Title: Twice-daily subcutaneous injection of kisspeptin-54 does not abolish menstrual cyclicity in healthy female volunteers

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Abstract

BACKGROUND: Kisspeptin is a critical hypothalamic regulator of reproductive function. Chronic kisspeptin administration causes profound tachyphylaxis in male monkeys and in women with functional hypothalamic amenorrhea (HA). The pharmacological effects of chronic kisspeptin exposure in healthy women with normal menstrual cycles have not been studied previously.

AIM: To determine the effects of follicular phase kisspeptin-54 treatment on menstrual cyclicity in healthy women.

METHODS: We performed a prospective, single-blinded, one-way crossover study. Healthy women received twice-daily subcutaneous injections of kisspeptin (6.4nmol/kg) or 0.9% saline during menstrual days 7-14 (N=5 per treatment arm). Serial assessments of basal reproductive hormones, ultrasound parameters, luteinising hormone (LH) pulsatility, and acute sensitivity to GnRH and kisspeptin-54 injection were performed.

RESULTS: Menstrual cyclicity persisted in all women following follicular phase kisspeptin-54 treatment. Chronic exposure to kisspeptin-54 did not abolish acute stimulation of LH following injection of kisspeptin-54 or GnRH. In addition, kisspeptin-54 treatment was associated with a shorter mean length of menstrual cycle (mean length of menstrual cycle in days: saline 28.6±1.4 *vs.* kisspeptin 26.8±3.1, $P<0.01$), advanced the onset of highest recorded serum LH (mean menstrual day of highest LH: saline 15.2 ± 1.3 *vs.* kisspeptin 13.0±1.9, $P<0.05$), and advanced the onset of the luteal phase (mean menstrual day of progesterone increase: saline 18.0±2.1 *vs.* kisspeptin 15.8±0.9, $P<0.05$).

CONCLUSION: Our data suggest that exogenous kisspeptin-54 does not abolish menstrual cyclicity in healthy women. Further work is needed to determine if kisspeptin could be used to treat certain anovulatory disorders.

Introduction

The kisspeptins are a group of arginine-phenylalanine (RF) amide peptides encoded by the *KISS1* gene, which are endogenous ligands for the kisspeptin receptor (KISS1R)[1-4]. *KISS1* and *KISS1R* are expressed predominantly in the hypothalamus, pituitary and placenta[3,5-7]. Kisspeptin signalling exerts powerful effects on the mammalian reproductive system. Mice and humans lacking kisspeptin or the kisspeptin receptor fail to undergo puberty and are infertile[8-10]. Central or peripheral administration of kisspeptin induces gonadotrophin and sex steroid release in all mammalian species investigated, including rats[11-13], mice[14,15], monkeys[16] and sheep[17,18].

Administration of kisspeptin-54 or kisspeptin-10 acutely stimulates gonadotrophin secretion in healthy male[19-22] and female volunteers[23,24], and women with functional HA (hypothalamic amenorrhea)[25,26]. However, twice-daily subcutaneous administration of kisspeptin-54 to women with HA causes profound tachyphylaxis within 24h of commencing treatment[25]. Furthermore continuous intravenous infusion of kisspeptin-10 to monkeys is associated with tachyphylaxis within 3h of commencing administration[16]. It has therefore been assumed that chronic treatment with kisspeptin-54 in healthy women may have limited therapeutic potential as a stimulator of human reproductive activity, due to tachyphylaxis observed following chronic administration in primates and women with HA. However, this hypothesis has not been tested previously.

In this study we aimed to determine whether chronic exogenous kisspeptin was sufficient to alter menstrual cyclicity, using the exact dose previously demonstrated to cause tachyphylaxis within 24h in women with HA (6.4nmol/kg)[25,26]. We performed a single-blinded placebo-controlled one-way crossover study to determine the effects of twice-daily administration of kisspeptin for 7 days on the menstrual cycle in healthy human female subjects with regular menstrual cycles.

Methods

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. Five healthy female subjects with regular menstrual cycles were recruited through advertisements placed in local newspapers (age in years: 31.6±2.6, range 24-37; weight in kg: 60.4±2.5, range 50.4-63.8) as described previously[22,23]

Protocol

A single-blinded, placebo-controlled, one-way crossover design study of ten menstrual cycles was performed (Figure 1). During month 1 of the study protocol, five healthy female subjects self-administered twice-daily subcutaneous saline injections between days 7 and 14 of their menstrual cycle (see Subject Characteristics in Supplemental Table 1). During month 2 of the study protocol, the same five healthy female subjects self-administered twice-daily subcutaneous kisspeptin-54 injections (6.4nmol/kg; equivalent to 37mcg/kg[19]) between days 7 and 14 of their menstrual cycle; (see Supplemental Methods for kisspeptin-54 peptide synthesis and testing). Day 1 of each month was defined as the first day of menstrual bleeding. *Kisspeptin injections:* All subjects were trained in self-administration of subcutaneous 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 unlabelled vials of freeze-dried saline (month 1) or vials of freeze-dried kisspeptin-54 (month 2), alcohol wipes, saline ampoules for reconstitution of freeze-dried vial contents, 0.5ml insulin syringes with needles, and needle disposal bins was given to each subject. For injection, vial contents were reconstituted in 0.5ml of 0.9% saline. A 0.5ml insulin syringe was then used to inject saline alone (month 1) or 6.4nmol/kg of kisspeptin-54 (month 2) into the lower anterior abdominal region subcutaneously. This kisspeptin-54 dose was the same used in our previous work in healthy

women and women with HA[25,26]. Volunteers were instructed to prepare and perform injections in the morning after breakfast (unless attending for a study in which case it was done by the volunteer at time=0 of the study) and in the evening before bed. The volume of the saline or kisspeptin injections was identical. Subjects were instructed to refrigerate vials stored at home. Prior to commencing study visits, injection sites were inspected, and numbers of returned vials, insulin syringes, and saline or kisspeptin vials were counted in order to monitor compliance. Plasma kisspeptin immunoreactivity (-IR) was also assessed throughout the study protocol to confirm compliance.

Baseline period: During menstrual days 1 to 6 of each month of the study protocol, pituitary responsiveness was assessed by a Gonadotrophin-Releasing Hormone (GnRH) test (see Supplemental Methods for protocol), and baseline reproductive hormones and ultrasound markers were measured. The collection, processing and analysis of blood samples are detailed in supplemental methods. The baseline period also allowed the acclimatisation of subjects to study conditions. No injections were administered during this period.

Treatment period: During menstrual days 7 to 14 of each month of the study protocol, subjects self-administered twice-daily, single-blinded subcutaneous injections of saline (month 1 of study protocol) or kisspeptin-54 (month 2 of study protocol) as above.

Post-treatment period: During menstrual days 15 to 28 of each month of the study protocol, subjects underwent a post-treatment observation period, in order to assess pituitary responsiveness (GnRH test), and measure circulating reproductive hormones and ultrasound markers. No injections were administered during this period.

4 hour blood sampling post-injection of saline or kisspeptin: All subjects underwent blood sampling during the 4 hour period immediately following the first injection of the saline (day 7, month 1) or kisspeptin-54 (day 7, month 2) treatment period in order to confirm that kisspeptin-54 acutely stimulated LH release as previously shown in healthy women[23]. Saline or kisspeptin-54 6.4nmol/kg was subcutaneously administered at 0 minutes by the subject,

and blood was sampled for serum LH, FSH, estradiol, and plasma kisspeptin-IR at -30, 0, 10, 20, 40, 60, 90, 120, 150, 180, 210 and 240 minutes.

8 hour blood sampling for assessments of LH pulsatility: Subjects underwent three assessments of LH pulsatility during month 1 and month 2 of the study protocol. Baseline assessment of LH pulsatility was performed on menstrual day 6 (1 day prior to commencing injections). LH pulsatility was also assessed following injection of saline or kisspeptin-54 on menstrual days 11 and 14. The saline or kisspeptin-54 was reconstituted using one of the vials given to each subject at the beginning of the treatment period for home storage. Blood was sampled every 10 minutes. Studies commenced between 0800h and 0900h.

Assessment of pituitary sensitivity before and after injections of saline or kisspeptin: Subjects each underwent two GnRH tests during month 1 and month 2 of the study protocol (see Supplemental Methods for protocol). A baseline GnRH test was performed on menstrual day 1 and was repeated in each subject 24 hours after final saline or kisspeptin injection (menstrual day 15).

Basal measurement of reproductive hormones: Basal measurements of serum LH, FSH, estradiol, progesterone, and plasma kisspeptin IR were taken from subjects during days 1, 6, 7, 11, 14, 15, 18, 21 and 28 during month 1 and month 2 of the study protocol. On days 7, 11 and 14 the basal blood sample was taken before the morning injection.

Ultrasound scans. Trans-abdominal ultrasound scans were performed on days 1, 7, 15, 18, 21 and 28 during month 1 and month 2 of the study protocol. Trans-vaginal scans were not used to minimise discomfort to volunteers during repeated examinations. The ultrasonographer was blinded to treatment for all subjects. During each scan the following parameters were measured: endometrial thickness in millimetres (mm); mean ovarian volume in cubic centimetres (cm^3); mean follicle number; maximum diameter of largest follicle in each ovary

(mm). Ovulation was defined as a rise in serum progesterone >10nmol/l together with suggestive radiological features (visualisation of a dominant follicle with subsequent appearance of a pre-ovulatory follicle and/or corpus luteum).

Data analysis:

Investigators performing the clinical studies were blinded to results until all subjects had completed the study protocol. JDV used an established, blinded deconvolution method with 93% sensitivity and specificity[27] to identify LH pulses, and calculate the secretory mass of LH pulses (integral of LH secretion over time during a secretory burst normalised per liter of distribution volume). Cumulative levels of basal, pulsatile and total (basal + pulsatile) LH secretion were also estimated during each study. Data are presented as mean±standard error of mean (SEM). Kisspeptin-IR data was log-transformed to normalise data prior to data analysis. All other analyses included data series, the majority of which had normal distributions assessed using the Komogorov-Smirnov test with Dallal-Wilkinson-Lillie analysis. Hormone profiles during 4 hour blood sampling studies were analysed using repeated measures 2-way ANOVA with Bonferonni post hoc correction. Pairs of means were analysed using the unpaired two-tailed t-test. Multiple means were compared using one-way ANOVA with Bonferonni's Multiple Comparison Test. In all cases, $P<0.05$ was considered statistically significant.

Results

Acute effects of saline or kisspeptin-54injection on plasma kisspeptin at the commencement, mid-point and end of twice-daily administration in healthy women:

(a) Acute changes in plasma kisspeptin-IR following injection of saline or kisspeptin-54: Plasma kisspeptin was unchanged at approximately 10pmol/l following injection of saline on the first (Figure 2A), fourth (Figure 2B) and final (seventh; Figure 2C) days of twice-daily administration. Kisspeptin-54 injection acutely elevated plasma kisspeptin-IR on the first day of administration, with peak mean kisspeptin-IR levels of 2421±392pmol/l at 45min post injection ($P<0.001$ vs. saline) (Figure 2A). Similar elevations of kisspeptin-IR were observed following injection of kisspeptin on the fourth and last injection days (Figures 2B-C).

(b) Plasma kisspeptin-IR pre-injection of saline or kisspeptin-54: In subjects receiving saline, plasma kisspeptin-IR measured prior to the morning saline injection remained approximately 10pmol/l throughout the study protocol (Figure 2D). In subjects receiving kisspeptin injections, plasma kisspeptin-IR was not elevated on menstrual days 1, 6 or 7 since these blood samples were taken prior to the first kisspeptin-54 injection. Plasma kisspeptin-IR was elevated on menstrual days 11 and 14 (during the twice daily kisspeptin treatment period but just prior to the morning kisspeptin injection), which suggested compliance with kisspeptin injection the previous evening. As expected, plasma kisspeptin-IR was not elevated on the morning of day 15, since it was approximately 24h following final kisspeptin-54 injection. Furthermore plasma kisspeptin-IR remained <10pmol/l on days 18-28 of the study protocol (Figure 1D).

Acute effects of saline or kisspeptin-54 injection on serum reproductive hormones at the commencement, mid-point and end of twice-daily administration in healthy women:

(a) Commencement of treatment period (menstrual day 7): Saline injection did not change serum LH levels when compared with baseline (Figure 3A, baseline LH 5.20±0.64IU/L). Kisspeptin-54 injection acutely increased serum LH levels in subjects when compared with saline (Figure 3A, $P<0.05$ at time-points 180 min to 210min, baseline LH 5.68±0.98IU/L).

The mean maximal increase in LH from baseline after kisspeptin injection was observed at 240 minutes, and was 8.6±3.4IU/l above baseline.

(b) Mid-point of treatment period (menstrual day 11): Saline injection did not change serum LH or FSH compared with baseline (Figure 3B, baseline LH 5.74±1.45IU/L). Kisspeptin-54 injection acutely increased serum LH levels in subjects when compared with saline (Figure 3B, $P<0.01$ at 210min, and $P<0.05$ at 220 to 240min, baseline LH 9.41±4.89IU/L). The mean maximal increase in LH from baseline after kisspeptin injection was observed at 240min, and was 8.3±2.4IU/l above baseline.

(c) End of treatment period (menstrual day 14): Saline injection did not change serum LH or FSH compared with baseline (Figure 3C, baseline LH 17.79±9.69IU/L). The mean maximal increase in LH from baseline after kisspeptin injection was observed at 220min, and was 12.7±8.1IU/l above baseline (baseline LH 6.01±1.60IU/L). Kisspeptin-54 injection showed a trend towards stimulating serum LH but this did not reach statistical significance compared with saline. During days 7, 11 and 14 of the treatment period, total LH secretion was increased significantly following kisspeptin-54 when compared with saline ($P<0.01$ using 2-way ANOVA) (Figure 3D).

Effects of twice-daily saline or kisspeptin injections on length of the menstrual cycle and biochemical markers of reproductive activity in healthy women

(a) Length of menstrual cycle: Subjects had the same menstrual cycle length prior to commencing the study when compared with menstrual cycle length during saline administration (mean menstrual cycle length in days: 28.6±1.1, prior to study commencement; 28.6±1.4, saline month; $P=1.00$). However all subjects had a shorter menstrual cycle (by approximately 2 days) during kisspeptin-54 treatment when compared with saline (mean length of menstrual cycle in days: saline 28.6±1.4 vs. kisspeptin 26.8±3.1, $P<0.01$) (Figure 4A).

(b) Timing of peak serum LH: During kisspeptin-54 treatment observed peak levels of serum LH and estradiol, but not FSH, were earlier during the menstrual cycle when compared with the saline group (Figures 4B-D). Furthermore the menstrual day of highest recorded serum LH was approximately 2 days earlier during kisspeptin-54 treatment when compared with saline treatment (mean menstrual day of highest recorded serum LH: saline 15.2±1.3 vs. kisspeptin 13.0±1.9, $P<0.05$) (Figure 4E).

(c) Timing of luteal phase of menstrual cycle: We examined the onset of the luteal phase which is characterised by release of a mature oocyte from the ovary and secretion of progesterone by the residual corpus luteum. During kisspeptin-54 treatment, levels of serum progesterone became elevated >10nmol/L earlier during the menstrual cycle when compared with the saline group (Figure 4F). The menstrual day of onset of luteal phase (defined as beginning when serum progesterone was elevated >10nmol/l) was approximately 2 days earlier during kisspeptin-54 treatment when compared with saline treatment (mean menstrual day of serum progesterone increase >10nmol/l: saline 18.0±2.1 vs. kisspeptin 15.8±0.9, $P<0.05$) (Figure 4G).

Effects of twice-daily saline or kisspeptin injections on radiological markers of reproductive activity in healthy women

A corpus luteum, indicating recent ovulation, was observed in all women following kisspeptin-54 treatment, and in 3/5 women following saline treatment. Elevated serum progesterone (>10nmol/l) was observed in all subjects receiving saline or kisspeptin-54 treatment. Immediately following 7 days of kisspeptin-54 treatment (on menstrual day 15), the mean diameter of the largest follicle seen during ultrasonography was significantly higher when compared with women during saline treatment (mean diameter of largest follicle in mm: saline 10.0±2.2 vs. kisspeptin 15.5±1.2, $P<0.05$) (Figure 5A). No significant differences in number of follicles or endometrial thickness were observed during kisspeptin-54 treatment when compared with saline (Figure 5B,C).

Effects of twice-daily saline or kisspeptin on LH pulsatility in healthy women

LH pulsatility was determined immediately prior to commencing saline or kisspeptin-54 treatment (menstrual day 6 of study protocol), and during saline or kisspeptin-54 treatment (menstrual days 11 and 14 of study protocol). Mean secretory mass was slightly lower before kisspeptin-54 treatment when compared with saline treatment (mean secretory mass in IU/L: saline 5.9±0.5 vs. kisspeptin 4.1±0.4, $P<0.01$) (Figure 6A). Despite this, mean secretory mass was increased significantly during menstrual day 11 (4^{th} day of treatment) during kisspeptin-54 treatment when compared with the saline treatment (mean secretory mass in IU/L: saline 3.4±0.4 vs. kisspeptin 14.5±3.6, $P<0.01$). On the last day of treatment (menstrual day 14) no significant differences in secretory mass were observed between saline or kisspeptin-54 treatment. No significant differences in pulse rate were observed between saline and kisspeptin-54 treatment (Figure 6B). Pulsatile LH secretion was increased significantly during menstrual day 11 (4^{th} day of treatment) in the kisspeptin-54 group when compared with the saline group (mean secretory mass in IU/L: saline 17.9±3.6 vs. kisspeptin 78.2±22.8, $P<0.05$) (Figure 6C). However basal and total LH secretion were not significantly different between groups (Figure 6 D-E).

Assessment of pituitary sensitivity before and after injections of saline or kisspeptin:

We examined sensitivity of all healthy female subjects to intravenous GnRH, both 6 days before (menstrual day 1 of study protocol) and 24h after saline or kisspeptin treatment (menstrual day 15 of study protocol). On menstrual day 1, no significant difference in pituitary sensitivity was observed following GnRH administration between subjects prior to commencing saline of kisspeptin treatment (mean maximal LH increase during first 2h post-GnRH injection in IU/L: 13.1±1.1; pre-saline, 13.4±1.1 pre-kisspeptin-54; P=ns) (Supplemental Figure 1A). Furthermore 24h following cessation of twice-daily saline or kisspeptin-54 injections, no significant difference in pituitary sensitivity was observed following GnRH administration between saline or kisspeptin-54 treatment (mean maximal LH

increase during first 2 hours post-GnRH injection in IU/L: 39.9±8.9; post-saline, 41.0±16.4 post-kisspeptin-54; P = ns) (Supplemental Figure 1B).

The expected physiological increase in pituitary sensitivity to GnRH during menstrual day 15 *versus* menstrual day 1[32] was observed in healthy female subjects, whether receiving saline or kisspeptin treatment (Supplemental Figure 1C).

Discussion

Genetic studies demonstrate that kisspeptin peptides are necessary for pubertal maturation in humans[8-10]. We and other investigators have recently demonstrated that exogenous kisspeptin acutely stimulates gonadotrophin secretion in women[23-26]. However the pharmacological effects of chronic kisspeptin exposure to healthy women with regular menstrual cycles have not been studied previously. Chronic kisspeptin administration causes profound tachyphylaxis in male monkeys and in women in functional HA[16,25,26]. We present novel data suggesting that menstrual cyclicity persists in healthy women following twice-daily kisspeptin-54 treatment during the follicular phase of the menstrual cycle.

In the current study, kisspeptin treatment was associated with an advanced timing of serum progesterone increase, and onset of menstrual bleeding by approximately 2 days when compared with saline. Animal studies demonstrate that kisspeptin stimulates gonadotrophin secretion in a GnRH-dependent manner, since its action is abolished by GnRH antagonist[14]. Exogenous GnRH is sufficient to trigger ovulation[28]. Further studies with daily follicle morphology assessment, and daily serum LH measurement would be required to confirm our findings. However our study data raise the possibility that kisspeptin-54 administration may advance the onset of ovulation in healthy women by stimulating endogenous hypothalamic GnRH secretion. In addition to stimulating GnRH, kisspeptin itself is implicated in generation of the LH surge needed for ovulation. In rodents, a subpopulation of hypothalamic anteroventral periventricular nucleus (AVPV) kisspeptin neurons are implicated in generating the LH surge needed for ovulation[29], and are positively rather than negatively regulated by estradiol[30]. Central administration of a monoclonal antibody to kisspeptin is sufficient to block ovulation in rats[31]. Furthermore, administration of kisspeptin-10 has been shown to stimulate ovulation in the musk shrew[32], rat[33] and sheep[17]. Humans have no anatomical equivalent of the AVPV. Nevertheless, it is possible that exogenous kisspeptin-54 may have advanced the onset of luteal phase in our female subjects by increasing kisspeptin signalling in a subpopulation of hypothalamic kisspeptin neurons which stimulate the LH surge. Several

lines of evidence suggest that in humans unlike in lower species, a change in pituitary responsiveness to GnRH rather than an actual GnRH surge is responsible for the LH surge[34,35]. It is therefore possible that kisspeptin-54 advances ovulation in women by increasing the prevailing levels of estradiol, which are needed to trigger the LH surge at a pituitary level.

Chronic kisspeptin administration has also been implicated as a potential novel therapy for inhibiting reproductive hormone secretion in contraception or the treatment of hormone sensitive cancer; our data suggest that kisspeptin-54 may have limited therapeutic potential in this regard, at least in women at the dose of kisspeptin tested. However our observation that kisspeptin may advance the menstrual cycle, raises the possibility that women with certain forms of infertility could be treated with kisspeptin. A recent study suggests that kisspeptin is sufficient to restore ovulation in a mouse model of anovulatory hyperprolactinaemia[36]. Further studies are required to determine if kisspeptin-54 treatment could be used to stimulate ovulation in women with anovulatory reproductive disorders other than HA.

Traditional ovulatory drugs such as clomiphene citrate have low pregnancy rates[37,38]. Although efficacious, in vitro fertilisation (IVF) confers a risk of ovarian hyperstimulation[39]. Kisspeptin acts by stimulating endogenous, rather than pharmacological, levels of reproductive hormone secretion. Kisspeptin might therefore offer a fertility treatment with lower risk of OHSS when compared with IVF.

Comparison of our data with other clinical studies of kisspeptin administration is complicated by the investigation of its two peptide forms (kisspeptin-54 and -10), and sexual dimorphism of the effects of kisspeptin-10; intravenous bolus administration consistently stimulates LH men[20,21,22], but women are much less sensitive to the effects of kisspeptin-10 during the follicular phase of menstrual cycle when compared with the preovulatory phase[22,24]. Although kisspeptin-54 stimulates LH in both sexes, its effects have never been compared directly between men and women[19,23]. Consistent with our findings in healthy women, George et al. recently observed that 22.5 hour intravenous infusion of the shorter form of kisspeptin, kisspeptin-10, stimulates LH secretion without apparent tachyphylaxis in healthy men[21]. However in contrast to the current findings, twice daily kisspeptin-54 treatment has been

shown to rapidly cause tachyphylaxis in women with HA. Women with HA are acutely 4-fold more sensitive to the effects of kisspeptin-54 when compared with healthy women in the follicular phase of their menstrual cycle. A higher dose of kisspeptin-54 may therefore be necessary to achieve tachyphylaxis in healthy female volunteers[23,25]. Nevertheless, our data suggest that menstrual cyclicity is able to persist in healthy women using the currently tested regimen of follicular phase kisspeptin-54 treatment. Preliminary data has emerged suggesting that chronic administration of a kisspeptin analogue causes tachyphylaxis in healthy men[40]. It would be interesting to determine if tachyphlyaxis to kisspeptin-54 is also sexually dimorphic, or dependent on the dose and precise form of kisspeptin receptor agonist administered.

It is interesting to note that while intravenous bolus injection of the shorter kisspeptin fragment, kisspeptin-10, acutely stimulates LH secretion within an hour of administration, we observed that sc injection of kisspeptin-54 acutely stimulated peak LH secretion 3-4h following administration despite a rapid elevation of kisspeptin-IR within minutes of administration. Kisspeptin-54 is a much more potent stimulator of LH secretion when compared with kisspeptin-10; it might therefore take longer for kisspeptin-54 to stimulate higher levels of LH secretion. It is also possible that greater levels of estradiol stimulation following kisspeptin-54 when compared with kisspeptin-10, lead to 'auto-priming' of the pituitary to respond even more to stimulation by GnRH.

It is interesting to appraise the evidence that exogenous kisspeptin stimulates basal and pulsatile GnRH secretion. Although kisspeptin stimulates rat pituitary gonadotrophin secretion *in vitro*[41], animal studies using GnRH antagonists support the view that exogenous kisspeptin stimulates basal LH secretion through a GnRH-dependent mechanism[42], which is possibly mediated through GnRH nerve terminals at the median eminence[43]. However it is less clear whether exogenous kisspeptin stimulates endogenous GnRH pulsatility. Sustained exposure to exogenous kisspeptin-10 or -54 stimulates pulsatile LH secretion for several hours in healthy men[21], and patients with inactivating mutations of the NKB signalling pathway[44]. However since these studies merely measure LH pulsatility, it is not possible to exclude that effects reflect increasing circulating oestrogen and pituitary responsiveness to

unchanged, endogenous GnRH pulsatility. Chan and colleagues recently demonstrated that intravenous bolus kisspeptin-10 increased the time to the next endogenous LH pulse in healthy men[20]; this may represent the only current data suggesting that exogenous kisspeptin can directly modulate endogenous GnRH pulsatility in humans (by increasing the latency period to next LH/ GnRH pulse).

It is important to recognise that the current study is based on observations within a small number of subjects, so may have insufficient statistical power to reveal subtle effects of kisspeptin-54 administration on menstrual cyclicity or LH pulsatility. Furthermore, gonadotrophin response to kisspeptin treatment is known to alter with the phase of menstrual cycle in healthy women[23,24]. A larger study is needed to confirm our findings and determine what factors influence the variability in response of subjects to kisspeptin treatment. It is possible that chronic kisspeptin-54 treatment might have effects which last beyond the treatment period. For this reason, we designed a one-way crossover protocol in which subjects self-administered saline during the first month followed by kisspeptin-54 during the second month. It therefore remains possible that an order effect might have contributed to our results. However it is noteworthy that LH pulse secretory mass was marginally lower at the start of the second month when compared with LH pulse secretory mass at the start of month one (when greater stress levels would be expected). Furthermore, subjects had the same menstrual cycle length prior to commencing the study when compared with menstrual cycle length during saline administration. We also recognise that although estradiol measurements were compared with placebo-controlled values, the automated platform assay used to analyse serum estradiol can cross-react with other steroids.

Mean peak levels of LH during the menstrual cycle were lower during kisspeptin treatment when compared with saline treatment. We therefore cannot exclude that kisspeptin-54 treatment caused partial tachyphylaxis in healthy female volunteers. Alternatively, the short baseline sampling period involving just two blood samples may have reduced our power to detect stimulation of LH following kisspeptin-54 injection on day 14. LH levels were highly variable during menstrual day 14; this may have been caused by some but not all subject

experiencing an LH surge, and by the potential effect of kisspeptin-54 to advance the onset of LH surge in subjects.

It is noteworthy that all subjects had peak progesterone levels ≥ 21mmol/l following kisspeptin-54 treatment, which is highly suggestive of ovulation. Furthermore a corpus luteum was observed in all five subjects during the month of kisspeptin-54 treatment (Supplemental Table 2). Our data therefore suggest that all five subjects had ovulatory cycles during kisspeptin treatment. Further studies are required to determine if menstrual cycles during kisspeptin therapy have subtle physiological differences when compared with natural menstrual cycles, and to determine if anovulation is observed at a different frequency during kisspeptin treatment when compared with placebo treatment.

In summary, our data have important pharmacological implications; menstrual cyclicity persists in healthy women during a treatment regime of kisspeptin-54 previously demonstrated to cause tachyphylaxis in women with HA. Furthermore, kisspeptin-54 treatment is associated with an advanced timing of the luteal phase of menstrual cycle when compared with placebo. Further studies are required to determine if kisspeptin-54 therapy could have potential to treat patients with anovulatory reproductive disorders.

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Figure Legends

Figure 1. Study protocol diagram.

Five healthy women underwent a one-way crossover protocol. Twice-daily injections of saline and kisspeptin-54 were administered between days 7 and 14 during month 1 and 2 of the protocol, respectively. During each month of the protocol, subjects underwent a baseline GnRH test (day 1) and 8h blood sampling (day 6) prior to commencing injections. During the injection period (days 7-14), subjects underwent a 4h study (day 7) and two further 8h studies (days 11 and 14) immediately following injection of saline or kisspeptin-54. A GnRH test was performed 24h after cessation of saline or kisspeptin treatment (day 15). 'USS' denotes ultrasound examination and measurement of reproductive hormones. Day 1 was defined as the first day of menstrual bleeding.

Figure 2. Acute changes in plasma kisspeptin levels in healthy women receiving twice-daily injections of saline or kisspeptin-54.

A-C: Time-profiles of plasma kisspeptin immunoreactivity (IR) following sc bolus injection of saline or kisspeptin-54 at time 0min on menstrual days 7 (**A**), 11 (**B**) and 14 (**C**) of the study protocol. **D:** Bar graph comparing mean pre-injection kisspeptin IR following saline or kisspeptin-54 throughout the study protocol. All subjects commenced twice-daily treatment with saline or kisspeptin-54 on the morning of menstrual day 7 following their basal blood sample, and finished treatment on the morning of day 14. Data are presented as mean±SEM. *, $P<0.05$; **, $P<0.01$.

Figure 3. Acute changes in serum reproductive hormones in healthy women receiving twice-daily injections of saline or kisspeptin-54.

A-C: Acute increases in serum LH following sc bolus injection of saline or kisspeptin-54 at time 0min on menstrual days 7 (**A**), 11 (**B**) and 14 (**C**) of the study protocol. All subjects underwent treatment during menstrual days 7 and 14 with twice-daily sc bolus of saline or kisspeptin-54 injections. **D:** Summary bar graph comparing mean area under curve (AUC) LH

increase following saline or kisspeptin-54, on days 7, 11 and 14 of the study protocol. Data are presented as mean±SEM. *, P<0.05; **, P<0.01.

Figure 4. Levels of serum reproductive hormones throughout the menstrual cycle in healthy women receiving twice-daily injections of saline or kisspeptin-54.

A: Comparison of cycle length in individual healthy women undergoing twice-daily sc bolus injections of saline or kisspeptin-54 between menstrual days 7 and 14 of the study protocol. **B-D:** Levels of serum LH (**B**), FSH (**C**) and estradiol (**D**) during the menstrual cycle in healthy women undergoing twice-daily sc bolus injections of saline or kisspeptin-54 between menstrual days 7 and 14. **E:** Comparison of peak serum LH in individual healthy women undergoing twice-daily sc bolus injections of saline or kisspeptin-54 between menstrual days 7 and 14 of the study protocol. **F-G:** Levels of serum progesterone (**F**), and comparison of menstrual day of onset of luteal phase (**G**) in individual healthy women undergoing twice-daily sc bolus injections of saline or kisspeptin-54 between menstrual days 7 and 14 of the study protocol. The luteal phase of menstrual cycle was defined as beginning when serum progesterone was elevated >10nmol/l. *, P<0.05; **, P<0.01. Data are presented as mean±SEM.

Figure 5. Changes in radiological markers during the menstrual cycle of healthy women receiving twice-daily injections of saline or kisspeptin-54.

Mean values for diameter of largest ovarian follicle (**A**), number of ovarian follicles (**B**) and endometrial thickness (**C**) in healthy women undergoing twice-daily sc bolus injections of saline or kisspeptin-54 (6.4nmol/kg) between menstrual days 7 and 14. Data are presented as mean±SEM.

Figure 6. Changes in LH pulsatility during the menstrual cycle of healthy women receiving twice-daily injections of saline or kisspeptin-54.

All women underwent frequent blood sampling for 8h on menstrual days 6, 11 and 14 of the study protocol. Twice-daily sc bolus injections of saline or kisspeptin-54 (6.4nmol/kg) were self-administered between menstrual days 7 and 14. **A-B:** Mean values for secretory mass (A), and estimated pulses per 24h (B) are presented. **C-E:** Levels of pulsatile (**C**), basal (**D**) and total (**E**) LH secretion during each 8h sampling study are presented. Data are presented as mean±SEM.*, $P<0.05$; **, $P<0.01$.

Figure 1

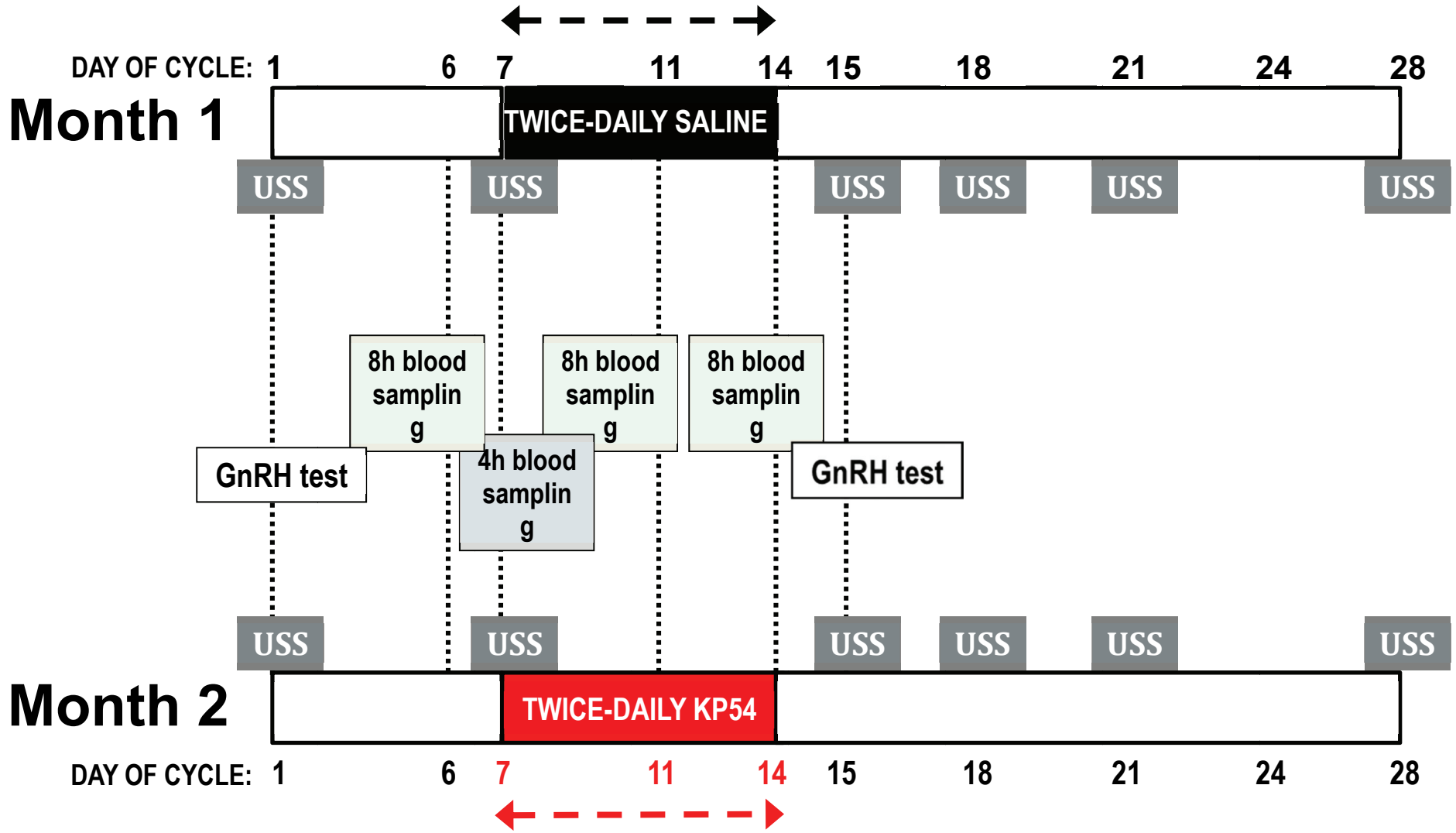

DAY OF CYCLE: 1 6 7 11 14 15 18 21 24 28
Month 1
TWICE-DAILY SALINE
USS
USS
USS
USS
USS
USS
8h blood samplin g
8h blood samplin g
8h blood samplin g
GnRH test
4h blood samplin g
GnRH test
USS
USS
USS
USS
USS
USS
Month 2
TWICE-DAILY KP54
DAY OF CYCLE: 1 6 7 11 14 15 18 21 24 28

Figure 2

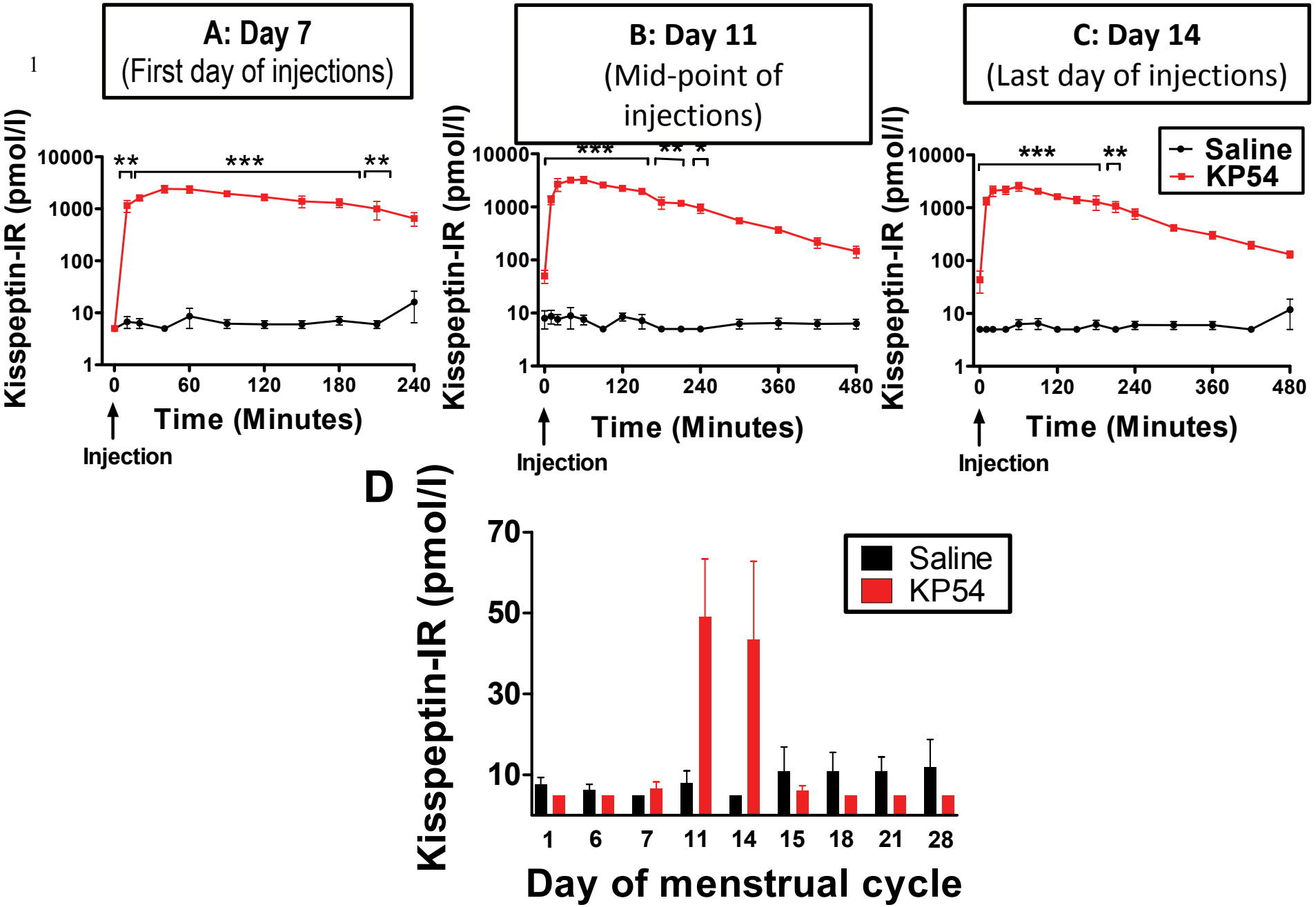

A: Day 7
(First day of injections)
B: Day 11
(Mid-point of injections)
C: Day 14
(Last day of injections)
Kisspeptin-IR (pmol/l)
10000
1000
100
10
1
**
***
**
0
60
120
180
240
Time (Minutes)
Injection
***
**
*
0
120
240
360
480
Time (Minutes)
Injection
***
**
Saline
KP54
0
120
240
360
480
Time (Minutes)
Injection
D
Kisspeptin-IR (pmol/l)
70
50
30
10
Saline
KP54
1
6
7
11
14
15
18
21
28
Day of menstrual cycle

Figure 3

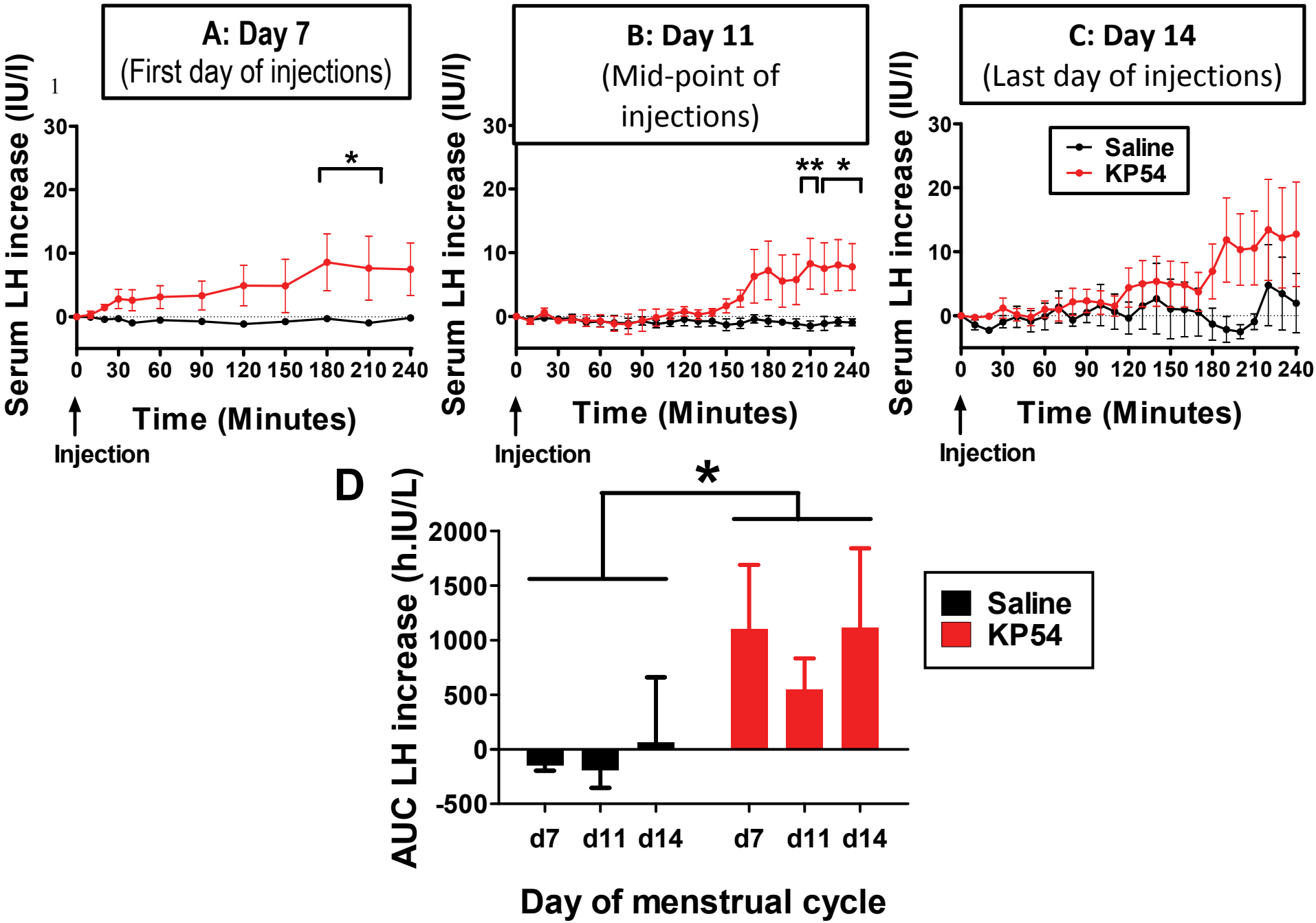

A: Day 7
(First day of injections)
B: Day 11
(Mid-point of injections)
C: Day 14
(Last day of injections)
Serum LH increase (IU/l)
Time (Minutes)
Injection
Saline
KP54
*
**
D
AUC LH increase (h.IU/L)
d7
d11
d14
Day of menstrual cycle

Figure 4

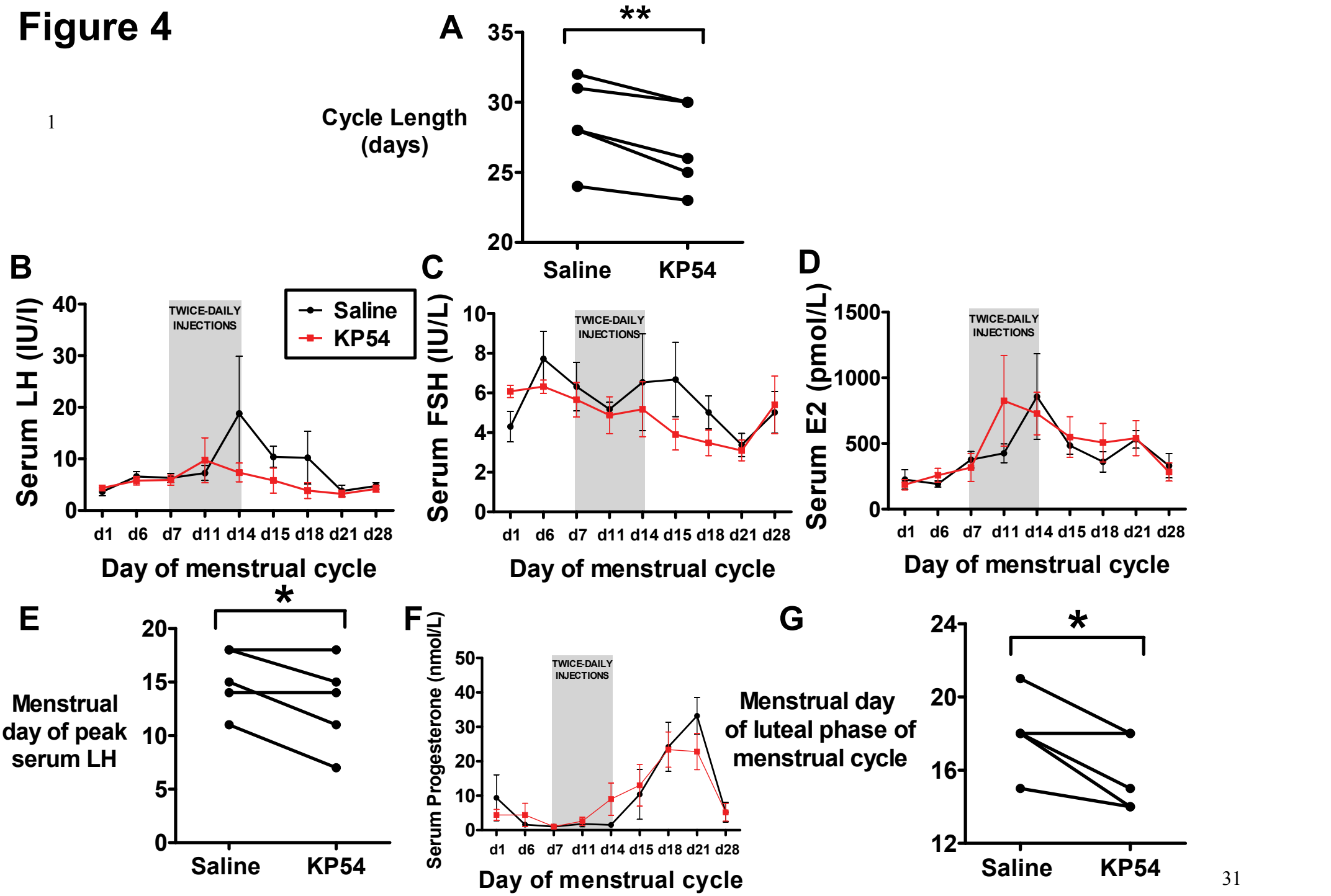

A
**
Cycle Length (days)
35
30
25
20
Saline
KP54
B
Serum LH (IU/l)
40
30
20
10
0
TWICE-DAILY INJECTIONS
Saline
KP54
d1 d6 d7 d11 d14 d15 d18 d21 d28
Day of menstrual cycle
C
Serum FSH (IU/L)
10
8
6
4
2
0
TWICE-DAILY INJECTIONS
d1 d6 d7 d11 d14 d15 d18 d21 d28
Day of menstrual cycle
D
Serum E2 (pmol/L)
1500
1000
500
0
TWICE-DAILY INJECTIONS
d1 d6 d7 d11 d14 d15 d18 d21 d28
Day of menstrual cycle
E
*
Menstrual day of peak serum LH
20
15
10
5
0
Saline
KP54
F
Serum Progesterone (nmol/L)
50
40
30
20
10
0
TWICE-DAILY INJECTIONS
d1 d6 d7 d11 d14 d15 d18 d21 d28
Day of menstrual cycle
G
*
Menstrual day of luteal phase of menstrual cycle
24
20
16
12
Saline
KP54

Figure 5

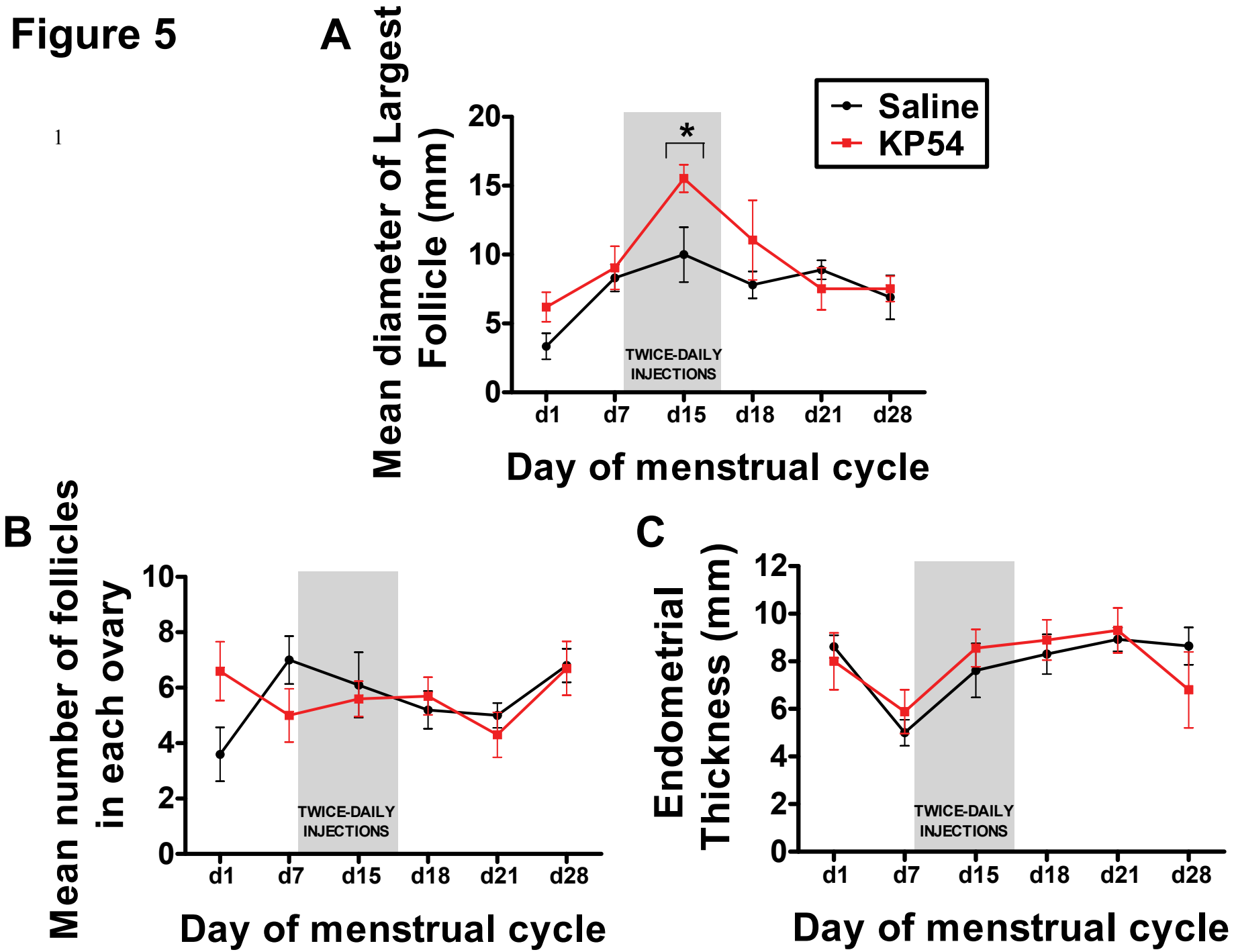
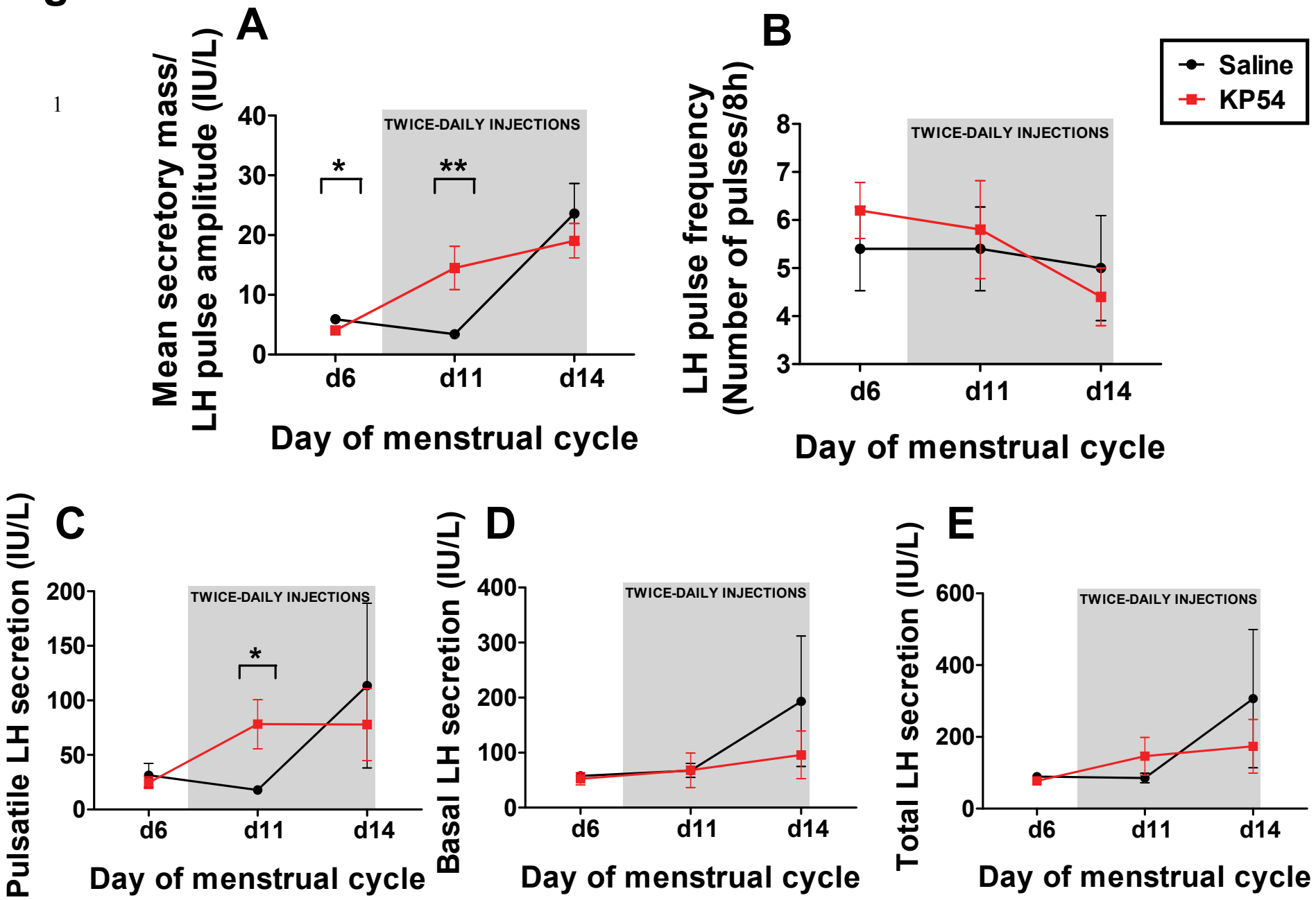

A
Mean diameter of Largest Follicle (mm)
Saline
KP54
*
TWICE-DAILY INJECTIONS
Day of menstrual cycle
B
Mean number of follicles in each ovary
TWICE-DAILY INJECTIONS
Day of menstrual cycle
C
Endometrial Thickness (mm)
TWICE-DAILY INJECTIONS
Day of menstrual cycle

Figure 6


A
Mean secretory mass/
LH pulse amplitude (IU/L)
40
30
20
10
0
TWICE-DAILY INJECTIONS
*
**
d6
d11
d14
Day of menstrual cycle
B
LH pulse frequency
(Number of pulses/8h)
8
7
6
5
4
3
TWICE-DAILY INJECTIONS
d6
d11
d14
Day of menstrual cycle
Saline
KP54
C
Pulsatile LH secretion (IU/L)
200
150
100
50
0
TWICE-DAILY INJECTIONS
*
d6
d11
d14
Day of menstrual cycle
D
Basal LH secretion (IU/L)
400
300
200
100
0
TWICE-DAILY INJECTIONS
d6
d11
d14
Day of menstrual cycle
E
Total LH secretion (IU/L)
600
400
200
0
TWICE-DAILY INJECTIONS
d6
d11
d14
Day of menstrual cycle