The role of kisspeptins in vernal transition management in mares
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by Scott Norman, Christopher Scott, Briony McGrath, Jaymie Loy, Tonya Collop, Jennifer Clulow

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Foreword

The spring transition period is a phase all mares progress through annually as they emerge from winter anoestrus and commence regular reproductive cycles through spring and summer. The period is characterised by displays of oestrus that are not coordinated with ovulation, usually lasting 6 to 8 weeks. The transition period currently requires significant managerial input to monitor mares if early pregnancies and efficient semen use is to be achieved. Currently, the most effective treatment to bring the transition period forward is to place mares under artificial lighting from mid-July, followed by trans-rectal examination and hormonal treatments once follicular activity commences. However, no treatments reduce the duration of the transition period or make it easy to predict when the first fertile ovulation of the season might occur.

The hypothalamic hormone, gonadotrophin-releasing hormone (GnRH) has traditionally been considered the central driver of pituitary gonadotrophin secretion. More recently, a hormone called kisspeptin has been found to be involved in modulating the hypothalamic-pituitary-gonadal (HPG) axis of various species, including the horse. Kisspeptin may have a role in governing the equine seasonal transitional period, suggesting a potential role for the use of kisspeptin in spring transition management of the mare and a need to initiate investigations in this area.

This report provides scientific information on the general role of kisspeptin in mare reproduction, and specifically the role of kisspeptin during the spring transition period. The findings of this preliminary investigation will assist the development of future research into the use of kisspeptin as a tool to manage spring transition in the mare.

Improving management of the spring transition period will increase the efficiency and safety within the equine stud industry. This improvement is due to anticipated reductions in the need for teasing, palpating and scanning mares, improved timing of inseminations and earlier foals. All members of the equine stud industry may benefit from information contained in this report. In the day-to-day management of a stud utilising natural service, improved management of spring transition can reduce the labour and risks associated with identifying mares for breeding and getting them bred. It can also assist stud managers to gain more efficient use of stallion services.

The project was funded in part from industry revenue which is matched by funds provided by the Australian Government. Additional financial support was provided by Charles Sturt University.

This report is an addition to RIRDC’s diverse range of over 2000 research publications and it forms part of our Horse R&D program, which aims for the Australian horse industry to be nationally and internationally recognised for its excellence as a reputable user and supplier of quality horses, products and services, and for the industry to expand in the global market by having the requisite skills and knowledge for efficient, profitable and sustainable production.

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Abbreviations

°C  degrees Celsius

¹²⁵I  Iodine-125 (radioisotope of iodine)

eLH  equine Luteinising Hormone

FSH  Follicle Stimulating Hormone

GnRH  Gonadotrophin-Releasing Hormone

GPR54  G protein-coupled receptor 54 (kisspeptin receptor)

HPG  Hypothalamic-pituitary-gonadal

i.v.  intravenous

Kiss1  the gene encoding kisspeptin

LH  Luteinising Hormone

m  metres

MBq  megabecquerels

µg  micrograms

mg  milligrams

mL  millilitres

mm  millimetres

n  number of observations or animals

ng  nanograms

ng/mL  nanograms per millilitre

nmol  nanomoles (10⁻⁹ moles)

%  percent

RIA  radioimmunoassay

rKP-10  rodent kisspeptin decapeptide

rpm  revolutions per minute
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Executive Summary

What the report is about

The spring transition period is a phase all mares progress through annually as they emerge from winter anoestrus and commence regular reproductive cycles through spring and summer. The period is characterised by displays of oestrus that are not coordinated with ovulation, usually lasting 6 to 8 weeks. The main influences on the duration of the spring transition period and timing of the first fertile ovulation for the season are: day length, body condition, and ambient temperature, in decreasing order of significance. The transition period currently requires significant managerial and financial input to stimulate and monitor mares if early pregnancies and efficient semen use are to be achieved.

Information from the literature indicates that kisspeptin is likely to play an important role in the control of reproduction in the mare. It is thought that kisspeptin may act as a gatekeeper to environmental influences on the hormones that control reproductive function, known collectively as the HPG axis.

This report describes a significant development in the search for a practical approach to managing spring transition in the mare. Preliminary findings on the effects of kisspeptin infusion on follicle and hormonal dynamics in transitional mares are described, and recommendations made that may lead to improvements in the efficiency of equine breeding management.

Who is the report targeted at?

The primary target audiences for this report are equine, animal and veterinary scientists. It provides preliminary data on the influence of kisspeptin on follicle and hormonal dynamics in transitional mares to inform pathways for future research into the management of spring transition. However, anyone associated with any form of equine breeding may have an interest in this report. The report is designed to inform horse owners, managers, and other equine breeding industry stake-holders about the findings of our research, providing them with information on emerging options for spring transition management. Members of the Thoroughbred and Standardbred industries may stand to gain the most benefit from this research, as their industries depend heavily upon the management of spring transition in order to gain early foals. In particular, the Thoroughbred breeding industry may benefit due to its requirement for a 1st of September breeding season commencement, and current focus on natural service.

Where are the relevant industries located in Australia?

The equine breeding industry covers the majority of populated Australia with areas of larger financial interest situated in the Darling Downs region of Queensland, the Hunter Valley region of New South Wales, multiple sites throughout Victoria and multiple sites mainly in the south-west of Western Australia. Within the equine breeding industry, the breeding of Thoroughbreds generates the majority of economic activity.

Background

Spring transition is a physiological process that all mares undergo annually at the commencement of the breeding season. This period marks the transition between the physiological states of winter anoestrus, where ovaries are inactive, to fertile oestrous cyclicity during the warmer months. During the 6 to 8 weeks duration of this transition period, mares cycle erratically and it is difficult to predict when the first fertile ovulation may occur. As a consequence, there is the need for intensive management of mares to ensure that stallions are not overworked, semen is not wasted and susceptible mares are not over-exposed to uterine contamination associated with excessive breeding. Current industry “gold standards” for the management of the spring transition period variably include; placing mares under artificial lighting for six to eight weeks commencing from mid-July; rugging mares for
warmth; teasing mares for behavioural oestrus every second day commencing in late August; transrectal palpation and ultrasound examination; and the administration of hormones to either suppress oestrus or induce ovulation. Importantly, no current treatments reduce the duration of the transition period or make it easy to predict when the first fertile ovulation of the season might occur. Regardless of the method, significant costs in labour and infrastructure are incurred to effectively manage the spring transition period.

Importantly, at the commencement of this study there were no practical techniques to shorten the transition period of the equine breeding cycle, or to provide reliable predictions of the first fertile ovulation for the breeding season.

The neuropeptide, kisspeptin has been identified in all studied mammalian species, including horses. There is strong evidence that it modulates metabolic and environmental influences on reproductive function. Kisspeptin also plays a critical role in the secretion of reproductive hormones, as well as the onset of puberty and ovulation. However, the role of kisspeptin in regulating the vernal transition period of the mare is unknown.

This project was based on the hypothesis that kisspeptin is capable of influencing the vernal transitional period in mares by directly influencing the hormones that control follicular development and ovulation.

**Aims/objectives**

The aim of this study was to test the hypothesis that intravenous infusion of kisspeptin in the seasonally transitional mare will advance the timing of ovulation, and/or assist with the prediction of the first ovulation of the season. In order to test this hypothesis, it was necessary to investigate the effect of a prolonged administration of kisspeptin on luteinising hormone (LH) release, follicular development and ovulation, in the early transitional mare. An additional objective was to determine the safety of kisspeptin administration on mare health.

**Methods used**

Seven Standardbred mares in seasonal transition received a constant infusion of kisspeptin diluted in normal saline (three mares received 66.7 nmol/hour, and four mares received 100 nmol/hour) for 30 hours. Two groups of four Standardbred mares received an equivalent volume of normal saline infused over 30 hours, to serve as controls. The study ran over two spring transition periods, with four control and three treatment mares infused during August/September 2010 and four control and four treatment mares infused during August/September 2012.

Blood samples were obtained to assay for LH concentrations throughout the infusion. A commercially available radioimmunoassay was used to measure the amount of LH within the blood.

Throughout the study, mares were teased for detection of oestrus and trans-rectal ultrasound was used to monitor ovarian follicular development and ovulation.

**Results/findings**

While the LH assay was not a specific requirement of this study, preliminary results found that kisspeptin infusion enhanced LH release from the pituitary. Further research is required to clarify the ideal dose and administration regimen for kisspeptin; however, we can be confident that kisspeptin administration in the transitional mare will achieve the goal of enhancing LH release.

Kisspeptin infusion at the trialled dose rate and administration regimen did not shorten the transition period. However, in the treatment mares, a pattern emerged suggesting that the larger the follicle present at the commencement of treatment, the shorter the duration to ovulation. In particular, a pattern appeared suggesting that follicles of a size between 19 to 22 mm in diameter at the commencement of treatment would ovulate in a temporal window between 38 to 42 days after completion of the infusion. Additionally, in the majority of the treatment mares (5/7), the follicle that was present at the
commencement of treatment was the follicle that went on to ovulate. In contrast, no such pattern was apparent in the control mares.

No adverse effects of treatment with kisspeptin infusion were noted in any of the mares throughout the study.

**Implications:**

The finding that kisspeptin infusion enhanced LH release from the pituitary gland is significant in that it confirms a mechanism by which kisspeptin may achieve the goal of becoming a tool to assist with the management of the transition period in the mare. This finding provides incentive for industry policy makers to continue research in this area with particular emphasis on refining the kisspeptin dose and method of administration.

The finding that kisspeptin infusion results in a more tightly controlled ovulation period in transitional mares is significant in that it suggests that with further refinement, a kisspeptin administration protocol may be developed which allows close prediction of the first fertile ovulation for the breeding season. The finding that the follicle that went on to ovulate was typically the same follicle that was present at the commencement of kisspeptin treatment is noteworthy. It suggests that in addition to stimulating pituitary LH release, kisspeptin infusion was capable of stimulating local ovarian or follicular factors necessary for follicle maturation and ovulation.

These findings have potential to dramatically improve the management of mares during the spring transition period. The implications of such a result are that it could significantly improve the financial efficiency of the Australian equine breeding industry due to reduced labour, reduced agistment costs, improved efficiency of stallion and semen use, and reduced treatment costs for mares susceptible to post-breeding uterine infections.

The finding that kisspeptin is safe for use in the mare provides confidence that further work in developing a kisspeptin administration protocol will not be hampered by the occurrence of adverse reactions.

**Recommendations**

Further research into the use of kisspeptin to manage vernal transition in mares is warranted.

A comprehensive benefit-cost analysis of the effects of either shortening or improving the management of the spring transition period via kisspeptin administration is justified. This would help to inform research funding.

Further research is required to:

i) Determine the ideal time to commence kisspeptin treatment in the vernal transition mare

ii) Determine the optimal kisspeptin dosage and administration regimen;

iii) Optimise kisspeptin dosages to ensure efficacy but prevent down-regulation of the HPG axis.

iv) Fully elucidate the events surrounding follicular development and maturation in the period immediately prior to the first ovulation of the season.
Introduction

Mares are seasonal breeders with a mean duration of gestation of approximately 335 days. Reproductive seasonality is a phenomenon seen as an adaptation designed to prevent the birth of foals during harsh winter months in more temperate climates. As day length shortens with the approach of the autumn equinox (21st of March in this hemisphere), reproductive cycles become erratic and in most mares they eventually cease during the winter months surrounding the winter solstice of 21st June (Sharp, 2011). Their reproductive cycles recommence (spring, or vernal, transition from anoestrus to cyclicity) as the days lengthen with the approach of the spring equinox at the 21st of September, with the peak of the breeding season centred on the summer solstice of the 21st of December in the southern hemisphere (Sharp, 2011).

The spring transition period is a phase all mares progress through annually as they emerge from winter anoestrus and commence regular reproductive cycles through spring and summer. The period is characterised by displays of oestrus which are not coordinated with ovulation, usually lasting 6 to 9 weeks (McCue & Ferriss, 2011). The main influences on the duration of the spring transition period and when the first fertile ovulation for the season will occur are day length, body condition, and ambient temperature, in decreasing order of significance (McCue & Ferriss, 2011).

The equine birthday in Australia is traditionally set at August 1st. Thus, to produce foals at the most competitive age possible for athletic pursuits such as racing, it is considered ideal to breed mares during September. Foals conceived during September are born in August, and are therefore close to one year old on August 1st the following year. In contrast, foals conceived in December (arguably the most fertile period of the equine breeding season) are born in November, making them only nine months of age by August 1st. Unfortunately, September conceptions require breeding mares that may still be undergoing the spring transition period. Therefore, the transition period currently requires significant managerial and financial input to stimulate and monitor mares if early pregnancies and efficient semen use is to be achieved.

At the commencement of this study there were no practical techniques to shorten the vernal transition period of the equine breeding cycle, or to provide reliable predictions of the first fertile ovulation for the breeding season. Current industry “gold standards” for the management of the vernal transition period include; placing mares under lights for 6 to 8 weeks commencing in mid-July; teasing mares for behavioural oestrus every second day commencing in late August; trans-rectal palpation and ultrasonography of mares displaying oestrus; and the administration of hormones to either suppress oestrus or induce ovulation (Sharp, 2011).

Information from the literature indicates that kisspeptin may play an important role in the control of reproduction in the mare, with suggestions that it may act as a gatekeeper to external influences on the HPG axis (Roa and Tena-Sempere, 2007). Importantly, we hypothesise that the influence of day length on the equine reproductive cycle may be modulated by kisspeptin. Therefore, the study presented in this report was designed to investigate the effect of kisspeptin infusion on follicular and hormonal dynamics in transitional mares.

The transitional mare - endocrine and follicular characteristics

Prior to the completion of this study, the reproductive endocrine physiology of the transitional mare had been well reviewed (Sharp, 2011) and can be summarised as follows:

During the latter part of winter anoestrus, there is reduced gonadotrophin-releasing hormone (GnRH) storage and secretion from the hypothalamus. There is also an inability of the pituitary gland to produce LH, resulting in a marked reduction in pituitary LH content. Importantly, the pituitary content of follicle-stimulating hormone (FSH) does not appear to change throughout the year, remaining high during anoestrus (Sharp, 2011). During the initial emergence from anoestrus into the transitional
period there is increased GnRH storage and secretion from the hypothalamus. This increase can be induced to occur after only two weeks of artificially increased day length (Sharp, 2011), suggesting that one of the earliest endocrine events associated with progressing from anoestrus into the vernal transition is an increase in GnRH release.

During anoestrus there is no significant follicular activity on the ovary despite there being adequate pituitary stores of FSH. This reduction in follicular activity is presumably associated with the diminished hypothalamic secretion of GnRH. As GnRH secretion returns during the early transition period there is associated pituitary FSH secretion which induces a return to follicular development within the ovary. However, due to the lack of pituitary LH production, the follicles that develop as a result of this FSH secretion, while often growing to greater than 30 mm in diameter, fail to progress to ovulation. In fact, a number of studies suggest that during the vernal transition period there is an average of 3.7 anovulatory follicular waves with an interval of 12 days between the initial detection of the largest follicle of each wave (Sharp, 2011).

The unanswered question associated with the reproductive endocrine characteristics of the vernal transition mare is: what mechanisms induce the return of pituitary LH production and receptivity to allow the follicle to become steroidogenically active, mature and eventually ovulate? It is our hypothesis that kisspeptin has a significant role in inducing LH production and receptivity. We also hypothesise that kisspeptin may act locally at the level of the ovary or follicle to induce the requirements necessary for follicle maturation and ovulation.

**Kisspeptin and female reproduction**

Kisspeptins are recently discovered neuropeptides, produced by the Kiss1 gene, which have been identified as major players in the brain control of reproduction (Caraty & Franceschini, 2008; Gianetti & Seminara, 2008; Roa & Tena-Sempere, 2007). Research using other species has attempted to determine the precise mode of action of kisspeptins. The current research indicates that the KiSS1 system is localised within the hypothalamus of the brain, and appears to be involved in the initiation of puberty, as well as the secretion of GnRH and subsequently gonadotrophins, such as LH and FSH (reviewed in Pineda et al., 2010). Treatment of various species, including humans, with kisspeptin increases circulating LH and FSH concentrations (Dhillo et al., 2007; Gottsch et al., 2004; Navarro et al., 2005). These hormones are particularly important in the female reproductive cycle (Kinoshita et al., 2005; Magee et al., 2009; Roseweir & Millar, 2009).

Kisspeptins are regulated by a combination of feedback actions from sex steroids, metabolic/nutritional signals and environmental cues (Roa & Tena-Sempere, 2007), including photoperiod. While research in this area is incomplete, there is some evidence that photoperiod, via melatonin, modulates kisspeptin to switch on the gonadotrophic axis (reviewed in Tena-Sempere, Felip, Gomez, Zanuy, & Carrillo, 2012). Thus, we proposed that manipulation of the kisspeptin pathway may be capable of influencing gonadotrophic function, and subsequently ovulation in seasonally transitional mares.
Objectives

The objective of this project was to test the hypothesis that intravenous administration of kisspeptin in the seasonally transitional mare will stimulate luteinising hormone release and advance the timing of the first ovulation for the breeding season. To achieve this objective the specific aims were to investigate:

- The influence on LH release of constant rate infusion of kisspeptin in transitional mares.
- Follicular activity in transitional mares after constant rate infusion of kisspeptin.
- Potential adverse effects of kisspeptin infusion.
Methodology

This study was designed to compare the follicular and endocrine responses of untreated controls with transitional mares treated with a 30 hour infusion of kisspeptin. The nature of the problem being investigated required the trials to be run during August/early September in each year of the study.

Infusion of kisspeptin in seasonally transitional mares

A randomised, controlled, block design was used, with the study being performed over the spring transition periods (August/September) of the 2010 and 2012 breeding seasons. The study included four cohorts, each comprising four clinically normal Standardbred mares (n = 16), aged between 3 and 15 years, with a reproductive tract consistent with seasonal transition. Each cohort consisted of two treatment and two control mares. Mares were paired as treatment or control based on trans-rectal palpation and ultrasonographic evaluation of the ovaries showing similar stages of follicular development. All mares were evaluated within 48 hours prior to commencement of the study to allow these group allocations to be made. Mares were considered to be in seasonal transition if they met the criteria of: no ultrasonographically visible corpus luteum on either ovary; each ovary having several follicles, none being greater than 20 mm in diameter; and an endometrial oedema score less than two on a scale of 0 to 3, indicating little or no endometrial oedema. These criteria were waived in one pair of mares in order to provide a preliminary insight into the influence of kisspeptin infusion when follicles greater than 35 mm were present. Mares were housed in 4m x 4m box stalls with ad libitum water. Fresh lucerne hay was fed twice daily for the duration, with the exception of mare 8 who was prone to laminitis and was provided with oaten hay.

Following administration of lignocaine hydrochloride, jugular catheters were placed on both sides of the neck of each mare. An infusion pump (T34 syringe driven pump, Caesera Medical Electronics Ltd, Germany) was attached to one side, while venous blood samples were collected from the other side throughout the study. The pumps were housed in pockets sewn into the side of horse neck-rugs, allowing the horses to move around freely without interfering with the infusion. The kisspeptin formulation, KP-10 was used, incorporating amino acids 112-121 of the full length human kisspeptin molecule, obtained from Phoenix Pharmaceuticals. The exact sequence of equine kisspeptin is unknown, but the sequence is highly conserved across mammalian species and this human peptide has stimulated LH secretion in all mammalian species studied. The kisspeptin infusion dose and rate were informed by the literature; and as there was no information on effective dose rates for kisspeptin in mares, dose rate calculations were extrapolated from studies in sheep (Caraty et al., 2007). A kisspeptin dose of either 66.7 nmol /hour (n=3) or 100 nmol/hour (n=4), diluted in normal saline, was infused over a 30 hour period for this study. Two groups of control mares (n=4 per group) received an equivalent volume of normal saline infused over the 30 hour period of the study. Blood samples were collected every fifteen minutes for between 90 to 120 minutes prior to the start of the infusion; and for the following 6 hours after infusion commencement. Sampling frequency was then decreased to every 3 hours until 72 hours after the commencement of infusion. Samples were centrifuged (3000rpm/4°C, 10 minutes) and plasma stored in aliquots at -20°C until radioimmunoassay for LH concentrations was performed.

The influence on LH release of constant rate infusion of kisspeptin in transitional mares.

The first aim of this study was to assess the effects of kisspeptin infusion on LH concentrations in seasonally transitional mares. At the commencement of this study there was no laboratory in Australia able to assay equine plasma LH concentrations. In a separately funded project running parallel to the
study reported here, we have set up and validated an equine LH assay in our laboratory and can report some preliminary LH concentration results from this project.

Concentrations of LH in blood plasma were determined by radioimmunoassay (RIA) techniques using a kit provided by the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases (USA). This assay used \(^{125}\text{I}\) (specific activity of 16MBq; Perkin Elmer, USA) as a tracer and a specific equine LH antibody raised in rabbit. The sensitivity of the assay was approximately 0.3ng/mL.

Once the assay had been validated for use in equine plasma, the objective was to assess LH concentrations in serial samples that were obtained prior to, during and following infusion of kisspeptin, described above. Luteinising hormone is released in both short duration pulses, as well as longer duration surges (approximately 5 days in the mare) prior to ovulation (the “pre-ovulatory surge”). The initial sampling frequency chosen in this project was deemed necessary for accurately determining the short duration LH pulses.

**Follicular activity in transitional mares after constant rate infusion of kisspeptin.**

The second aim of this project was to assess follicular activity in transitional mares following kisspeptin infusion. Trans-rectal palpation and ultrasonographic evaluation of the ovaries and reproductive tract was performed at both the start and the conclusion of the infusion protocol. Mares were then palpated and scanned every second day until an ovulation was detected or until six weeks had elapsed post infusion, whichever came first. Follicles were measured by freezing the image on the ultrasound machine to allow the inbuilt callipers to be utilised. Two experienced operators performed all palpations and follicle measurements.

**Potential adverse effects of kisspeptin infusion**

The final objective of the project was to identify any potential adverse effects of kisspeptin infusion. A general physical and reproductive examination was performed on all mares prior to commencement of the study, and again following infusion. This was repeated every second day until ovulation or until six weeks had elapsed. The general physical examination consisted of visual assessment of body condition and measurement of body temperature, pulse and respiration. Mares were also observed in the paddock to assess for normality of behaviour and grazing. The reproductive examination consisted of visual assessment of the vulva and the perineal region. Speculum examination of the vaginal vault was performed as mares were enrolled into the study, within 24 hours after conclusion of the kisspeptin infusion, and at the final ovarian palpation.

**Statistical analysis**

Due to the preliminary nature of the study, only descriptive representations of these data are presented.
Results

The influence on LH release of constant rate infusion of kisspeptin in transitional mares.

Preliminary LH data for two treatment mares and two control mares is presented. An LH pulse is defined as the peak being greater than the nadir of the previous two samples (Goodman & Karsch, 1980).

Figure 1: Binding of iodinated equine luteinising hormone (eLH) in blood plasma from horses infused with kisspeptin (Horses 6 and 8) compared with Controls (Horses 5 and 7). LH pulses (indicated by stars) occurred in all animals three hours post the start of the infusion, and were more distinct following kisspeptin infusion (higher percentage binding when compared to earlier peaks).
Pilot data from the LH RIA (Figure 1) showed a trend for an LH pulse to occur between hours 1.5 and 3.5 during the intravenous (i.v.) kisspeptin infusion (horses 6,8), suggesting that there is an initial effect of kisspeptin on pituitary gonadotrophs. Control animals (horses 5,7) also exhibited LH pulses in the first 3 hours of the infusion, but importantly, these pulses had a much smaller rise to peak than the treatment animals.

**Follicular activity in transitional mares after constant rate infusion of kisspeptin.**

By assessing follicular activity, we were able to track follicle size and subsequent ovulation of the animals. Two treatment mares showed follicular waves, but the size of follicles regressed and they did not ovulate. This suggests that the follicles were not steroidogenically competent, and therefore not able to respond to the gonadotrophins or circulating sex steroids, and proceed to ovulation. The mares whose follicles regressed were part of the treatment group that received a higher dosage of kisspeptin (100 nmol/hour compared to 66.7 nmol/hour). Conversely, the other two mares that were infused 100 nmol/hour demonstrated the shortest time from start of the infusion until ovulation (Figure 2). Of the remaining treatment mares, the ovulatory follicle was present at the initiation of treatment. In contrast, in the control mares it was between 1 to 3 follicular waves prior to emergence of the follicle resulting in the first ovulation for the season. There was a trend for kisspeptin infusion to result in tighter control of time to ovulation (38 to 42 days for follicles between 19.5 and 22mm, respectively; Figure 2). Conversely, Control mares showed more variation in the relationship between size of initial follicles and time to ovulation with follicles between 17 and 20mm ovulating 38 and 29 days later, respectively.
Figure 2: The initial size of the ovulating follicle compared to the time taken until ovulation in mares infused with kisspeptin and control mares. The yellow circled data points received a higher dosage of kisspeptin (100 nmol/hour compared to 66.7 nmol/hour).

Control mares showed a variable downward trend in the duration to ovulation when related to the largest follicle present at first palpation, whilst treatment mares mimiced the results observed for the initial ovulating follicle (Figure 3). This suggests that the kisspeptin infusion was able to rescue steroidogenically competent follicles and push them towards ovulation.
Figure 3: Comparison of the size of the largest follicle at first palpation (mm) with the time taken until ovulation for control and kisspeptin treatment mares.

Potential adverse effects of kisspeptin infusion

Based on assessments of systemic and reproductive health, no abnormalities were detected in any of the mares throughout the duration of the study. Follow-up assessment of the mares in the cycle following the first ovulation post-infusion showed no abnormalities in health or cyclicity.
Discussion

A constant rate infusion of kisspeptin did not appear to shorten the transition period in this study. However, in the mares treated with kisspeptin, there was a trend indicating that the larger the follicle present at the commencement of treatment, the shorter the duration to ovulation. Additionally, follicles between 19 to 22 mm in diameter at the commencement of treatment ovulated in the temporal window between 38 to 42 days after completion of the infusion. Except for two mares that did not ovulate at all, in all of the kisspeptin treated mares the follicle that went on to be ovulated was present at the commencement of the study. This was not the case for the control mares, and indeed there appeared to be no pattern in time to ovulation in control mares, whether the largest follicle will be ovulated, or whether the ovulatory follicle will come from a subsequent follicular wave. Thus, there appeared to be a trend for kisspeptin infusion to result in tighter control of folliculogenesis and time to ovulation. Whilst we still await the full set of hormonal data, the results of this small study provide initial evidence that kisspeptin treatment is able to influence ovarian function in mares during the seasonal transition, and encourage us to further explore the role of kisspeptin during the seasonal transition period and the potential for a kisspeptin based therapy.

Preliminary LH data from two control mares and two treatment mares suggested an increase in the pulsatility in LH secretion in the first hours after the commencement of infusion. Only the data from the rapid sampling period (the period prior to the commencement of treatment through to the first 6 hours after start of treatment) are meaningful for consideration of the incidence and size of LH pulses. Nevertheless, these data are consistent with a previous study where intravenous injections of kisspeptin in mares resulted in an increase in LH secretion, with sustained incidence of LH pulses being generated (Magee et al., 2009), albeit with doses much higher than in our study (bolus injections of 500µg or 1mg, which equates to about 400-800 nmol, compared with our upper dose of 100nmol/hour). Indeed, in all species studied so far, both central and peripheral kisspeptin treatment has rapidly stimulated LH secretion, sometimes at remarkably low doses (reviewed in Oakley, Clifton, & Steiner, 2009). The ability of kisspeptin to have physiological effects at low doses highlights the need to carefully study doses to ensure over stimulation does not occur. In particular, the effect of longer term treatments with kisspeptin, such as those with the goal of stimulating ovulation needs to be carefully assessed.

Continuous intravenous infusion of kisspeptin was very effective in stimulating ovulation in anoestrous sheep (Caraty et al., 2007). The evidence from that study indicated that the kisspeptin infusions were not directly stimulating a pre-ovulatory surge in LH, but were doing so indirectly by increasing gonadotrophin secretion in a way that ovarian follicles were induced to grow and produce oestrogen. Over a two day period, this increase in oestrogen then had a positive feedback action in the brain to trigger a natural LH surge and hence ovulation. Our study was modelled on that research, and we reasoned that it would be superior to previous pharmacological methods of inducing ovulation in mares, as it stimulated the development of the ovary, including the expression of LH receptors on granulosa cells, to the point where it would be ready to respond to an ovulatory signal. Interestingly, Schauer et al (2011) observed that LH treatment during early transition resulted in dominant follicles (20 mm diameter) progressing to ovulation, rather than regressing.

On the other hand, continuous infusions of kisspeptin in rats and monkeys (Ramaswamy et al., 2007; Seminara, 2006; Thompson et al., 2006) have resulted in an initial increase in LH secretion, followed by a suppression of LH secretion. It is not clear why continuous infusion of kisspeptin would be effective in stimulating ovulation in one species but not others, but it is suggestive of down-regulation in the Kiss1 receptor, although this has not yet been measured. In our study, after the first round of infusions, where we did not see a strong, clear effect of kisspeptin, we increased the kisspeptin dose by 50% for the second round. In this group we did see two mares ovulate relatively quickly, but this might reflect their greater follicle size at the start of the treatment. By contrast, in two mares the largest follicle regressed and there was no ovulation. This raises the possibility of down-regulation of the HPG axis, presumably acting at that level of the Kiss1 receptor (sometimes also called GPR54). It
has been argued (Caraty, Decourt, Briant, & Beltramo, 2012) that this may be related to dose of kisspeptin used, with higher doses inducing desensitisation. Notably, the dose of kisspeptin used in the monkey studies where continuous kisspeptin infusion caused a suppression in LH was up to one hundred fold greater than that used in the sheep studies, despite being a smaller animal. Additionally, reproductive status may be a factor, with response to kisspeptin being greater in sheep during anoestrus (Caraty et al., 2007) than in the breeding season, and greater in early follicular phase of the oestrous cycle in some species (reviewed in Caraty et al., 2012). It is clear that if kisspeptin is to be a useful therapeutic option in horses or any other species, further work is required to investigate the effect of kisspeptin dose and administration regimen on gonadotrophin secretion, follicle growth, and ultimately, ovulation.

In an effort to avoid any possible Kiss1 receptor down-regulation, some studies have employed repeated i.v. injections of kisspeptin instead of continuous infusion. This has been effective in stimulating LH secretion in rats (Tovar et al., 2006) and juvenile monkeys (Plant, Ramaswamy, & Dipietro, 2006). The strategy has not been effective in stimulating ovulation in either dioestrus or oestrus mares (Magee, 2010). This is likely due, in part, to the frequency of injections; hourly versus four hourly, as the commercially available form of kisspeptin (KP-10) is a short peptide and is likely to have a very short plasma half life. Thus for repeated injections given at intervals greater than one hour, long acting analogues would be necessary. Such analogues are not yet commercially available but a great deal of work is currently being undertaken into their development, especially in Japan.

Although, as a starting point, we modelled our 30 hour infusion protocol on that used successfully to stimulate ovulation in sheep (Caraty et al., 2007), we did not observe ovulation during our treatment period or shortly after its completion. The follicular phase in a sheep is only 2-3 days, which is much shorter than the corresponding period in the mare, and accordingly it is likely that a longer treatment period would be needed to induce ovulation in the mare. This being the case, it is likely that alternative delivery methods will need to be developed and validated, such as osmotic mini-pumps. A similar endocrine situation exists with the dog and the control of ovulation in that species. We are currently developing delivery systems that we hope will allow us to treat bitches with kisspeptin for 10 days or more.

There is evidence that in transitional mares, progesterone treatment will facilitate follicle development. In one study, a single injection of a long acting progestogen lead to ovulation between 10 and 24 days after treatment in 83% of transitional mares with 20-25 mm follicles, compared with 25% in control mares with similar sized follicles (Staempfli et al., 2011). Hanlon et al (2012) also observed a marked increase in follicle size following 10 days of intravaginal progesterone treatment in transitional mares with 20-25 mm follicles. While our kisspeptin treatment did appear to similarly stimulate follicle growth, the treatment may prove more effective if we pre-treat mares with progesterone for 10 days, prior to kisspeptin administration.

Assessing the patterns of FSH, progesterone and oestradiol secretion may provide information regarding the steroidogenic competency status of the follicles. Tracking the relationship between the secretion of these hormones and follicle growth may help elucidate why some follicles regressed and others were able to proceed to ovulation. Comparing FSH concentrations from mares whose follicles regressed with mares that ovulated would also provide an interesting view of the hormonal environment of transitional mares. This may enable us to determine whether a specific follicle will ovulate, or regress.

An important component of our study was to determine if there were any adverse effects of our kisspeptin treatments on the health of the mares. No adverse effects were observed in any of the mares as a result of kisspeptin infusion. This indicates that the possible use of kisspeptin treatments as a method for advancing the timing of the first ovulation for the breeding season would be a safe option.

Overall, these results provide preliminary evidence that kisspeptin treatment can influence ovarian follicle development in the mare during seasonal transition, through an increase in gonadotrophin secretion. Further research is required to confirm this and refine our understanding of the mechanisms.
Similarly, the ideal dose and timing of treatment need to be determined in order to ensure a predictable ovulation without potentially stimulating down-regulation of the reproductive endocrine system.
Implications

Findings from this preliminary study support the concept that a kisspeptin administration protocol may be developed to assist with management of the spring transition period in the mare. The data presented in this report indicate that this treatment regimen is safe for use in the mare, with no adverse reactions observed in any of the mares.

There is scope for further research to determine the optimal kisspeptin dosage and administration regimen. Knowledge gained from the development of GnRH as an ovulation induction agent provides insight into the need for careful assessment and optimisation of kisspeptin dosages. If the dosage is too low, the desired response may not be achieved; however, if the dosage is too high, down-regulation of the HPG axis may occur. Additionally, further work is required to fully elucidate the events surrounding follicular development and maturation in the period immediately prior to the first ovulation of the season. For example, this study suggests the possibility that kisspeptin may be responsible for the induction of LH receptors on the granulosa and theca cells of the follicle.

A comprehensive benefit-cost analysis of the effects of either shortening or improving the management of the spring transition period is beyond the scope of this report. However, by improving the management of spring transition in the mare, and potentially shortening the transition period, kisspeptin administration would save the Australian equine breeding industry substantial sums of money annually as a result of: reduced labour requirement for teasing, palpating and breeding mares; reduced agistment costs and feed requirements during breeding; reduced drug and electricity costs associated with current transition management programmes; and improved occupational health and safety for both horse and horse handler.
Recommendations

Further research is required to assess the optimal kisspeptin dose to reliably induce ovulation in the transitional mare.

Further research is required to determine the most appropriate and practical administration route and frequency in the light of subsequent LH data, which we will have available shortly.

Further research is required to characterise the role of FSH and progesterone on follicle maturation and to assist with the identification of mares likely to respond to kisspeptin treatment during the transitional period.

Investigations into the use of kisspeptin administration protocols commencing immediately after the winter solstice (late July) are recommended as this may mimic the effects of photoperiod increase and be cheaper than providing lighting systems.
Glossary:

Gonadotrophins – hormones which stimulate the development, maturation and ovulation of follicles on the ovary.

Vernal – youthful, young. Used in reference to the new life of spring. Spring and vernal transition are interchangeable terms.
References


The role of kisspeptins in vernal transition management in mares

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The spring transition period is a phase all mares progress through annually as they emerge from winter anoestrus and commence regular reproductive cycles through spring and summer.

This report describes a significant development in the search for a practical approach to managing spring transition in the mare. Preliminary findings on the effects of kisspeptin infusion on follicle and hormonal dynamics in transitional mares are described, and recommendations made that may lead to improvements in the efficiency of equine breeding management.

The primary target audiences for this report are equine, animal and veterinary scientists.

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