significantly increase plasma LH levels.[16] All these findings suggest that kisspeptin/GPR54 system is very important in fertility control, and kisspeptin is the main triggering factor for puberty onset.

In the present experiment, peripheral administration of kisspeptin on puberty onset was investigated in immature female rats. Although central administration of kisspeptin has been shown to advance puberty onset in immature female rats,[15] it is not known whether chronic peripheral administration of kisspeptin has a similar effect on puberty onset. For this aim, kisspeptin-10 was peripherally given to immature female rats until the beginning of puberty. Vaginal opening, ovarian and uterus weights, serum LH and estradiol levels were determined as the signs of pubertal development. Although there are many studies on the modulatory effects of leptin on kisspeptin, there is no study on the effect of kisspeptin on leptin secretion. Therefore, serum leptin levels were also measured following peripheral administration of kisspeptin. Investigating peripheral effects of kisspeptin on pubertal maturation may be useful for its possible therapeutic potential.

MATERIALS AND METHODS

ANIMALS AND DRUGS

Wistar female rats were used in the study. The day the litters were born was considered as day of 1 of age. They were housed under constant conditions of temperature (22 °C) and light (12 h light/ 12 h dark from 07.00 h). They were weaned on day 21, and accomodated individually after then. Food and water were supplied ad libitum. The experimental protocol was approved by the Firat University Ethical Committe, and carried out according to "Guide for the Care and Use of Laboratory Animals www.nap.edu/catalog/5140.html). Kisspeptin-10 was obtained from Phoenix Pharmaceuticals Ltd (Belmont, CA, USA).

EXPERIMENTAL DESIGN

The rats were divided into two groups. Controls (n=10) received saline only (1 ml/kg). This group will be referred as "Vehicle". Experimental group (n=9), which will be referred as "Kisspeptin", was intraperitoneally injected with daily 100 nmol kisspeptin-10 between 09.00h-10.00h a.m. Body weight and food intake were daily determined, and vaginal opening (VO) was daily monitored starting from day 26. The animals were decapitated when the first diestrus was determined by vaginal smears. Upon decapitation, serum was separated and stored at -20 °C until measurement of leptin, LH and estradiol. Uteri and ovaries were dissected out and weighed.

HORMONE ASSAYS

Serum leptin, LH and estradiol levels were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturers' (LINCO Research, Cat. # EZRL-83K for serum leptin, Shibayagi C., Ltd., Code No.: AKRLH-010 for serum LH

and BioSource, Cat. # KAP0621 for serum estradiol) instructions. The lowest levels of rat leptin and LH that can be detected by Rat Leptin and LH Elisa Kits used in these assays were 0.04 ng/ml and 0.3 ng/ml, respectively, and minimum detectable concentration of estradiol was 5 pg/ml.

Statistical analysis

Data are expressed as median (min-max). Differences between medians were evaluated using Mann-Whitney U test with Origin 6.0 software (Microcal, Northampton, USA). The difference was accepted as significant when $P< 0.05$.

RESULTS

Intraperitoneal injection of 100 nmol kisspeptin-10 did not change median VO ages, being 38 days in the Vehicle and Kissppetin groups (Figure 1, A), with a median body weights of 87.8g (min- max, 83.8g- 91.6g) and 87.2g (min- max, 80g- 93g). (Figure 1B), respectively. There were no differences in food intake (Figure 2, A) and percentage of body weight change (Figure 2, B) between Vehicle and Kisspeptin groups during the experimental period. As shown in Figure 3, kisspeptin administration elicited significant ($p= 0.001$) increases in uterus weight over Vehicle values, being 143.2 mg/100g BW (min- max, 129-153 mg/100g) and 85.0 mg/100g BW (min- max, 72-97mg/100g), respectively. The median value of ovarian weights increased slightly from 37.1 mg/100g body weight (min-max, 31.6- 54 mg/100g) in vehicle group to 43.5 mg/100g body weight (min- max, 35.4-57.3 mg/100g) in Kisspeptin group (Figure 4), which was

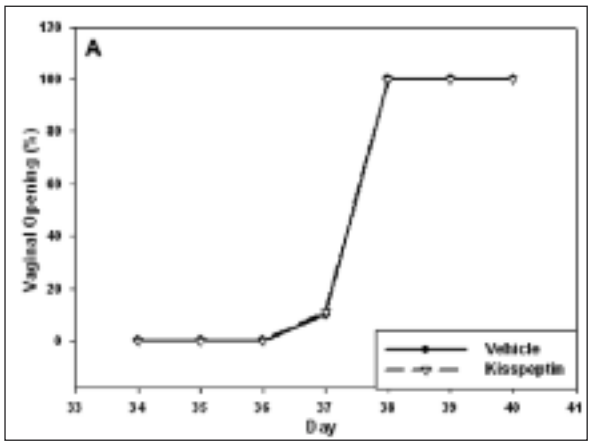

FIGURE 1: A) Cumulative percentage of animals showing vaginal opening.

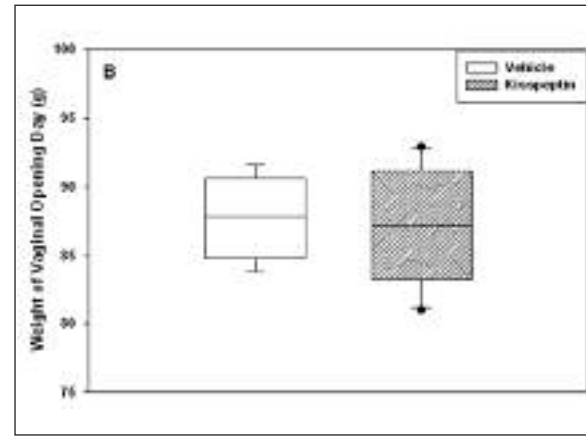

FIGURE 1: B) Median body weights on the day of vaginal opening in vehicle (n=10) and kisspeptin-treated (n=9) animals.

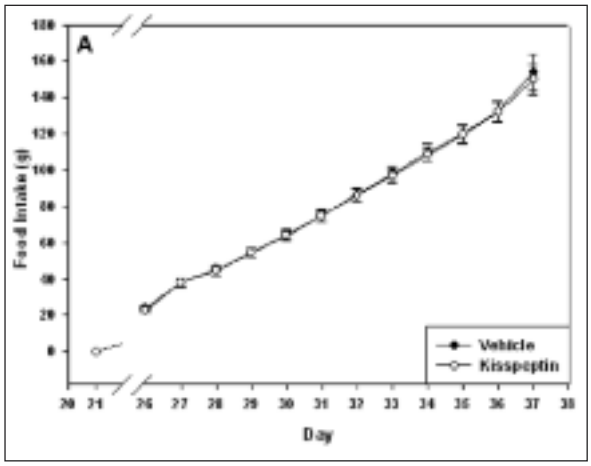

FIGURE 2: A) The daily amount of consumed food.

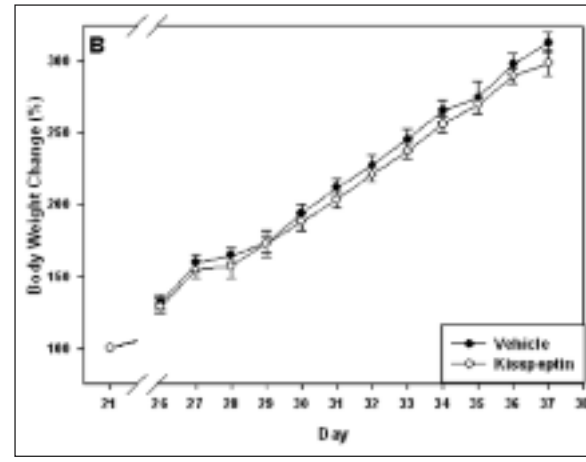

FIGURE 2: B) Body weight changes (%) in vehicle (n=10) and kisspeptin-treated (n=9) animals.

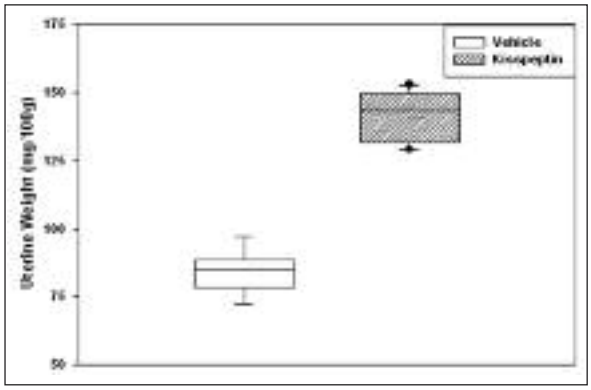

FIGURE 3: Uterus weights in vehicle and kisspeptin-treated animals. P= 0.001 versus vehicle-injected group.

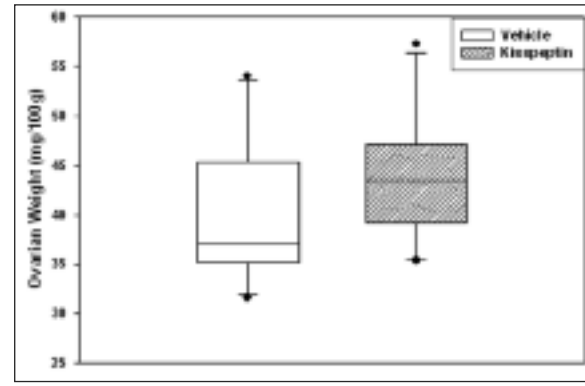

FIGURE 4: Ovarian weights in vehicle (n=10) and kisspeptin-treated (n=9) animals.

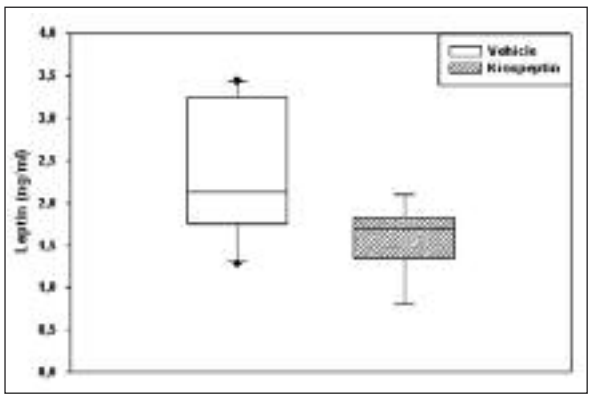

FIGURE 5: Serum leptin levels in vehicle (n=10) and kisspeptin-treated (n=9) animals. p= 0.030 versus Vehicle group.

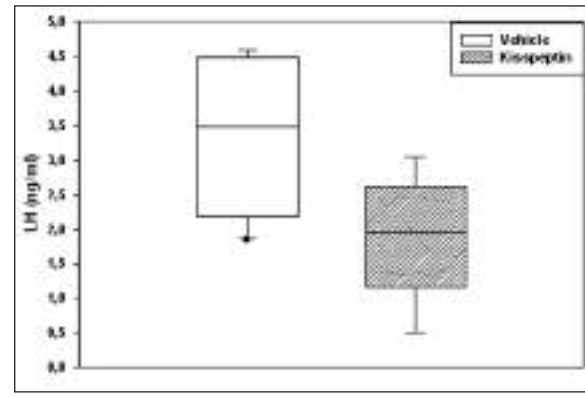

FIGURE 6: Serum LH levels in vehicle (n=10) and kisspeptin-treated (n=9) animals. p= 0.027 versus Vehicle group.

not statistically significant. As shown in Figure 5, serum leptin levels were significantly lower (P=0.03) in Kisspeptin group (1.7 ng/ml (min-max, 1.3-3.4 ng/ml) compared to Vehicle group (2.1 ng/ml (min- max, 0.8- 2.1 ng/ml)). Kisspeptin administration increased serum LH (p=0.027) and estradiol levels (p=0.033) from 2.0 ng/ml (min- max, 0.5-3.1 ng/ml) and 73.3 pg/ml (min- max, 59-115.4 pg/ml) in Vehicle group to 3.5 ng/ml (min-max, 1.8-4.6 ng/ml) and 96.3 pg/ml (min-max, 67-116 pg/ml) in Kisspeptin group, respectively, (Figure 6, 7).

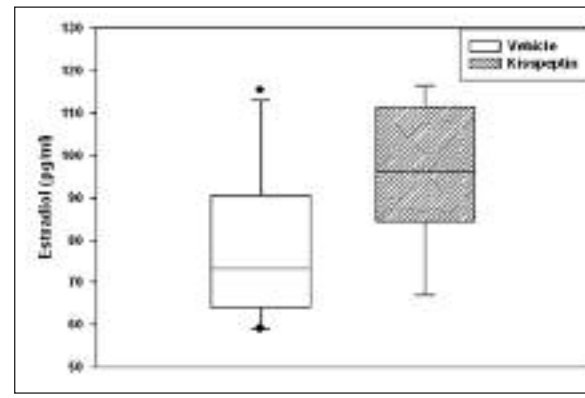

FIGURE 7: Serum estradiol levels in vehicle (n=10) and kisspeptin-treated (n=9) animals.
.P= 0.033 versus Vehicle group.

DISCUSSION

Puberty is one of the most complex biological events in mammals. Puberty onset is not simply determined by aging, but also depends on nutritional status, weight and environmental contaminants.[17-19] From the neuroendocrinological point, maximal pulsatile release of GnRH is essential for gonadotropic axis to maturate enough to initiate puberty.[20] The transition from the prepubertal dormant state is believed to result from the the concerted decre-

ase in the activity of the inhibitory factors and the increase in the activity of stimulatory signalling at the same time.[21] During the past decade, early pubertal development and an increased incidence of sexual precocity have been noticed in children, primarily girls,[22] which may cause psychosocial disorders and mammary cancer resulting from early exposure to estrogenic effect.[23] Therefore, more attention has been paid to the understanding of the mechanisms related to puberty onset nowadays. Kisspeptin has been recently suggested to be an essential regulator stimulating GnRH secretion, which is necessary for puberty to begin.[13] Therefore it is essential to know the effects of kisspeptin on the biological mechanisms related to the onset of puberty.

Chronic central administration of kisspeptin-10 to immature female rats was reported to cause precocious activation of HPG axis as estimated by early vaginal opening, elevated uterus weight and increased serum levels of LH and estrogen.[15] Subcutaneous administration of metastin also elevated follicle-stimulating hormone (FSH) and LH in immature female rats.[14] Intraperitoneal (i.p.) injection of kisspeptin-10 was also reported to elicit LH secretion at early stages (neonatal-to juvenile) of postnatal development.[13] All these studies show that kisspeptin has an ability to potently activate the reproductive axis in immature females.

In the present experiment, the effects of peripheral administration of an effective dose (100 nmol) of kisspeptin-10 on puberty onset were investigated in the female rats. This dose of kisspeptin-10 was chosen on the basis of previous studies on the systemic administration of kisspeptin on gonadotropin secretion in pubertal and adult animals.[14-16] Chronic peripheral administration of kisspeptin to female rats from 26 days at the juvenile period did not change the age of vaginal opening as an external sign of pubertal maturation, but elicited a significant increase in uterus weight and serum LH and estradiol levels. According to these results, peripheral administration of kisspeptin at a dose of 100 nmol seems to be sufficient to increase uterus weight and serum levels of estrogen but not enough to trigger puberty onset.

Although there is much evidence that kisspeptin directly activates GnRH neurones, it is not certain whether it also has direct effects on pituitary gland and reproductive organs. KiSS-1 and GPR54 genes are expressed in male and female rat gonadotrophs.[24,25] The incubation of rat and bovine pituitary cells with kisspeptin-10 was reported to elicit a significant increase in LH secretion.[25,26] In our experiment, ovulation, which is accepted to be a definitive proof of complete puberty onset, was not monitored. However, ovarian-derived serum estrogen, and its biomarker, uterus weight, show that puberty begins in all the rats. One finding of this experiment is that peripheral chronic administration of kisspeptin does not have any effect on puberty onset. The fact that kisspeptin-treated females have greater uterus weight and higher serum estrogen may result from the effect of kisspeptin on LH secretion from the pituitary. Intraperitoneal injection of 100 nmol kisspeptin-10 was shown to significantly increase plasma LH,[16] which is consistent with our findings. Thus, the increase in serum estrogen levels and uterus weight in our experiment may result from the increased LH secretion although it does not advance puberty onset. The finding that kisspeptin-IR and GPR54-IR were detected in rat ovary, with strong signals in theca layers of growing follicles, corpora lutea, and interstitial gland[27] suggests that peripheral kisspeptin may have a direct effect on ovary besides its central effects.

There is evidence that sustained changes in leptin occur during pubertal development,[28] and it is known that kisspeptin expression increases in hypothalamic regions related to pubertal development.[29] Therefore, there may be a mutual interaction between kisspeptin and leptin to initiate puberty. Therefore, serum leptin levels were also measured following peripheral administration of kisspeptin. In the present experiment, peripheral administration of kisspeptin caused a significant reduction in serum leptin levels, which is a new finding. KiSS-1 mRNA has been recently found to be expressed in adipose tissue,[30] but there is no evidence that adipose tissue, which is responsible for secreting leptin, expresses GPR54. Therefore, the mechanism by which kisspeptin decreases leptin secretion is not

known. Leptin is known to have a facilitatory effect on puberty onset[31] by increasing kisspeptin expression in hypothalamus.[32] It is difficult to explain the physiological importance of the decrease in leptin secretion caused by kisspeptin since kisspeptin and leptin seem to have synergic effects on puberty onset. An increase in leptin secretion resulting from increase in adipose tissue is suggested to have an inhibitory effect on gonadal activity.[33,34] Therefore, negative feedback effect of kisspeptin on leptin secretion may be important in terms of prevention of leptin induced gonadal inhibition. Kisspeptin has been reported to dramatically increase in pregnancy.[35] There may be a negative feedback regulation between kisspeptin and leptin secretion. Further studies are needed to determine physiological role of kisspeptin-induced leptin reduction.

We studied the effects of peripferal administration of kisspeptin on food intake and body weight, since GPR54 and kisspeptin mRNAs have been detected in the hypothalamic nuclei such as arcuate nucleus related to energy metabolism.[36] Kisspeptin-10 had no effect on food intake and body weight, which shows kisspeptin does not seem to be involved in the regulation of feeding behavior.

CONCLUSION

We have shown here that chronic peripheral administration of kisspeptin-10 does not accerate puberty onset as estimated by the date of vaginal opening, but potentiates other conventional indices of maturation of reproductive axis such as elevated uterus weight and increased serum level of LH and estrogen. We report for the first time the ability of kisspeptin-10 to decrease leptin secretion in vivo. The reduction in leptin secretion may prevent exogenous peripheral kisspeptin from advancing puberty onset. Therefore, there may be a reciprocal relationship between kisspeptin and leptin.

Acknowledgements

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