The Kisspeptin analogue C6 induces ovulation in jennies

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Abstract:
Kisspeptins (KPs) are the most potent stimulating neurotransmitters of GnRH release, and consequently KP administration triggers LH and/or FSH release. In small ruminants, KP or its analogs induced an LH surge followed by ovulation in both cyclic and acyclic animals, while in the mare KP only increased LH plasma levels but failed to induce ovulation. This study in jennies compares the endocrinological effects, ovulatory and pregnancy rates of the KP analog C6 and the GnRH analog buserelin acetate. The ovarian activity of nine Amiata jennies was monitored daily by transrectal ultrasound for three complete estrous cycles. Jennies in estrus were assigned, to one of three treatment groups: 50 nmol of the KP analog C6 (injected twice, 24 hours apart, C6 group); 0.4 mg buserelin acetate (injected once, Bu group); and 2 mL of saline (injected once, CTRL group). Blood samples were collected at Day-1 (-24h) Day0 (h0, before treatment), h2, h4, h6, h8, h10, h24 (before second treatment with C6), h26, h28, h30, h32, h34, h48 and every 24 hours until ovulation. Jennies were inseminated once at h24 with fresh extended semen from a donkey stallion. Pregnancy diagnoses were performed 14 days after ovulation. On days 5, 10, and 14 after ovulation, for every CL the cross-sectional area (CSA) and the vascularized area (VA) were recorded by color Doppler ultrasound and measured. Significantly higher plasma LH levels were found after induction between the Bu and CTRL groups at h6 and h8 ($P<0.05$), while tendentially higher differences were found between the Bu/C6 groups and CTRL at h10. Five/9, 4/9, and 2/9 jennies ovulated between 24 and 48 hours after induction from the Bu, C6, and CTRL groups respectively, ($P>0.05$). Correlations between corpora lutea CSA and VA with serum progesterone concentration were $r=0.31$, $P=0.01$, $r=0.38$, $P=0.01$, respectively. Pregnancy rates after artificial insemination did not differ among groups (CTRL: 6/9, 66.7%; C6: 7/9, 77.8%; Bu: 6/9, 66.7%; $P>0.05$). Ovulation rates after C6 treatment were comparable to that of Bu, although not different from the CTRL. Pregnancy rates were comparable to the literature in terms of fresh extended donkey semen in every group. This study suggests that stimulation of the Kp system in jennies, in contrast to findings observed in mares, induces ovulation. Further studies using higher doses and/or more animals are needed to better characterize the efficacy of C6 in jennies.

Keywords: Kisspeptin, buserelin acetate, LH, induction of ovulation, donkey, doppler ultrasound.

1. Introduction

The discovery of the kisspeptin (KP) system profoundly modified our understanding of the neuroendocrine mechanisms involved in reproduction control. Kisspeptins (KPs) are a family of neurotransmitters derived from a unique precursor, encoded by the KISS1 gene, which binds and activates a specific receptor, KISS1R [1–5]. Ten amino acids in the C-terminal position, named KP10, are common to all KPs. KPs are the most potent stimulating neurotransmitter of GnRH release from GnRH neurons. Due to their pivotal role in stimulating GnRH release, KPs have been implicated in key aspects of reproduction such as the modulation of puberty onset, the regulation of gonadotropin release, the metabolic control of fertility, sexual behavior, and seasonal reproduction [6-20].

Particular attention has focused on the potential of using KPs to trigger ovulation in livestock. In several farm species (sheep, goats, cattle, pigs, and horses), the administration of KP, either as a bolus injection or prolonged perfusion, stimulates the release of LH and/or FSH [21–28]. In small ruminants (goats and sheep), the perfusion with KP10, or bolus injection of one of its analogs TAK-683 or C6, was shown to induce an LH surge followed by ovulation in both cyclic and acyclic animals [22,25,29–33]. These results provide further evidence that stimulation of KISS1R provides an interesting opportunity to manage reproduction in livestock and that KP analogs offer advantages (single injection vs perfusion) for field applications.

Concerning equids, few data on the effects of KP on reproduction are available. In the mare, administration of KP10 led to an increase in the plasma level of gonadotrophins. However, regardless of the dose used, perfusion duration, and mare physiological status, it was unable to trigger ovulation [27,34]. Recently a new set of experiments using the C6 analog were performed, however also in this case, treatment was unable to induce ovulation [unpublished observations, 35]. Does this indicate that the KP system is not a viable alternative to trigger ovulation in equids? The only other domestic equid is the donkey. However, to the best of our knowledge, there are no published studies on the action of KP in the donkey. Although jennies and mares share several aspects of reproductive physiology, there are also significant differences between the two species [36]. The oestral cycle in jennies is longer (between 25 and 30 days) compared to mares (21-22 hours). In jennies ovulation occurs when the follicle reaches 25-32 mm in diameter, conversely in the mare, the size of an ovulatory follicle is highly variable and can range between 35 and 55-60 mm in diameter. In addition, jennies seem to be less seasonal than mares, with a lower percentage showing seasonal anoestrus and of short duration, between 39 to 72 days [37]. With respect to ovulation induction, the jenny ovulates after lower doses of GnRH (at least 0.04 mg) [38] analogs, whereas mares require a larger dose (at least 0.125 mg) [39].

To further explore whether there is also a difference upstream of the GnRH system, we investigated the role of the KP system in the jenny. We thus used the KP analog C6 and compared its effectiveness in triggering ovulation to that of current treatment, i.e., the administration of GnRH analogs (buserelin acetate).

2. Materials and methods

2.1 *Ethical statement*: The study was approved with authorization no. 857/2018-PR, issued on 02/11/2018, pursuant to art.31 of Legislative Decree no. 26/2014, by the Directorate General of Animal Health and Veterinary Medicines of the Italian Ministry of Health.

2.2 *Animals*: Experiments were performed using nine Amiata jennies aged between 3 and 10 years, which were cyclic, in good general health, and with a body condition score of 3/4 [40]. Animals were maintained in paddocks and fed with hay and water. Ovarian activity was monitored daily by transrectal ultrasound for three complete estrous cycles. The presence of a 28 mm diameter follicle, along with ultrasound imaging and behavioral manifestations typical of estrus, was considered as day -1 (d-1), while the presence of a 32 mm diameter follicle was considered as d0. The jennies were tested daily with a teaser donkey stallion from d-1 to the day of ovulation.

Experiments were performed in three consecutive cycles and jennies were assigned to one of the three treatment groups:

I. "C6" Group: 50 nmol of the KP analog C6 diluted in 2 mL of saline at 37°C. This group was injected intramuscularly at 8:30 Day 0 (d0, hour 0 = h0) and again at 8:30 of g1 (h24).98
II. "Bu" Group: this group was injected with 0.4 mg buserelin acetate, a GnRH analog (Suprefact®, AVAS Pharmaceuticals S.r.l, Italy) at D0 (h0). We have previously shown thatthere is no significant difference in the induction of ovulation after a single or double injection of busrelin acetate [38]. We thus chose to administer a single dose of the treatment.
III. "CTRL" Group: this group was injected with 2 mL of saline at D0 (h0).

For all groups, blood samples were collected as follows: d-1 (-24h) d0 (h0, before treatment), h2, h4, h6, h8, h10, h24 (before second treatment with C6), h26, h28, h30, h32, h34, h48 and, if the jennies did not ovulate, one sampling per day until ovulation.

On days 5, 10, and 14 after ovulation, for every CL, three images were taken in color flow mode at a standard depth of 10 cm, with a 5.0 MHz frequency, 70% gain, and with a pulse repetition frequency of 2.8, at maximum cross-sectional diameter. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda). Cross-sectional areas of the CL (CSA) were measured, and all lacunas were excluded. Images were cropped and colored zones selected (vascularized area, VA). For each image, the CSA and VA areas of the CL were measured and evaluated by counting the number of gray and color pixels, respectively. Using the same software, the number of pixels resulting from the measurement was converted into mm2 for CSA and VA. The mean CSA and VA areas were calculated using ImageJ (National Health Institutes, Bethesda), and the mean ratio of VA to CSA was taken as an index of vascularization (IV) [41].

Blood samples between g0 and g1 were collected from a jugular vein catheter. To prevent stress and unnecessary pain to the animal, the venous catheter was inserted into the left jugular vein after general sedation with 0.03 mg·kg-1 of acepromazine (Prequillan, Fatro, Italy) and local subcutaneous injection of 1 mL of lidocaine hydrochloride (Zoetis, Italia). g1 blood sampling was then performed by jugular venipuncture.

Before ovulation, blood was collected in tubes containing lithium heparin, the plasma was separated by centrifugation, and frozen at -20°C until the evaluation of LH plasma concentrations. To evaluate the progesterone (P4) concentration, serum blood samples were collected on days 5, 10, and 14 after ovulation in tubes without anticoagulants. The serum was separated and frozen at -20°C.

2.3 *Hormone assays*: Plasma LH concentration was assayed by RIA following a procedure adapted from Guillaume et al. [42]. All samples were run in duplicate. Standards were prepared using plasma from a mare vaccinated against GnRH with an equine reference hormone (eLH, NHPP AFP 5130A; Dr.A.F. Parlow). The eLH was labeled with 125I (PerkinElmer NEN Radiochemicals (500µCi) using 10µg of Iodogen (Pierce, Interchim). The antibody (anti-LH AFP-240580; Dr. A.F. Parlow) was used at a final dilution of 1:440,000. The detection limit of the assay was 0.25 ng·mL-1. The mean intra- and interassay coefficients of variation (CV) for plasma containing 3 ng·mL-1of LH (corresponding to 50% bond) were 4% and 10%, respectively.

Plasma concentration of progesterone (P4) was measured using an ELISA sandwich following the procedure previously described [43]. The detection limit was 0.25 ng·mL-1. The intra-assay CV for PBS containing 3.5 ng·mL-1 of P4 was 3.7%.

2.4 *Artificial insemination and pregnancy diagnosis*: The jennies were artificially inseminated (AI) with fresh semen (1x109 total spermatozoa/dose) only once at d1. Induced ovulations were considered to occur between 24 and 48 hours after treatment. Pregnancy diagnoses were made 14 days after ovulation, when the jennies were inoculated with a PGF2alpha analog (3 mg/im Alfaprostol, Gabbrostim, CEVA VETEM, Italy) and assigned to a new cycle of treatment, at the beginning of which (g-1) they underwent uterine washing and cytological examination.

2.5 *Statistical analyses*: JMP®, Version Pro 16 (SAS Institute Inc., Cary, NC, 1989–2021) was used to perform the statistical analyses.

The number of ovulations occurring between 24 and 48 hours and pregnancy rates at 14 days were evaluated by Fisher's exact test.

Data (LH, CSA, VA, IV and P4) were analysed with the following linear model:

$$y_{ijz} = m + Treatment_i + Time_j + Treament_i \times Time_j + Animal_z[Treatment_i] + e_{ijz}$$

where

y_{ijz} = variables;

m= mean;

$Treatment_i$= fixed effect of the i^{th} Treatment (CTRL, C6, Bu);

$Time_j$ = fixed effect of the j^{th} Time (14 levels for LH and 3 levels for CSA, VA, IV and P4);

$Animal_z$ = random effect of the z^{th} animal (7 levels);

e_{ijz}= random error.

Orthogonal contrasts were performed as post-hoc analysis for the $Treament_i \times Time_j$ effect.

A Pearson's product-moment correlation was run to assess the relationship between corpora lutea ultrasonographic characteristics (CSA, VA, and IV) vs serum progesterone concentration.

3. Results

Mean ovulation times among groups were not statistically different (CTRL: 93.3 ± 36.8 hours; Kiss:61.3 ± 27.1 hours; Bu: 66.7 ± 33.5 hours; P>0.05).
Table 1 reports the ovulation rates among jennies included in the CTRL, C6, and Bu groups.

Table 1. Ovulation rates after induction with saline (CTRL), kisspeptin analog C6 (C6), or buserelin acetate (Bu).

Group	**Ovulation time**		
	0-24 h	*24-48 h*	*> 48 h*
Bu (%)	1/9 (11.1%)	5/9 (55.6%)	3/9 (33.3%)
C6 (%)	1/9 (11.1%)	4/9 (44.4%)	4/9 (44.4%)
CTRL (%)	0/9 (0.0%)	2/9 (22.2%)	7/9 (77.8%)

P>0.05

Figure 1 shows differences among timepoints and treatments (CTRL, Bu, C6) of LH plasma concentration.

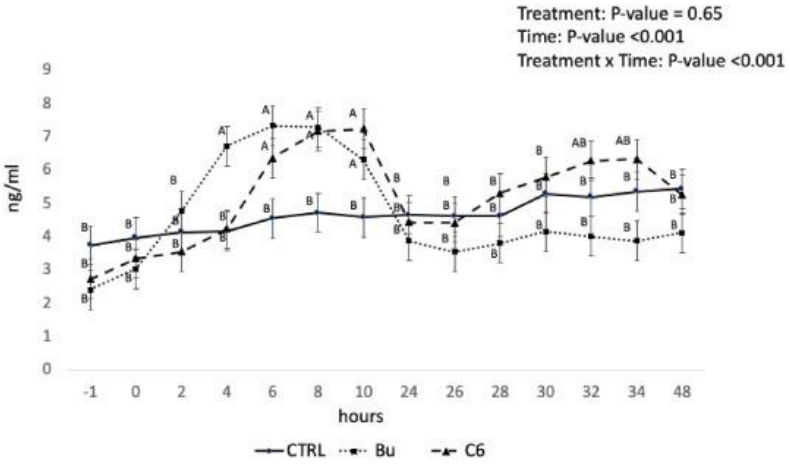

Figure 1: Differences among [LH] (Mean ± SE) for treatment (CTRL, Bu and C6) and time (hour: -1, 0, 2, 4, 6, 8 10, 24, 26, 28, 30, 32, 34 and 48). Different letters (A,B) indicate significant differences (P<0.05).

Pregnancy rates after artificial insemination did not differ among groups (CTRL: 6/9, 66.7%; C6: 7/9, 77.8%; Bu: 6/9, 66.7%; P>0.05).

Correlations between corpora lutea CSA, VA and IV with serum progesterone concentration were r=.31, P=0.01, r=.38, P=0.01 and 0.04 P=0.73, respectively.

Tables 2, 3, 4 and 5 report the differences among corpora lutea: CSA, VA, IV, and serum progesterone concentration for the treatment group (CTRL, Bu, and C6).

Table 2: Corpus luteum CSA (mean ± SE; mm2) at days 5, 10 and 14 after ovulation (D5, D10, D14), in Amiatina jennies, after induction of ovulation with saline (CTRL), buserelin acetate (Bu) or the kisspeptin analog C6 (C6).

Group	CTRL	Bu	C6	SE	P-value		
Day after ovulation					Treatment	Time	Treatment x Time
D5 (mm^2)	1186	1135.7	1025.2	143.9	0.861	0.317	0.637
D10 (mm^2)	1180.9	1187.7	1123.6				
D14 (mm^2)	1065.7	1171.0	1045.8				

Table 3: Corpus luteum VA (mean ± SE; mm2) at days 5, 10 and 14 after ovulation (D5, D10, D14), in Amiatina jennies, after induction of ovulation with saline (CTRL), buserelin acetate (Bu) or the kisspeptin analog C6 (C6).

Group	CTRL	Bu	C6	SE	P-value		
Day after ovulation					Treatment	Time	Treatment x Time
D5 (mm^2)	116.0	115.5	89.5	25.0	0.679	0.029	0.241
D10 (mm^2)	155.4	143.9	155.9				
D14 (mm^2)	113.9	181.4	128.1				

Table 4: Corpus luteum IV (mean ± SE, ratio) at days 5, 10 and 14 after ovulation (D5, D10, D14), in Amiatina jennies, after induction of ovulation with saline (CTRL), buserelin acetate (Bu) or the kisspeptin analog C6 (C6).

Group	CTRL	Bu	C6	SE	P-value		
Day after ovulation					Treatment	Time	Treatment x Time
D5	0.11	0.12	0.09	0.04	0.619	0.085	0.253
D10	0.13	0.13	0.15				
D14	0.12	0.23	0.14				

Table 5: Serum progesterone concentration (mean ± SE; ng/mL) at days 5, 10 and 14 after ovulation (D5, D10, D14), in Amiatina jennies, after induction of ovulation with saline (CTRL), buserelin acetate (Bu) or the kisspeptin analog C6 (C6).

Group	CTRL	Bu	C6	SE	P-value		
Day after ovulation					Treatment	Time	Treatment x Time
D5 (ng/mL)	17.1	14.3	15.2	1.6	0.950	0.002	0.313
D10 (ng/mL)	20.4	19.3	19.3				
D14 (ng/mL)	16.0	18.5	18.7				

4. Discussion

The use of KP, or its analogs, is promising for the management of reproduction in livestock due to their capacity to trigger ovulation. However, to date, few data on equids are available and not always concordant with the results obtained in other species. The stimulation of the KP system in the mare was found to increase the gonadotropin level but did not induce ovulation. Our results in jennies, which showed that the KP analog C6 induced ovulation in a similar way to buserelin acetate, suggest that regarding the action of the kisspeptin system, the horse could be an exception also among equids.

In these experiments with C6, we used two injections at 24-hour intervals. A double injection was used for two reasons. Firstly, a single injection of kisspeptin [27,44,45] has been shown not to trigger ovulation in the mare. Secondly, our unpublished experiments with C6 in the ewe suggest that two or three injections at 24-hour intervals can induce ovulation during the non-breeding season in the absence of progestogen priming. We thus decided that a double injection might be a good strategy for a first attempt at inducing ovulation in the jenny. We decided to compare the effect of C6 with that obtained with a standard treatment: buserelin acetate. The use of a single injection of buserelin acetate was dictated by our previous observation of the efficacy in inducing ovulation with the same dose after a single administration. [38]

Our results showed that injection of the KP analog C6 induces fertile ovulations in jennies between 24 and 48 hours with an efficacy (44%) comparable to that of buserelin (56%) and larger than the control (22%). In our study, ovulation rates obtained with buserelin were lower than those reported in the literature. In a similar study exploring the effect of various doses, buserelin at the same dose used in our study, resulted in an 81% ovulation rate [37]. It should be noted that higher (0.8 mg) or lower (0.2 mg) doses were shown to be less effective with a success rate of 67% and 61% respectively. Two other studies used lecirelin, another GnRH analog, to stimulate ovulation in jennies. The use of a 100-µg dose resulted in 65% to 69% of ovulation between 25- and 48-hours post-injection depending on the follicle diameter at treatment. In another study, lecirelin at a dose of 50 µg, triggered ovulation within 48 hours in 73% of cases. However, it should be noted that two jennies ovulated before 24 hours after induction of ovulation. If, as in our study, only animals ovulating between 24 and 48 hours were considered, the success rate decreased to 54% [44]. The subjective response to ovulation induction, which has already been shown in a previous study on jennies [38], probably conditioned the lower ovulation rate we obtained in the present study.
Regardless of the difference in success rate, the results we obtained with this first attempt using C6 indicate the capacity of this molecule to trigger ovulation in jennies.

Concerning the hormonal response, as expected, the injection of buserelin triggered a sudden increase in LH which peaked at around 8 hours post-injection. Compared to buserelin, the

increase in LH after the C6 treatment took longer to develop, and the LH peak occurred with a delay of 8-10 hours after treatment. In ewes and goats, an intramuscular injection of C6 triggered a much faster increase in LH which also peaked at an earlier time, 4-5 hours post-injection [31,33]. We recently tested the effect of C6 in mares [unpublished observations, 35], and again the response to C6 after intramuscular injection was faster than that observed in the present study. This therefore clearly shows that the time course of the response in donkeys is different from that observed in other species.

The reasons for this difference remain unclear. One possibility is the difference in the diffusion rate of the molecule from the site of injection into the blood, thus taking longer to reach an effective C6 blood concentration. On the other hand, particular characteristics of the kisspeptin system of the donkey may also be involved in this slower response. To address this issue, it would be interesting to verify whether an intravenous injection of C6 would result in a faster increase in LH plasma concentration.

In mares, the stimulation of the kisspeptin system with the endogenous ligand KP10 at a dose of up to 6 mg did not advance ovulation although an LH peak was induced [27]. We obtained similar results using an intravenous injection of C6 at a dose of 150 nmol per mare [unpublished observations, 35].

In our study, the ultrasonographic characteristics (CSA, VA, and IV) of the corpus luteum obtained after C6-induced ovulation were similar to those obtained with buserelin or after natural ovulation. As has been previously reported for jennies, a positive correlation was found between corpora lutea CSA, VA but not IV with serum progesterone concentration [47]. Plasma progesterone concentrations were also similar between treatments.

The induction of ovulation with C6 showed no differences in pregnancy rates compared to GnRH or placebo. Pregnancy rates were comparable to those reported for jennies inseminated with fresh extended semen [48–51] and were obtained with single insemination, although in about half of the cases, the jennies were found to be pregnant even after ovulating between 72 and 144 hours after insemination.

Both the luteogenesis characteristics and pregnancy rates observed in this study show that treatment with C6 produces the same overall physiological response observed after buserelin treatment or a natural ovulatory cycle.

5. Conclusions

In conclusion, no significant differences were found after C6 intramuscular administration in jennies in terms of ovulatory rates, luteogenesis, and fertility, compared to buserelin. One drawback of the present study is the limited number of jennies included and their already known physiological reproductive differences. Nevertheless, the results obtained are promising. It is reasonable to suppose that a higher dosage of C6 would further increase gonadotropin release and probably the ovulation rate. This first study on the C6 effect on ovulatory rates and fertility in jennies opens an interesting perspective for possible applications in the field.

6. References

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Highlights

- Kisspeptin C6 analogue was administered to determine its efficacy in inducing ovulation in jennies, compared to placebo and GnRH analogue buserelin acetate.
- LH plasma concentration patterns showed significant increases after induction of ovulation in C6 and GnRH treated jennies, compared to placebo.
- No differences on ovulation, pregnancy rates and corpora lutea functionality, after insemination among C6, GnRH, placebo treatments were found, respectively.
- This study showed intermediate results for ovulation induction between the mare and small ruminants.