—Technology Report—

The efficacy of a newly developed neurokinin 3 receptor agonist B21-750 on luteinizing hormone secretion in cycling goats

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Abstract. This study aimed to investigate the efficacy of a newly developed NK3 receptor agonist (B21-750) on the secretion of luteinizing hormone (LH) in association with ovarian steroid hormones during the follicular phase (FP, n = 5) and luteal phase (LP, n = 5) of Shiba goats. The FP group was treated with both prostaglandin $F_{2\alpha}$ and progesterone-controlled internal drug release (CIDR) inserts for 10 d, and B21-750 (200 nmol) was injected 12 h after removing the CIDR. Meanwhile, the LP group received B21-750 injections on a day during the mid-luteal phase. LH secretion increased at 1 h after B21-750 injection in both groups. The percent changes in the area under the curve of LH was higher during the hour after injection than during the hour before injection in both groups. Thus, this study demonstrated that B21-750 induces rapid LH secretion for a short period during both the follicular and luteal phases.
Key words: Estrous cycle, Goats, Luteinizing hormone, Neurokinin B

Neurokinin B (NKB) signaling controls reproductive development [1–3], and agonists of the neurokinin 3 receptor (NK3R), which preferentially binds to NKB, have been reported to regulate gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) secretion in rats [3], sheep [4, 5], monkeys [6], and humans [7]. In addition, the central administration of an NKB compound has been reported to stimulate the neural generator that governs pulsatile GnRH secretion in goats [8], and NK3R activity has been reported in both the hypothalamic-pituitary system and gonadal tissues (e.g., ovary) [9].

Based on the above mentioned studies, our pilot experiment has evaluated the effect of an NK3R agonist on the reproductive function of goats. The experiment revealed that intermittent intravenous administration of senktide, a known NK3R agonist, stimulates LH secretion and follicular development and causes ovulation within 96 h after treatment in anestrous goats [10], thereby demonstrating the potential value of NKB application in the treatment of ovarian dysfunction. However, several studies have suggested that the development of a more potent NK3R agonist is needed for the stimulation of LH secretion in clinical applications. This was demonstrated in our previous study, i.e. LH secretion is affected by the method of senktide administration. In goats, a continuous 6-h intravenous infusion of senktide during the follicular phase stimulated an im-mediate increase of LH secretion, whereas a single intravenous injection was insufficient to induce any significant increase in plasma LH concentration [11]. Continuous intravenous administration of senktide is considered less practical in the field. Study in ewes [4] have demonstrated that a single injection of senktide (1 nmol) into the third ventricle stimulates a rapid and sustained increase in LH concentration, followed by a surge-like release during the follicular phase. However, this stimulatory action disappeared during the luteal phase, and neither the frequency nor amplitude of LH changed after senktide injection; this suggests that the effect of senktide on LH secretion diminishes in the presence of progesterone in circulation.

Recently, a newly developed NK3R agonist, B21-750, was designed for prolonged residence time in the blood, with the aim of promoting LH secretion in goats. B21-750 is a polyethylene glycol (PEG)-peptide conjugate in which methoxy-poly(ethylene glycol) amine 750 (MPEG amine, MW 750) is conjugated with [Glu1]-senktide at the Glu1 side chain *via* an amide linkage [12].

To evaluate the effect of B21-750 on the reproductive function of goats, in the present study, we aimed to measure the response of LH secretion to a single intravenous injection of B21-750 in association with ovarian steroid hormone during the follicular and luteal phases in goats. The dose of B21-750 used was 200 nmol/head so that the results could be compared to our previous study, which evaluated the efficacy of senktide at the same dose [11].

Rapid increases in LH secretion were detected in both the FP and LP groups during the first 1 h after B21-750 injection (Fig. 1). In the FP group, the area under the curve (AUC) of LH concentration during the first hour after injection (1.07 ± 0.16 ng/ml) did not differ significantly from that during the hour preceding injection (0.59 ± 0.10 ng/ml), and a similar trend was observed in the LP group (0.17 ± 0.04 ng/ml before *vs.* 0.34 ± 0.19 ng/ml after). However, the percent

changes in the AUC of LH were significantly greater during the hour after injection than during the hour before injection, regardless of treatment group (Fig. 2). The percent changes in AUC of LH of the FP group (184.9 ± 33.4%) and LP group (215.4 ± 161.4%) during the hour after injection were similar.

In the LP group, plasma progesterone concentration after injection did not significantly differ with that during the hour before injection (Fig. 3, upper panel). However, the AUC of plasma progesterone during the hour after injection (13.4 ± 2.7 ng/ml) was significantly greater than that observed during the period before injection (8.6 ± 1.9 ng/ml; Fig. 3, lower panel). Meanwhile, in the FP group, no significant differences were detected in the plasma estradiol concentration (Fig. 4).

In the FP group, ovulation occurred at 3.0 ± 0.8 days after treatment. All goats from the FP group exhibited clear estrus behavior within 2 days after treatment, followed by ovulation on the next day. Meanwhile, all goats in the LP group exhibited clear estrus behavior within 9.2 ± 1.8 days after treatment, and the length of the estrous cycle during the treatment period was within the normal range (i.e., 20.2 ± 1.5 days). The length of the subsequent estrous cycle, which was calculated as the period from the first ovulation detected after treatment until ovulation from the next cycle, was within the normal range in both the FP and LP groups (18.3 ± 3.2 and 20.2 ± 1.5 days, respectively).

The results of the present study demonstrate the significant effects of B21-750 on the reproductive endocrine function of female goats. The dose of B21-750, which was the same as the amount of senktide used in a previous study [11], stimulated LH secretion during the follicular phase of goats. In addition, the present study also demonstrated that LH and progesterone secretion respond immediately to B21-750 during the luteal phase. Our pilot study of ovariectomized goats demonstrated that the stimulatory time after the treatment of B21-750 on the hypothalamic multi-unit activity governing GnRH neurosecretion is approximately three times longer than that of senktide [12]. This pharmacological characteristic may contribute to the duration of the drug's agonistic action since a rapid and significant increase in the AUC of LH secretion was detected during the first hour after injection.

In contrast to the results of the present study, senktide has been reported to elicit a weak LH response during the luteal phase in ewes [4]. The local administration of senktide into the retrochiasmatic region of ewe hypothalamus had no effect on LH pulse frequency or amplitude. Other researchers have also reported that more kisspeptin neurons were activated by GnRH neurosecretion in the hypothalamus during the follicular phase than during the luteal phase [13]. Furthermore, the non-significant difference between the percent LH changes of the FP and LP groups suggests that B21-750 potently stimulates LH secretion during both the follicular and luteal phases. Indeed, previous studies in ewes have suggested that increases in estradiol secretion during follicular phase are not essential for the stimulatory actions of NKB receptor activation [4]. It is possible that the pharmacological action of B21-750 reduces the inhibitory effect of progesterone on LH secretion. Previous research in ewes has demonstrated that the central injection of senktide induces a surge-like release of LH during the breeding season [4]. Thus, the stimulatory effect of B21-750 on LH secretion during the luteal phase
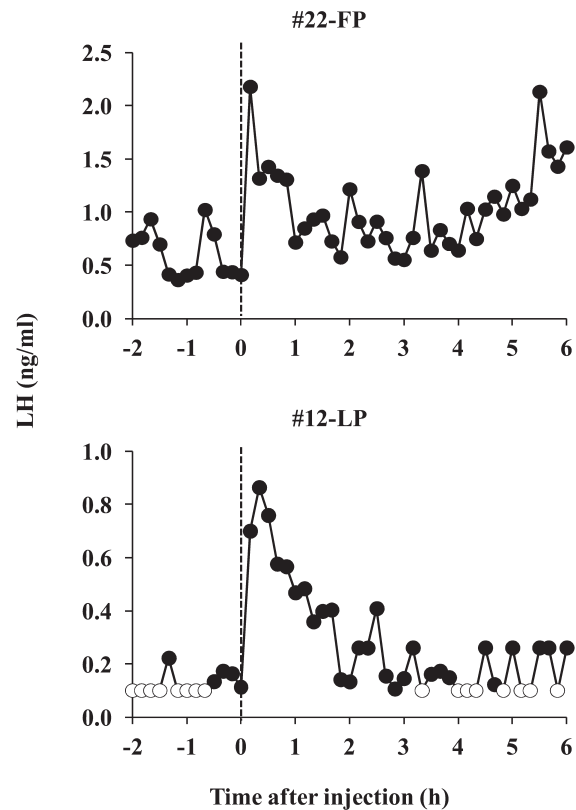

Fig. 1. Representative profile of luteinizing hormone (LH) from –2 to 6 h after B21-750 injection in the follicular (FP, upper panel) and luteal (LP, lower panel) phase groups. The dashed lines indicate the time of B21-750 injection. Open circles indicate values that were below the sensitivity of the assay (i.e., 0.1 ng/ml).

might be involved in the activation of the hypothalamic GnRH surge generator that governs LH surge.

In the FP group, it is unlikely that B21-750 treatment influenced the timing of ovulation, which occurred 3–4 days after injection, because ovulation generally occurs 3 to 4 days after the removal of CIDR inserts in the estrus-synchronized protocol of ruminants, including goats. However, the continuous intravenous infusion of senktide during the follicular phase can stimulate ovulation within 2 days after treatment [11]. The ability of senktide to induce early ovulation results from sustained increases in LH secretion, which accelerates follicular maturation. In the present study, B21-750 failed to elevate plasma estradiol concentrations. A transient increase in LH secretion, induced by a single injection of 200 nmol B21-750 during the follicular phase may be insufficient to stimulate follicular development.

In the LP group, an increase in progesterone level occurred coincidently with the elevation of LH concentration. *Foster et al.* [14] reported that, in cows, the injection of GnRH during the luteal phase resulted in an increase in LH that corresponded with an increase in progesterone. The simultaneous response of LH and progesterone also indicates that B21-750 treatment induced the secretion of progesterone from the corpus luteum. Indeed, it has
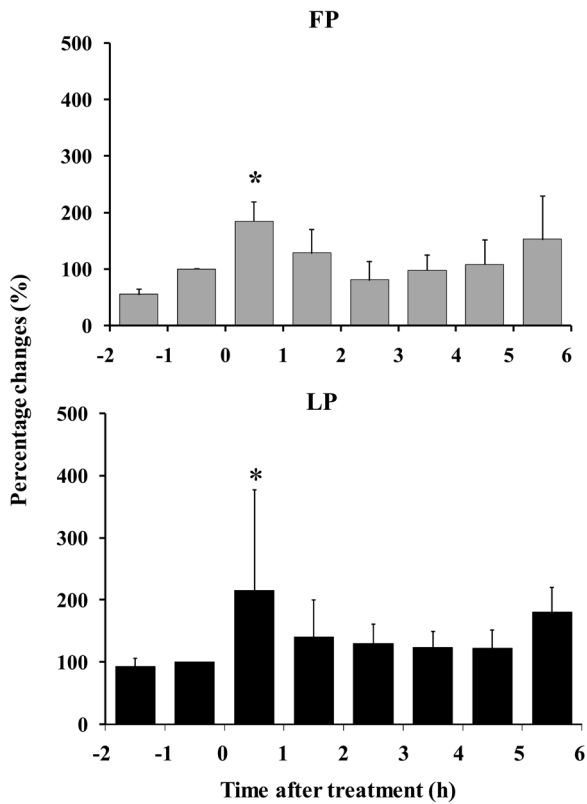
Fig. 2. The percentage changes in area under the curve (AUC) of LH after B21-750 treatment in the FP (upper panel) and LP (lower panel) groups. Asterisks indicate significant differences, when compared to the value for the period from −1 to 0 h preceding injection (P < 0.05, Dunnett's test).

Fig. 3. Effect of B21-750 injection on progesterone levels in the LP group. Plasma progesterone concentrations were measured at 10-min intervals (upper panel), whereas the area under the curve was measured at 1-h intervals (lower panel). Asterisk indicates significant differences, when compared to the value for the hour preceding injection (P < 0.05, Dunnett's test). Arrow indicates injection time.

been reported that NK3R mRNA is expressed in bovine corpus luteum [15]. In addition, Löffler et al. [9] detected NK3R mRNA in rat ovaries and reported that NK3R agonist treatment increased the number of corpora lutea; however, the direct effects of NK3R agonist or B21-750 on the ovary are still unclear.

In conclusion, the present study demonstrated that a newly developed NK3R agonist (B21-750) potently stimulates LH secretion in female goats during both the follicular and luteal phases. However, the effects of B21-750 on ovarian steroid hormones were dependent on the estrous cycle stage. Further studies are needed to determine the effective dose range of B21-750 for stimulating LH secretion and follicular development.

Methods

Animals and housing

Seven non-seasonal-breeding Shiba goats (5.0 ± 1.9 years, 28.8 ± 3.7 kg) were maintained and used at Tokyo University of Agriculture and Technology, Tokyo, Japan. All goats were kept under natural daylight in a paddock that allowed the goats to move freely and were fed alfalfa hay cubes (660 g dry matter/day) with clean water and mineral salt blocks available *ad libitum*. During the day of
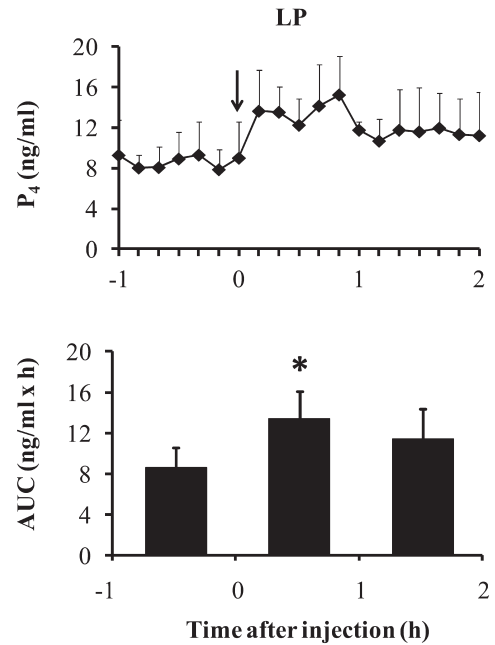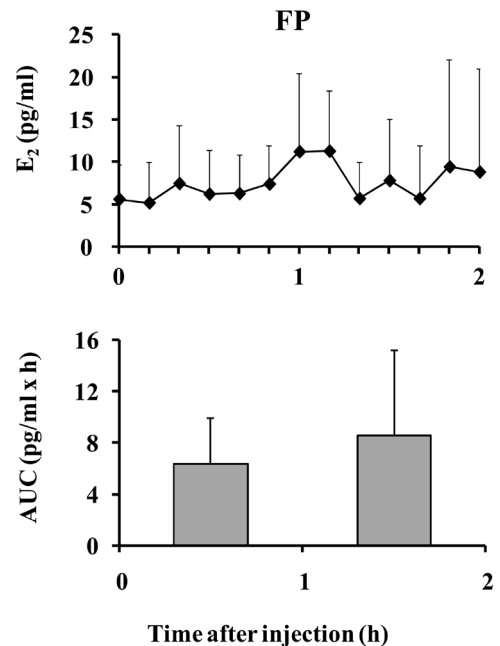

Fig. 4. Effect of B21-750 injection on estradiol levels of follicular-phase female goats. Plasma estradiol concentrations were measured at 10-min intervals (upper panel), whereas the area under the curve was measured at 1-h intervals (lower panel). Injection was performed at 0 h.

serial blood sampling, the goats were moved to individual cages. All procedures were approved by the University Committee for the Use and Care of Animals of the Tokyo University of Agriculture and Technology (No. 22-67).

Experimental design

The goats were assigned to follicular-phase (FP, n = 5) or luteal-phase (LP, n = 5) groups, and three goats were included in both groups. These three goats were kept untreated for at least one estrous cycle before being allocated to either phase. In the FP group, estrus was synchronized by the injection of 2 mg prostaglandin $F_{2\alpha}$, (Pronalgon F, contained dinoprost tromethamine; Zoetis, Tokyo, Japan) during the luteal phase, followed by the insertion of a progesterone-controlled internal drug release insert (CIDR-G EAZI-BREED; Pfizer, Auckland, New Zealand) for 10 days. B21-750 (200 nmol/head) was injected 12 h after removing the CIDR inserts, and blood was serially collected. In the LP group, the injection of 200 nmol B21-750 and serial blood sampling were conducted either 10 days (n = 2) or 11 days (n = 3) after ovulation.

Three milliliters of blood was collected from each animal using an indwelling catheter, at 10-min intervals, from 2 h before to 6 h after injection. The samples were centrifuged at 3000 rpm at 4°C, and the separated plasma was kept at –20°C until assayed for LH, progesterone, and estradiol. Transrectal ultrasounds (HS-1500V; Honda Electronics, Aichi, Japan), equipped with a linear probe, were conducted at 24- or 48-h intervals, in order to determine the time of ovulation, which was defined as the disappearance of a large follicle that had been observed the previous day and was reconfirmed by the development of a corpus luteum at the same location [16].

Hormone assay

Plasma LH concentrations were measured in duplicate using radioimmunoassay, as described previously [17]. Intra- and inter-assay coefficients of variation were 9.8 and 8.3%, respectively, and the average sensitivity of the assay was 0.1 ng/ml. Plasma estradiol concentration was measured using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan, USA), following extraction by dichloromethane (Wako Pure Chemical Industries, Osaka, Japan). The intra-assay coefficient of variation was 10.8%, and the sensitivity of the assay was 0.68 pg/ml. Plasma progesterone was extracted as described previously [18], using diethyl ether (Wako Pure Chemical Industries), and progesterone concentrations were measured by enzyme immunoassay. The intra-assay coefficient of variation was 14.1%, and the sensitivity of the assay was 0.84 ng/ml.

Statistical analysis

All experimental data are expressed as mean ± SD values. The data were analyzed using a XLSTAT software package (Addinsoft, Long Island, NY, USA). Data were analyzed using one-way ANOVA, followed by Dunnett's or Tukey's tests, using a significance level of P < 0.05.

Acknowledgments

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