# Subcutaneous Injection of Kisspeptin-54 Acutely Stimulates Gonadotropin Secretion in Women with Hypothalamic Amenorrhea, But Chronic Administration Causes Tachyphylaxis

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**Background:** Kisspeptin is a critical regulator of normal reproductive function. A single injection of kisspeptin in healthy human volunteers potently stimulates gonadotropin release. However, the effects of kisspeptin on gonadotropin release in women with hypothalamic amenorrhea (HA) and the effects of repeated administration of kisspeptin to humans are unknown.

**Aim:** The aim of this study was to determine the effects of acute and chronic kisspeptin administration on gonadotropin release in women with HA.

**Methods:** We performed a prospective, randomized, double-blinded, parallel design study. Women with HA received twice-daily sc injections of kisspeptin (6.4 nmol/kg) or 0.9% saline (n = 5 per group) for 2 wk. Changes in serum gonadotropin and estradiol levels, LH pulsatility, and ultrasound measurements of reproductive activity were assessed.

**Results:** On the first injection day, potent increases in serum LH and FSH were observed after sc kisspeptin injection in women with HA (mean maximal increment from baseline within 4 h after injection: LH, 24.0 $\pm$ 3.5 IU/liter; FSH, 9.1 $\pm$ 2.5 IU/liter). These responses were significantly reduced on the 14th injection day (mean maximal increment from baseline within 4 h postinjection: LH, 2.5 $\pm$ 2.2 IU/liter, $P < 0.05$; FSH, 0.5 $\pm$ 0.5 IU/liter, $P < 0.05$). Subjects remained responsive to GnRH after kisspeptin treatment. No significant changes in LH pulsatility or ultrasound measurements of reproductive activity were observed.

**Conclusion:** Acute administration of kisspeptin to women with infertility due to HA potently stimulates gonadotropin release, but chronic administration of kisspeptin results in desensitization to its effects on gonadotropin release. These data have important implications for the development of kisspeptin as a novel therapy for reproductive disorders in humans. **(*J Clin Endocrinol Metab* 94: 4315–4323, 2009)**

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Abbreviations: HA, Hypothalamic amenorrhea; IR, immunoreactivity; KISS1R, kisspeptin receptor; RFRP, RF amide-related peptide.

Hypothalamic amenorrhea (HA) is defined as the cessation of menstruation due to abnormal signaling between the hypothalamus and the pituitary gland (1), and it accounts for approximately 30% of cases of amenorrhea in women of reproductive age (2). Functional HA is defined as HA occurring in the absence of a structural lesion and often results from a relative energy deficit within the body (low body weight or weight loss) (3–7). Although current treatments for women with HA such as clomiphene, gonadotropin injections, and GnRH pump therapy are efficacious, each has associated limitations (8, 9).

The kisspeptins are a group of arginine-phenylalanine (RF) amide peptides, encoded by the *KISS1* gene, that have been identified as potential novel agents for treating reproductive disorders. They act as endogenous ligands for the kisspeptin receptor (KISS1R, alternatively known as G protein-coupled receptor 54) (10–12). *KISS1* and *KISS1R* are expressed in the hypothalamus, pituitary, and placenta (10, 12–14). Kisspeptin signaling exerts powerful effects on the mammalian reproductive system. Mice lacking kisspeptin or the kisspeptin receptor fail to undergo puberty and are infertile (15, 16). In humans, inactivating mutations of *KISS1R* cause pubertal failure (16, 17), and activating mutations lead to precocious puberty (18). Furthermore, central or peripheral administration of kisspeptin induces gonadotropin and sex steroid release in all mammalian species investigated, including rats (19–21), mice (22, 23), monkeys (24) and sheep (23, 25). We have previously demonstrated that iv infusion or sc bolus injection of kisspeptin-54 stimulates gonadotropin secretion in healthy human male and female subjects, respectively (26, 27). Kisspeptin may therefore be a potential novel therapy for treating reproductive disorders in humans. However, the effects of kisspeptin administration in patients with infertility have not been previously investigated.

Although it has been consistently demonstrated that acute administration of kisspeptin stimulates gonadotropin release (19–27), the effects of chronic administration of kisspeptin on reproductive function are less clear. Chronic administration of kisspeptin to nonhuman mammals causes either sustained or nonsustained stimulation of reproductive function, depending on the mode of administration used. Intermittent administration of kisspeptin-10 to juvenile female rats (twice-daily injections) for 5 d and juvenile male monkeys (hourly injections) for 2 d induces precocious reproductive maturation (28, 29). In contrast, continuous peripheral infusion of kisspeptin-10 to monkeys or rats increases LH release only during the first 3 h or the first day of administration, respectively; LH concentrations subsequently return to levels observed before infusion of kisspeptin-10 (30, 31). The long-term effects of administration of kisspeptin in humans have not been studied to date.

In prepubertal female rats, caloric restriction leads to reduced gonadotropin levels, delayed vaginal opening, and low hypothalamic kiss1 expression (32). Twice-daily administration of kisspeptin-10 to these animals restores vaginal opening and gonadotropin secretion (32). Based on these data, we hypothesized that repetitive administration of kisspeptin would restore gonadotropin secretion in human female subjects with HA.

A randomized, double-blinded, placebo-controlled, parallel design study was conducted to determine whether twice-daily administration of kisspeptin to human female subjects with HA would sustainably stimulate gonadotropin release.

## Subjects and Methods

### Kisspeptin-54

Kisspeptin-54 was synthesized by the Advanced Biotechnology Centre, Imperial College London, and purified by reverse-phase HPLC. Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide as previously described (26, 27). The peptide was tested for bioactivity and toxicity as previously described (26). The *Limulus* amebocyte lysate assay (Associates of Cape Cod, Liverpool, UK) was negative for endotoxin, and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital, London). Although kisspeptin-10, -13, -14, and -54 display similar potency *in vitro*, we used kisspeptin-54 due to its higher *in vivo* potency than the other kisspeptin fragments (31, 33).

### Subjects

Ethical approval was granted by the Hammersmith and Queen Charlotte's and Chelsea Hospitals Research Ethics Committee (registration number: 05/Q0406/142). Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki.

Subjects were recruited through advertisements placed in local newspapers. Responders to advertisements were evaluated with a detailed menstrual history, clinical examination, electrocardiogram, and blood tests. Screening blood tests performed were as follows: full blood count, renal profile, liver profile, bone profile, glucose, thyroid profile, LH, FSH, estradiol, progesterone, androstenedione, dehydroepiandrosterone, testosterone, SHBG, prolactin, 17-hydroxyprogesterone, and cortisol. Women were diagnosed with functional HA and included within the study if they fulfilled the following criteria: body mass index below 25 kg/m$^2$; stable body weight over the previous 6 months; age between 18 and 40 yr; secondary amenorrhea of at least 6 months duration; absence of oral contraceptive pill therapy for at least 1 yr; absence of systemic disease comorbidity; absence of active psychiatric illness; stable body weight; absence of therapeutic or recreational drug use; absence of clinical or biochemical hyperandrogenemia; structurally normal hypothalamopituitary region assessed by magnetic resonance imaging; structurally normal female reproductive tract visualized on ultrasound; absence

**TABLE 1.** Comparison of baseline characteristics of women with HA randomized to saline *vs.* kisspeptin-54

| Characteristic | Study group | | *P* value |
|---|---|---|---|
| | Saline | Kisspeptin-54 | |
| Age (yr) | 24.8 ± 0.5 | 26.8 ± 2.4 | 0.28 |
| Weight (kg) | 51.8 ± 3.3 | 54.5 ± 1.1 | 0.46 |
| Body mass index ($kg/m^2$) | 19.0 ± 0.7 | 19.9 ± 0.4 | 0.25 |
| Duration of amenorrhea (months) | 22.4 ± 9.9 | 23.2 ± 12.7 | 0.96 |
| Serum LH (IU/liter) | 4.5 ± 1.6 | 2.6 ± 0.9 | 0.32 |
| Serum FSH (IU/liter) | 6.6 ± 0.7 | 6.1 ± 1.0 | 0.69 |
| Serum estradiol (pmol/liter) | 105 ± 13.9 | 78 ± 4.9 | 0.10 |

Values are provided for subjects randomized to receive saline (n = 5) or kisspeptin-54 (n = 5). Data are shown as mean ± SEM.

of polycystic ovarian appearances on ultrasound; normal thyroid function tests; normal serum prolactin levels; and serum LH:FSH ratio less than 1.5. Ten subjects with HA were recruited to the study. We have previously published baseline clinical and biochemical data for healthy women in the follicular phase of the menstrual cycle and for their responses to kisspeptin-54 administration (27).

## Protocol

A randomized, double-blinded, placebo-controlled, parallel design study was performed. Ten subjects with HA (see Table 1 for baseline characteristics) were randomized to either saline or kisspeptin treatment groups (five subjects per group). Before commencement of the 8-wk study protocol, subjects were taught how to self administer sc injections of saline.

### *Baseline period*

This initial 4-wk control period (wk 1–4) allowed the measurement of baseline values of reproductive hormones and ultrasound markers and the acclimatization of subjects to study conditions. During wk 1–2 of the protocol, all women with HA self-administered twice-daily sc injections of saline (blinded to subjects only). No injections were administered during wk 3–4 of the protocol.

### *Treatment period*

During wk 5–6 of the protocol, women with HA either self-administered twice-daily, double-blinded sc injections of saline (five subjects) or kisspeptin-54 (five subjects), depending on the treatment group to which they had been assigned. The dose of kisspeptin administered was 6.4 nmol/kg [equivalent to 37 $\mu$g/kg (26)]. Twice-daily injections were self-administered at home by subjects during the treatment period, except on the last day (wk 6, d 7) when just one injection was administered in the morning. This final injection was administered within the investigation unit as part of a 4-h sampling study (see *4-h blood sampling after injection of saline or kisspeptin*).

### *Posttreatment period*

During wk 7–8, subjects underwent a posttreatment observation period to measure reproductive hormones and ultrasound markers. No injections were administered during this period.

### *Kisspeptin injections*

All subjects were trained in self-administration of sc injections by an investigator at the start of the study protocol. At the beginning of each week when injections were to be performed, a box containing unlabeled vials of freeze-dried saline or kisspeptin-54, alcohol wipes, saline vials, needles, and needle disposal bins was given to each subject. For injection, vial contents were reconstituted in 0.5 ml of 0.9% saline. Then a 0.5-ml insulin syringe was used to inject a weight-adjusted volume of dissolved vial contents into the lower anterior abdominal region. Subjects were instructed to refrigerate vials stored at home.

### *4-h blood sampling after injection of saline or kisspeptin*

All subjects underwent blood sampling in the 4-h period immediately after the first (wk 5, d 1) and final (wk 6, d 7) injection of saline (five subjects) or kisspeptin-54 (five subjects) of treatment period. These studies were done in an investigation unit. An unused vial returned by each subject from home storage was used for their final injection of kisspeptin or saline. Saline or kisspeptin-54 (6.4 nmol/kg) was sc administered at 0 min by the investigator, and blood was sampled for serum LH, FSH, estradiol, and SHBG, and plasma kisspeptin-immunoreactivity (IR) at −30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min. In one subject, the study was extended to include blood sampling at 270, 300, 330, 360, 390, 420, 450, and 480 min postinjection.

### *Assessments of LH pulsatility*

Subjects underwent assessment of LH pulsatility on the first study day (wk 1, d 1) and approximately 24 h after the final injection of the treatment period (wk 7, d 1). Blood was sampled sequentially every 10 min for serum LH over an 8-h period. These studies were commenced between the hours of 0800 h and 1200 h.

### *Basal measurement of reproductive hormones*

Twice-weekly basal measurements of serum LH, FSH, estradiol, progesterone, SHBG, and plasma kisspeptin-IR were taken from subjects throughout the 8-wk study protocol between 0800 and 1800 h. During weeks when injections were self-administered by volunteers (wk 1, 2, 5, and 6), these blood tests were performed a mean of 4.5 ± 0.4 h after the previous injection, depending on the availability of volunteers. These twice-weekly basal measurements were used to calculate mean values for serum LH, FSH, and estradiol during the baseline period (wk 1–4), treatment period (wk 5–6) and posttreatment period (wk 7–8) of the study protocol. Kisspeptin-IR was measured to confirm subject compliance to kisspeptin injections.

### *Ultrasound scans*

Transabdominal ultrasound scans were performed once a week throughout the 8-wk study period. During each scan, the following parameters were measured: endometrial thickness in millimeters; mean ovarian volume in cubic centimeters; mean follicles number; and maximum diameter of largest follicle in each ovary in millimeters. Ovulation was confirmed by satisfaction of all of the following criteria: visualization of a dominant follicle (diameter, 11 mm or greater); enlargement of dominant follicle into a preovulatory follicle (diameter, 18 mm or greater); subsequent collapse of preovulatory follicle or appearance of

internal echoes on ultrasonography; and a rise in serum progesterone to over 10 nmol/liter.

### *Other measurements*

Weight was measured on the first study day (wk 1, d 1) and subsequently every 2 wk during the 8-wk protocol. During each study visit, urine was tested to exclude pregnancy (Clearview easy-HCG; Inverness Medical Innovations Inc., Waltham, MA).

Diastolic and systolic blood pressure and heart rate were recorded every 30 min during the LH pulsatility studies, to compare mean values for each parameter before and after the treatment period (wk 5–6). Blood pressure and heart rate were also recorded during 4-h blood sampling studies performed after injection of kisspeptin or saline.

### *Response of HA subjects to GnRH before and after injections of kisspeptin*

A second group of five female subjects was recruited using identical inclusion criteria for HA described in this study, to determine whether sensitivity to the effects of GnRH was retained after desensitization to the effects of kisspeptin. A baseline GnRH test was performed in all subjects in the investigation unit. In brief, subjects were cannulated and given a 100-$\mu$g iv bolus injection of GnRH (HRF; Intrapharm Ltd., Kent, UK) at 0 min. Blood was sampled for measurement of serum LH, FSH, and estradiol at −30, 0, 15, 30, 45, 60, 90, and 120 min. Seven days after the GnRH test, all five women self-administered twice-daily kisspeptin injections (6.4 nmol/kg) for 2 wk. The GnRH test was repeated in each subject 8–12 h after their final kisspeptin injection.

### Collection and processing of blood samples

Blood samples for serum analysis were collected in plain serum Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot before centrifugation and separation of serum. Blood samples for plasma kisspeptin analysis were collected in lithium heparin tubes (Becton Dickinson) containing 5000 kallikrein inhibitor units of aprotinin (0.2 ml Trasylol; Bayer, Newbury, UK). Samples were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 10 min at 3000 rpm and then separated. Serum and plasma samples were stored at −20 C until analysis.

### Analytical methods

Serum LH, FSH, estradiol, and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). SHBG was measured using a solid-phase automated enzyme immunoassay (Immulite; Siemans, Llanberis, UK). Reference ranges for females were as follows: LH (follicular), 2–10 IU/liter; LH (midcycle), 20–60 IU/liter; LH (luteal), 4–14 IU/liter; FSH (follicular and luteal), 1.5–8 IU/liter; estradiol (early follicular), less than 300 pmol/liter; estradiol (midcycle), 400–1500 pmol/liter; estradiol (luteal), 200–1000 pmol/liter; and SHBG, 40–80 nmol/liter. Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; estradiol, 3.4%; progesterone, 1.8%; and SHBG, 5.6%. Limits of detectability for each assay were as follows: estradiol, 70 pmol/liter; FSH, 0.05 mIU/ml; LH, 0.07 mIU/ml; progesterone, 0.1 ng/ml; and SHBG, 0.1 nmol/liter.

Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established RIA (26, 27). The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43, neuropeptide FF, and neuropeptide AF. The limit of detectability was 2 pmol/liter, and the intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

### Data analysis

Data are presented as mean ± SEM. Hormone profiles during 4-h blood sampling studies and GnRH tests were analyzed using repeated measures two-way ANOVA with Bonferonni *post hoc* correction. Pairs of means were analyzed using the unpaired two-tailed *t* test. Multiple means were compared using one-way ANOVA with Bonferonni's Multiple Comparison Test. A previously described modified Santen and Bardin method was used to assess LH pulsatility (34, 35). In all cases, $P < 0.05$ was considered statistically significant.

## Results

### Characteristics of subjects recruited to the study

Baseline age, weight, and body mass index were not significantly different between kisspeptin and saline study groups (Table 1). Weight remained stable in both treatment groups during the study (mean weight change from beginning to end of study: saline, −0.5 kg; kisspeptin, −0.1 kg; $P = 0.09$). Subjects reported no increased incidence of nausea or other side effects after injection of kisspeptin or saline. Mean heart rate and systolic and diastolic blood pressure were similar before and after the treatment period (wk 5–6) in all participants (data not shown). Furthermore, no significant acute changes in heart rate or systolic and diastolic blood pressure were observed after kisspeptin administration when compared with saline control (data not shown).

### Kisspeptin immunoreactivity in plasma was raised after injection of kisspeptin

Baseline plasma kisspeptin-IR was below 2 pmol/liter and remained unchanged during the 4-h period after injection of saline (Fig. 1, A and B). Kisspeptin injection resulted in a rise in plasma kisspeptin-IR, with peak mean kisspeptin-IR of approximately 5000 pmol/liter at 45 min after injection (Fig. 1, A and B). Similar patterns of kisspeptin-IR were observed after injection of kisspeptin on the first and last injection days (Fig. 1, A and B).

In subjects randomized to receive saline injections, plasma kisspeptin-IR remained less than 2 pmol/liter during basal measurements taken throughout the 8-wk study protocol (Fig. 1D). In subjects randomized to receive kisspeptin injections, plasma kisspeptin-IR was raised

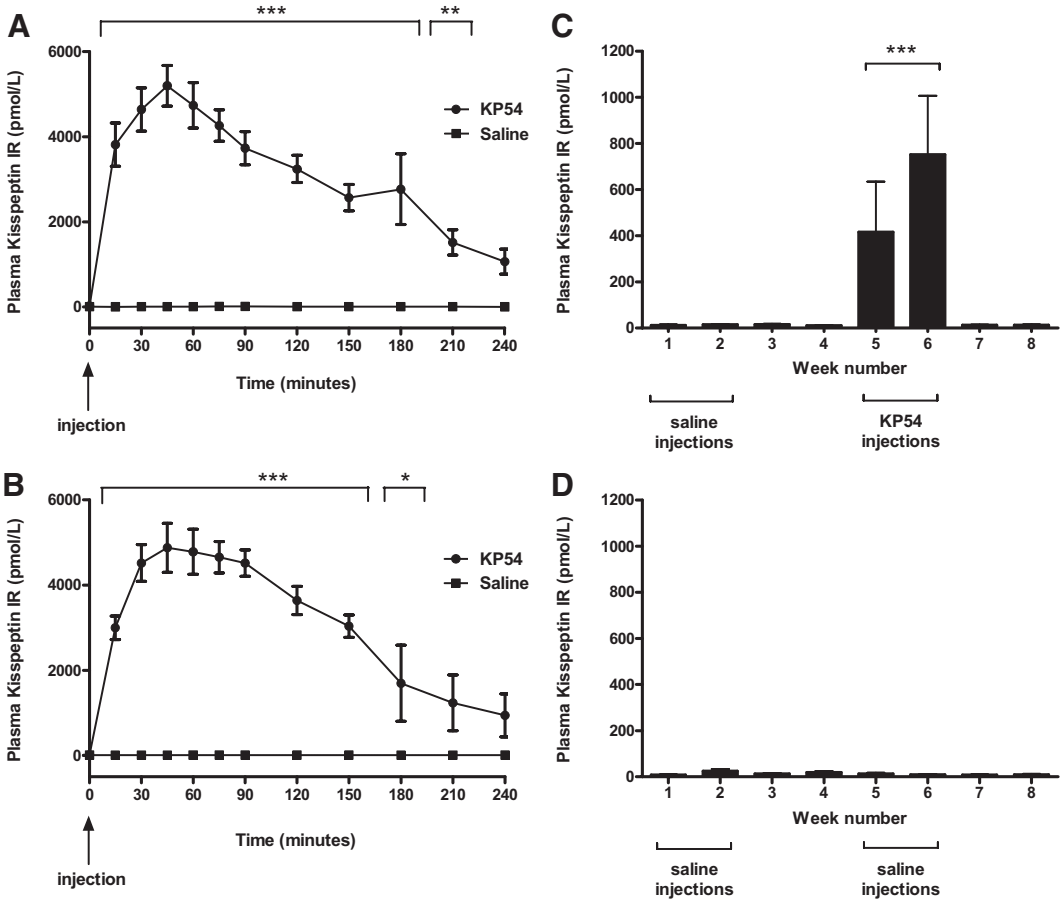


**FIG. 1.** Effect of injections of saline or kisspeptin-54 on plasma kisspeptin-IR in women with HA. A and B, Mean ± SEM plasma kisspeptin-IR after bolus sc injection of saline or kisspeptin-54 (KP54) 6.4 nmol/kg (n = 5 per group) on the first day (A) or last (14th) day (B). Injections were administered at 0 min. C and D, Mean ± SEM basal plasma kisspeptin-IR during each week of the 8-wk study protocol in subjects randomized to receive kisspeptin-54 (C) or saline (D) injections during wk 5–6. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

during the 2-wk kisspeptin treatment period (wk 5–6) but remained below 2 pmol/liter for the remainder of the study protocol (Fig. 1C). These twice-weekly blood samples were taken at various times of the day (determined by subject availability) between the twice-daily injections. Accordingly, the mean kisspeptin-IR values during the treatment period (wk 5, mean plasma kisspeptin-IR, 416 ± 217 pmol/liter; and wk 6, mean plasma kisspeptin-IR, 751 ± 252 pmol/liter) were lower than the peak kisspeptin-IR observed 45 min after kisspeptin injection.

### *Effects of first injection of saline or kisspeptin on serum reproductive hormones in women with HA*

On the first injection day of the treatment period (wk 5, d 1), saline injection did not change serum LH, FSH, or estradiol levels compared with baseline (Fig. 2, A–C). Kisspeptin-54 injection acutely and potently increased serum LH levels in subjects with HA in comparison to saline ($P < 0.001$ at time points 150 to 240 min; Fig. 2A). The mean maximal increase in LH from baseline after kisspeptin injection was observed at 240 min and was 24.0 ± 3.5 IU/liter above baseline. Kisspeptin-54 injection also potently increased serum FSH levels compared with saline ($P < 0.001$ at time points 180 to 240 min; Fig. 2B). After kisspeptin injection, the maximal FSH rise was observed at 240 min and was 9.1 ± 2.5 IU/liter above baseline. Estradiol levels after kisspeptin injection were initially similar to those following saline. However, estradiol levels significantly increased above baseline between 180 and 240 min after kisspeptin administration ($P < 0.05$; Fig. 2C).

### *Effects of last injection of saline or kisspeptin on serum reproductive hormones in women with HA*

On the last injection day of the treatment period (wk 6, d 7), saline injection did not change serum LH, FSH, or estradiol levels compared with baseline (Fig. 2, D–F). Kisspeptin administration resulted in a significant rise in LH only at 240 min after injection and no significant rises in FSH or estradiol. Responses of LH, FSH, and estradiol to the kisspeptin administration were all significantly reduced after the last injection (wk 6, d 7) when compared with the responses after the first kisspeptin injection (wk 5, d 1) ($P < 0.05$ for LH, FSH, and estradiol responses).

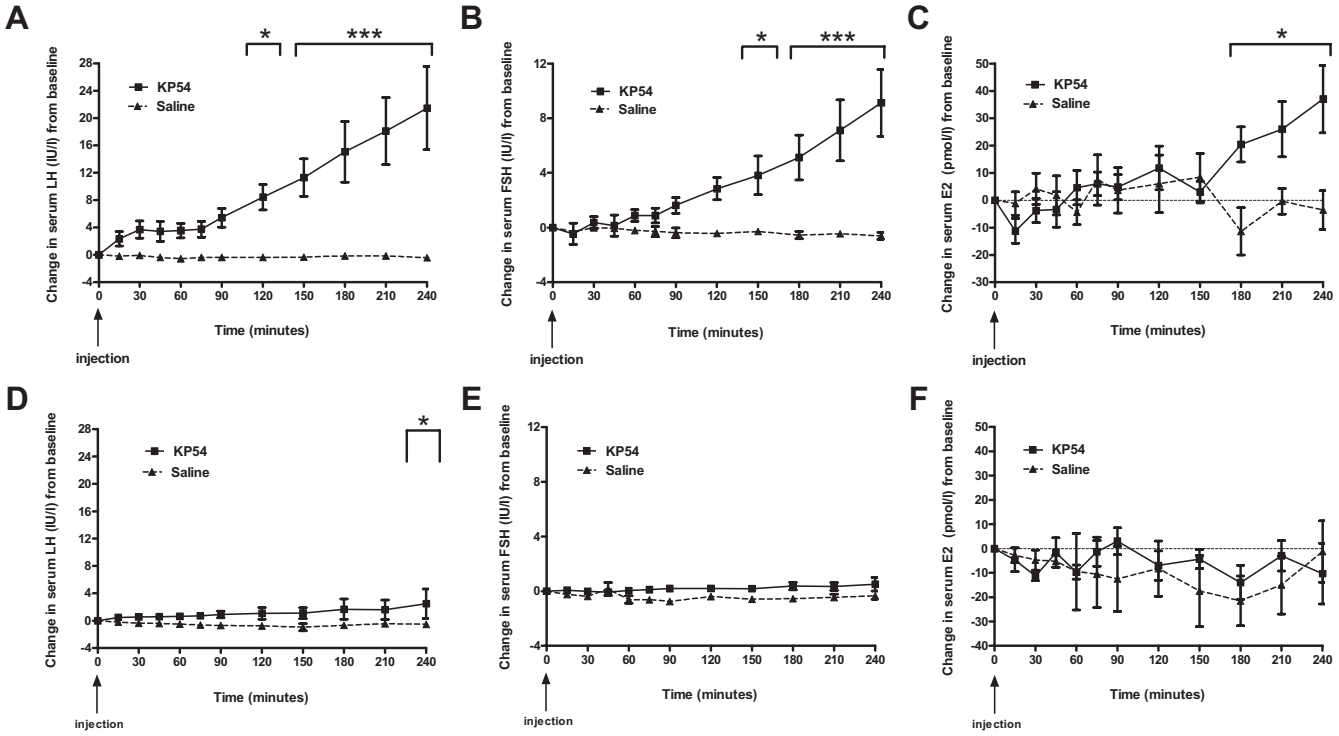


**FIG. 2.** Effects of the first and last injections of saline or kisspeptin-54 on serum reproductive hormones in women with HA. A–C, Changes in serum LH (A), FSH (B), and estradiol (C) after bolus sc injection of saline (n = 5) or 6.4 nmol/kg kisspeptin-54 (KP54, n = 5) on first day (wk 5, d 1) of treatment period are shown. D–F, Changes in serum LH (D), FSH (E), and estradiol (F) after bolus sc injection of saline or kisspeptin-54 on the last day (wk 6, d 7) of the treatment period are shown. Injections were administered at 0 min. Data are shown as mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$. E2, Estradiol.

After the observation of significantly reduced gonadotropin responses to kisspeptin on the last injection day, we decided to assess further the duration of response to kisspeptin injection in one volunteer in whom blood sampling was extended to 8 h after injection. In this volunteer, kisspeptin-IR was raised until 6 h after injection (data not shown). Furthermore, serum LH, FSH, and estradiol were still raised above baseline by the end of the 8-h sampling period (data not shown).

We also examined responsiveness to iv GnRH in five additional subjects with HA, both before and after kisspeptin treatment. We observed LH responses to GnRH in all subjects before commencing kisspeptin treatment (mean peak LH increase during first 2 h after GnRH injection, 14.4 ± 4.6 IU/liter) (Fig. 3). Furthermore these subjects remained responsive to GnRH injection 8–12 h after their final kisspeptin injection ($P = 0.23$ *vs.* baseline LH response, using two-way ANOVA) (Fig. 3).

## Reproductive hormones, LH pulsatility pattern, and radiological findings after saline or kisspeptin treatment

Mean LH levels from twice-weekly basal blood tests during wk 1 to 4 (the baseline period) were slightly lower in the kisspeptin group than the saline group [mean LH, saline 3.7 ± 0.6 *vs.* kisspeptin 1.8 ± 0.3; $P < 0.05$; Supplementary Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org]. Small rises in mean basal LH and FSH levels were detected during wk 5 to 6 (the treatment period) in women randomized to kisspeptin *vs.* saline (mean basal LH, saline −0.8 ± 0.7 *vs.* kisspeptin +1.2 ± 0.7; $P = 0.09$; mean basal FSH, saline −1.8 ± 0.4 *vs.* kisspeptin +0.6 ± 0.4; $P < 0.05$). Basal serum reproduc-

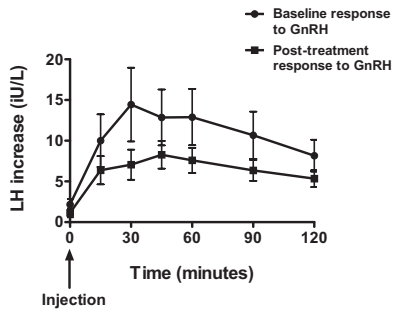


**FIG. 3.** Comparison of LH responses to GnRH administration before and after kisspeptin-54 injections in women with HA. Intravenous GnRH (100 μg) was administered 7 d before commencement of a 14-d, twice-daily regime of sc kisspeptin injections (6.4 nmol/kg) (baseline response to GnRH) (n = 5). The GnRH test was repeated 8–12 h after the last injection of kisspeptin (posttreatment response to GnRH). When comparing baseline and posttreatment LH responses to GnRH injection, overall responses were similar ($P = 0.23$), as were LH changes at each time-point after GnRH injection. Injections were administered at 0 min. Data are shown as mean ± SEM.

tive hormone levels were otherwise similar between kisspeptin and saline groups throughout the 8-wk study period.

There was no significant change in mean LH, number of LH pulses, or mean pulse amplitude observed in patients receiving saline or kisspeptin (Supplementary Table 2).

There was no significant change in mean values for endometrial thickness, ovarian volume, follicle number, or maximum follicle diameter observed after kisspeptin *vs.* saline treatment (Supplementary Table 3). One subject receiving kisspeptin developed radiological changes suggesting possible ovulation, with subsequent symptoms of premenstruation. Ultrasound scans of the subject revealed the rupture of a preovulatory follicle (19-mm diameter) together with subsequent appearance of a corpus luteum. However, this subject had no detectable rise in serum progesterone level and reported no menstrual bleeding.

## Discussion

We report the first study of kisspeptin administration in a human model of infertility and the first investigation of the effects of chronic administration of kisspeptin in humans. Our results show that acute administration of kisspeptin-54 increased serum gonadotropin levels in women with HA but repeated injections lead to reduced effect.

Subjects with HA display a reproductive hormone profile that resembles the follicular phase of the menstrual cycle (low circulating gonadotropin and estradiol levels) more closely than the other phases. Acute LH response to kisspeptin injection was approximately 4-fold greater in patients with HA than in previously studied healthy females in the follicular phase of the menstrual cycle given an identical weight-adjusted dose (mean area under curve LH increase during first 4 h post-kisspeptin injection in h.iU/liter: women with HA in the current study, 40.2; healthy females in follicular phase, 9.8; $P < 0.01$) (27). This is consistent with the observation that LH responses to kisspeptin-10 may be higher in undernourished female rats when compared with those fed *ad libitum* (32). Further work in a single study comparing women with HA and women with normal menstrual cycles is required to confirm the observation made in this study. If confirmed, it would be interesting to determine whether increased responsiveness of women with HA to kisspeptin is attributable to factors such as increased sensitivity to kisspeptin itself or increased pituitary sensitivity to GnRH.

In juvenile female rats, twice-daily intracerebroventricular administration of kisspeptin-10 induces precocious vaginal opening in *ad libitum* fed animals (28) and restores vaginal opening under conditions of caloric restriction (32). Furthermore, Plant *et al.* (29) found that hourly iv kisspeptin-10 pulses were sufficient to induce a train of GnRH discharges characteristic of puberty in juvenile monkeys. We were therefore surprised to observe that LH, FSH, and estradiol responses to the last kisspeptin injection were markedly lower than responses to the first injection. In addition, reproductive ultrasound and basal reproductive hormone parameters were similar between the two treatment groups. The last kisspeptin injection, which was reconstituted using peptide returned from home storage by each volunteer, led to similarly elevated plasma kisspeptin-IR to that observed after the first injection. This suggests that peptide degradation caused by home storage of kisspeptin did not account for the markedly reduced gonadotropin responses to kisspeptin on the last injection day.

Kisspeptin-10 was used during the animal studies of repetitive kisspeptin administration (28, 29), whereas the 54-amino acid form of kisspeptin was administered during this study. Our results reveal that plasma kisspeptin-IR is raised for up to 6 h after each sc injection of kisspeptin-54. Sustained exposure of monkeys and rodents to kisspeptin also leads to desensitization to its effects. Seminara *et al.* (30) demonstrated that continuous iv kisspeptin-10 administration to male rhesus monkeys for 98 h led to increased LH release lasting only 3 h, followed by a return of gonadotropin concentrations to levels similar to those observed before the kisspeptin-10 infusion. Similarly, Thompson *et al.* (31) observed increased LH levels only during the first day of a continuous 3-d sc kisspeptin-54 infusion to adult male rats. A recent publication by Keen *et al.* (36) demonstrates the pattern of kisspeptin release within the monkey hypothalamic median eminence to be pulsatile. Animal data suggest that a protocol using intermittent administration of kisspeptin (28–29, 32) may be less likely to result in desensitization than a protocol using continuous administration (30, 31). However, given the prolonged action of sc kisspeptin-54 injection on plasma kisspeptin-IR and reproductive hormone levels, our protocol of twice-daily kisspeptin-54 injections may have resulted in desensitization through prolonged and nonpulsatile kisspeptin exposure. An intermittent, iv method of kisspeptin administration might minimize or prevent the desensitization of gonadotropin responses observed in this study.

We observed GnRH administration to stimulate LH secretion in HA subjects even after 2 wk of kisspeptin treatment. A study by Ramaswamy *et al.* (37) demonstrated that responsiveness to GnRH bolus was maintained in adult male rhesus monkeys during an infusion of kisspeptin-10 delivered at 200 $\mu$g/h but was reduced at a higher infusion rate of 400 $\mu$g/h. In our study, a lower LH response to GnRH administration was observed after

kisspeptin treatment when compared with the baseline response; however, this difference was not significant. Our results therefore suggest that the protocol of kisspeptin administration used during this study led to desensitization upstream of the pituitary gland. It is possible that our observations are explained by KISS1R down-regulation, which has been previously demonstrated *in vitro* (38).

Kisspeptin antagonism has been shown to inhibit pulsatile GnRH release in pubertal female rhesus monkeys and pulsatile LH release in adult female sheep (39). Furthermore, Ramaswamy *et al.* (37) observed that LH pulse amplitude and frequency was reduced by infusion of kisspeptin-10 at 400 $\mu$g/h (but not 200 $\mu$g/h) in adult male monkeys. In the current study, neither LH pulse amplitude nor LH pulse frequency was significantly altered after kisspeptin treatment in women with HA. Our results might be explained by rapid recovery from kisspeptin exposure during the 24-h period between the final kisspeptin injection and the second assessment of LH pulsatility study. It is also plausible that a protocol using higher or more frequent doses of kisspeptin injections would have significantly altered LH pulsatility.

This study demonstrates that acute sc administration of kisspeptin-54 potently stimulates pituitary-gonadal function in human females with HA. However, significantly reduced gonadotropin responses to kisspeptin-54 administration were observed after 2 wk of twice-daily kisspeptin-54 injections, suggesting desensitization. These results have important implications for the therapeutic potential of kisspeptin to treat patients with reproductive disorders.

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