The Buserelin Enigma; How Does Treatment with this GnRH Analogue Decrease Embryo Mortality?

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Abstract

The use of the GnRH analogue buserelin administered in mid dioestrus has been shown to increase early pregnancy rate, reduce embryo loss and increase litter size in several species. The mechanism by which this works is unclear and may differ between species. In cattle it is believed that buserelin, by reducing oestrogen concentrations, causes the development of endometrial oxytocin receptors to be delayed. Luteal oxytocin binds with these receptors to promote the formation and secretion of prostaglandin. Any delay in the release of PGF2α would allow an underdeveloped embryo extra time to produce and release the maternal recognition of pregnancy signal, which might otherwise be lost following luteolysis. This may also be the mechanism in sheep and pigs although an increase in progesterone concentrations could also be important. However in the mare, following buserelin administration at 9 or 10 days after the detection of ovulation, pregnancy rates are already increased by 12-13 days compared with untreated controls, i.e. before the time of luteolysis, without any change in progesterone concentrations. In addition to an increased pregnancy rate, the twin embryo rate is also increased and subsequent embryo loss rate reduced. The possible mechanisms by which this may result in the mare are discussed.

Keywords: Embryo; Pregnancy; Embryo loss; Mare; Cow; Progesterone; GnRH; Buserelin

Introduction

Field studies in several domestic species have consistently shown that the administration of a single treatment with buserelin or other GnRH analogues in mid to late dioestrus, results in increases in pregnancy rate and/or litter size. Temporary support of luteostasis during the time of maternal recognition is believed to be the mechanism for this increase in cows. Treatment of dioestrous mares with buserelin has shown improvements in pregnancy rate of up to 10%. However, because pregnancy diagnosis using ultrasound can be made in mares before the time of luteolysis, temporary luteostasis cannot be the mechanism in this species. This review explores and compares the relationships between progesterone, luteolysis and GnRH (buserelin) in the mare and the cow and suggests other possible avenues for future investigation.

The Role Of Progesterone In Luteolysis In The Cow

The maintenance of the corpus luteum (luteostasis; CL) in the cow is a balance between luteotropic and luteolytic mechanisms. Luteolysis is caused by pulsatile release of PGFM from the endometrium which is driven by pituitary and/or luteal oxytocin [1]. Development of oxytocin receptors in the endometrium is dependent upon oestradiol-17β and progesterone stimulation [2] but these receptors may not be present until Day 16 or 17 [3]. Whilst the luteal phase of the cycle is effectively defined by the secretion of progesterone, any delay in rising oestradiol-17β concentrations, delays the development of these receptors and thus delays luteolysis [4].

In the ruminant, secretion of interferon τ by the conceptus inhibits the synthesis of the luteolysin PGF2α [5] and is therefore considered to be the signal for the maternal recognition of pregnancy (MRP). It is thought that much infertility in dairy cows is due to insufficient or delayed production of this anti-luteolytic signal from the conceptus with the consequent failure to block luteolysis [6]. Any delay in the release of PGF2α may allow a slightly immature conceptus with delayed secretion of interferon τ, to develop further, to a stage of maturity at which it can produce sufficient interferon to successfully block luteolysis. About 80% of embryo and foetal loss is thought to occur before 25 days [7] with the majority of this before Day 17, partly due to failure of the anti-luteolytic signal. Therefore embryo loss occurring at this time does not affect cycle length such that these cows return to oestrus at the normal interval of around 21 days.

There is uncertainty in the literature as to when progesterone profiles in pregnant and non-pregnant cows start to diverge. For example [8] milk progesterone profiles were similar up to about Day 12 but then started to fall in those not pregnant. However luteolysis per se is not initiated until Day 16 or 17. It has therefore been suggested that the CL is sensitive to low levels of PGF2α as early as Day 11 or 12 [7]. However other studies [9] have shown that a single pulse of PGFM (metabolite of PGF2α) occurs before the start of luteolysis with only a transient effect upon peripheral progesterone concentrations, since this is followed by an LH pulse which restores progesterone to previous levels. Full luteolysis is initiated by a second PGFM pulse some 8 hours after the first, at about 17 days after ovulation [9]. From then on, luteolysis is essentially completed within 16-24 hours. The fall in progesterone after Day 11 is therefore probably not due to PGF2α release but more likely a result of reduced LH secretion or loss of some other luteotrophic support. Conversely progesterone concentrations in pregnant cows are maintained due to elevated LH on which the CL becomes dependent in order to maintain the pregnancy. Up to 90% of well-managed, inseminated cows may have embryos at Day 16, but embryos were...
found to be larger in cows which had a normal postovulatory rise in progesterone, while smaller under-developed embryos were found in those cows which had a slower rise in progesterone to sub-optimal peak levels [10-12]. Thus not only is progesterone essential to maintain the pregnancy in cows but adequate concentrations in early dioestrus, are necessary for optimal embryo development [13].

**Gnrh and Early Pregnancy in the Cow**

Treatment with GnRH between Day 11 and 13 after insemination appears to depress the luteolytic drive via reduced plasma oestradiol-17β concentrations and the oestradiol:progesterone ratio [14,15]. Treatment with buserelin either induces ovulation, luteinisation or atresia of the dominant follicle depending on its stage of development [16,17]. It was reported [18] that buserelin given four times daily from Day 9 to 12, extended the cycle length by 6 days and increased progesterone concentrations. Treatment with either of two GnRH agonists, gonadorelin (Fertagyl) or buserelin (Receptal) from Day 3 to 17, delayed luteolysis for up to 6 days in heifers challenged with 15 mg of the PGF2α agonist luprostiol on Day 13 [19]. Similarly in another study [20] where PGF2α and GnRH were given simultaneously on Day 11 after insemination and was difficult to reconcile with the lack of effect on overall litter size. Any difference between overall litter size and live litter size is due to the release of interferons, oestrogen or other steroids by the embryo [42]. However one study [43] reported that the

**Gnrh and Pregnancy in Sheep and Pigs**

Buserelin has also been used to improve fertility in sheep [34]. These New Zealand authors injected ewes and ewe lambs on either Day 10, 11, 12 or 13 after mating and showed improved pregnancy rates particularly in those injected on Day 12. This approach was repeated [35] under Welsh conditions in which there was a marginal but non-significant increase in non-return rate (controls: 76% versus treated: 83%) and litter size (1.51 versus 1.77) in 3 of 4 flocks. However, there was a significant increase in the twinning rate (20% versus 40%) and litter size (1.44 versus 1.68) in one flock of ewe lambs. A group of animals was slaughtered on Day 31 of pregnancy revealing a higher ovulation rate in treated ewes, suggesting that any beneficial effects of this treatment might be due to increased progesterone from accessory CL. In a further study to examine the possible mechanism [36], ewes were injected with buserelin 12 days after mating and plasma concentrations of plasma progesterone and oestradiol-17β measured. The animals were slaughtered 48 h after treatment. The ovulation rate was significantly higher in treated ewes (2.76 versus 1.87) and 41 of the 113 CL observed in the GnRH-treated group, had formed during the previous 48h compared with 2/71 of the controls. Plasma progesterone concentrations were higher and oestradiol-17β concentrations lower in the treated ewes. Thus this picture was reasonably consistent with that in cattle and it is thought likely that a similar mechanism was operating [36].

The effect of buserelin (8 μg) on the fertility of outdoor sows has also been examined [37]. A total of 1231 mixed parity sows from five farms were randomly assigned to either buserelin treatment within 24h of service, or on Day 11 or 12 after service, or were left untreated as controls. There was no significant effect on farrowing rates or on overall litter size. However, there was a significant increase in the number of pigs born alive, averaging approximately 0.5 pigs per litter, in both buserelin treated groups. This was an interesting and unexpected result and was difficult to reconcile with the lack of effect on overall litter size. Any difference between overall litter size and live litter size is due to foetal death either at or immediately after parturition. It was apparent that neither treatment with buserelin had any immediate effect on embryo survival. Therefore it was tentatively proposed [37] that the buserelin treatment resulted in an improved quality or viability of the embryos and subsequent foetuses, by improving the synchrony between service and ovulation (Day 1) or possibly by increasing progesterone concentrations (Day 11 or 12). To our knowledge there has been no further work to confirm, refute or extend this work in pigs.

**Luteolysis and the Role of Progesterone in the Mare**

Luteolysis in non-pregnant mares is the result of a positive feedback between pituitary [38] and/or endometrial oestrinocin and PGF2α [39]. Exogenous PGF2α causes luteolysis in both pregnant and non-pregnant mares. Exogenous oestrinocin results in PGF2α release but only in non-pregnant mares [40]. The significant increase in oestrinocin receptor site concentration which occurs between Days 12 and 14 does not occur in pregnant mares [41]. While the mechanisms for recognition of pregnancy are known in ruminants and the pig, in the mare, it does not now appear to be due to the release of interferons, oestrogen or other steroids by the embryo [42]. However one study [43] reported that the
The embryo does appear to release an anti-luteolytic signal to most if not all parts of the endometrial surface during its migratory phase, which can inhibit the synthesis and/or release of PGF2α following oxytocin challenge. Induction of Cox-2 expression in the surface epithelial cells in late dioestrus of cyclic mares results in the pulsatile release of PGF2α which is blocked by the presence of a conceptus [44]. It has been shown [45] that prostaglandin synthase 2 is the target for the anti-luteolytic signal with PGF2α concentrations being either increased or reduced in endometrial explants, by oxytocin or conceptus secretions respectively. By restriction of the embryo and its secretions to one horn and body of the uterus, sufficient PGF2α was released from the other horn to effect luteolysis [46]. However the role of the embryonic secretions in inhibiting luteolysis or promoting luteostasis, has been brought into question by a recent study [47] where Day 10 embryos were successfully transferred into Day 3 uteri. By the time that MRP was needed to suppress luteolysis at Days 10-14, the embryo was already 17-21 days old and had vacuolated in utero at a much earlier stage. These observations suggest that although there is an endocrinological signal to promote luteolysis, the conceptus continues to develop for several days at a normal rate and in the absence of a viable embryo up to Day 16 which is too late to be responsible for pregnancy recognition.

In further work [48], it was found that luteolysis in non-pregnant mares could be blocked by intrauterine administration of both peanut and coconut oils but not by mineral oil. It had been shown many years previously [49] that intrauterine infusion of sodium benzyloxycarbonyl penicillin during dioestrus caused luteal persistence in 46% of 13 pony mares. Inanimate objects have also been shown to cause luteostasis. Glass balls inserted at ovulation extended luteal function in 39% of mares [50] although other authors [51,52] failed to find that this caused any modification of behaviour or luteal function in pony mares. Water-filled plastic balls inserted at oestrus [53], prevented luteolysis in 75% of 12 treated mares despite the fact that the balls remained in one location and did not migrate through the uterus.

The above reports suggest that neither an embryo-produced MRP endocrine signal nor intrauterine migration is necessary to promote luteolysis. It is possible therefore that luteolysis is not necessarily the result of an endocrinological signal, but that it possibly results from the physical interaction between the endometrium and the early embryo, marble, plastic ball or oil (S. Wilshere, personal communication).

Luteal persistence in non-pregnant cows is unusual although it is associated (24%) with higher milk yielding post-partum cows [51]. It is relatively common in horse mares although not so in native ponies [W. E. Allen, personal communication]. The CL of the mare therefore appears to be comparatively less susceptible to luteolysis, or alternatively luteolysis is more susceptible to disruption. The equine CL is however more sensitive to exogenous prostaglandin than the cow [54]. Doses as small as 12.5μg of d-clomifoprostenediol [55] and 3.75μg d-clomifoprostenediol (J Newcombe, unpublished data) have been shown to be luteolytic when the CL is fully mature. In contrast however, it can be rendered insensitive by repeated 8 hourly treatment with exogenous d-clomifoprostenediol (Genesan) given during the first few days of dioestrus (Newcombe and Cuervo-Arango, Poster ISER 2014).

Although early pregnancy loss is a major cause of infertility in mares, losses due to failure of the anti-luteolytic signal and consequent luteolysis are infrequent [56]. While some embryos are significantly small for age by about 2 days growth (8mm) or more, only those which are the most retarded fail to block luteolysis [57]. Average reported embryo loss rates between Days 11 to 15 and 40 to 50 are 7.7% [56] and are highest between the first ultrasonic pregnancy examination at Day 12 to 14 and the next examination at around 21 days after service. Loss rates in the following weeks are much lower. Unlike the cow where luteal survival is dependent on luteotrophic support from the conceptus, it is uncommon for embryo failure to be either caused by, or to be followed by luteolysis [56,58]. Usually the CL will persist for its normal lifespan of about 60 to 110 days in the absence of a viable embryo [59,60].

Most reports suggest that pregnancy rates at Days 12 to 14 in the mare are around 60-65% and yet fertilisation rates are considered to be much higher; at least 85% for older mares and nearer 95% for young mares [61]. Thus a considerable degree of embryo loss, in the order of 25% to 35%, must occur between fertilisation and the first ultrasonic examination for pregnancy between Day 12 and 14.

There is little or no evidence that progesterone supplementation during the first two weeks of dioestrus results in increased pregnancy rates in the mare. In fact there is much evidence that the early equine conceptus can survive and develop at a normal rate for several days during low [62] or even sub-luteal plasma progesterone concentrations. When exogenous PGF2α is given during early pregnancy resulting in luteolysis, the conceptus continues to develop for several days at a normal rate until it suddenly disappears. Pregnancy failure may be due to evacuation through the cervix as the mare comes into oestrus, rather than embryonic death. Later embryos, following PGF2α administration, may be seen still with a heartbeat even though the conceptus has become displaced by endometrial oedema and fluid secretions. Embryos in 10 pregnant mares, given a single dose of clomifoprostenediol between Day 8 and 26, survived and developed normally for between 2 and 8 days after treatment (mean 4.7 days; J Newcombe, unpublished data). At the next examination after the last one in which the viable embryo was still present (2 to 5 days later), the vesicle had either disappeared or the embryo appeared dead.

Two cases of pregnant mares which returned to oestrus yet retained their pregnancy following a spontaneous ovulation, have been reported [63,64] and a third unreported (J Newcombe, unpublished data). Early pregnancies with clinical evidence of spontaneous luteal regression can be saved by progesterone supplementation [65]. Inevitably a spontaneous ovulation occurs at about Day 21 or 22 after which progesterone supplementation can be withdrawn (J Newcombe, unpublished data). It has even been suggested [66] that only a relatively low threshold progesterone level is necessary to maintain pregnancy. Indeed it has been confirmed by the present author that pregnancy can survive low progesterone concentrations from soon after ovulation. Fourteen inseminated mares were treated with multiple luteolytic doses (37.5 μg) of d-clomifoprostenediol every 8 hours starting as early as 28 hours post ovulation and continued to be at 12 hours. NinEight (57%) were diagnosed pregnant at 12 to 13 days and were still pregnant at 20 days although several had ovulated without any clinical evidence of oestrus (Newcombe and Cuervo-Arango, Poster ISER 2014).

Embryo Loss in the Mare

There is little experimental evidence as to when embryo losses occur before the time of the first ultrasonic pregnancy detection which is usually between Day 11 and 14. Any evidence has to be based on the percentage of embryos flushed from either the uterine tubes or the lumen on different days after ovulation. In one study [67] significantly fewer embryos per ovulation were recovered following unilateral twin ovulations versus bilateral ovulations (34% v. 66%). In a large field study, less than half the mares pregnant after multiple ovulations were found.
The effects of buserelin given in mid-dioestrus on pregnancy rates have been studied in thoroughbred mares in field trials [76,77]. In a series of trials, 40 μg was given by a single subcutaneous injection to mares on Day 9, 10, 11 or 12 (initially) then later, either 20 μg or 40 μg buserelin was given either on Day 10 or on Day 11. Later field trials were done with either 20 μg or 10 μg. Improvements in Day 13-15 pregnancy rates after first service were found in every trial when mares were treated on Day 10 or 11. From 1994 to 2004, the margins of improvement in pregnancy rates over untreated controls in 14 trials were 6.0%, 14.2%, 7.3%, 12.2%, 12.5%, 2.2%, 16.5%, 2.4%, 7.3%, 4.2%, 9.3%, 9.1% and 2.5% (latter two both in 2003) and 2.2% in 2004 (P<0.0001 by meta-analysis [78]). From 2008 to 2010 using either 20 or 10 μg buserelin, improvements were 2.2%, 16.5% and 13.9% respectively, an average of 7.6% [78]; J. Newcombe unpublished data). A 2.86% increase in the number of mares with multiple embryos was also found in buserelin treated mares 17.32% (n=2540) v. 14.46% (n=1452). This represented a significant (P<0.001) increase of 19.8% in the percentage of mares with multiple embryos. The effect of buserelin even extended beyond the time of first pregnancy examination. Embryo loss from that time and up to 40 days was reduced from 8.22% in 1314 pregnancies to 6.06% of 2427 pregnancies (P=0.014). In those mares which did not conceive at the first cycle, treatment in the second or subsequent cycles improved pregnancy rates regardless of whether or not they had been treated with buserelin in the first or previous cycle (J. Newcombe, unpublished data).

In other work [79], 40 μg buserelin was administered on Day 10 to half of 171 warm blood mares inseminated with either fresh or frozen/thawed semen. The pregnancy rate in treated mares was 46% compared with 36.4% for untreated mares (P=0.22). Although progesterone concentrations were unaffected, the authors found that LH concentrations were significantly elevated. Others [80] giving 40 μg buserelin on Day 10 also found no evidence of raised progesterone concentration, secondary ovulations or prolonged luteal life. In another study [81] mares were challenged on Day 12 with oxytocin after giving 40 μg buserelin to mares on Day 10. There was no difference between pregnant and non-pregnant treated mares in their PGFM response. Buserelin was therefore unable to suppress luteolysis in the non-pregnant mare.

The effect of 40 μg buserelin given 10 days after AI was investigated in a group of subfertile mares inseminated with either fresh or frozen/thawed semen [82]. Half the mares were treated and half remained as untreated controls. Pregnancy rates over 136 untreated cycles averaged 34.5%, with 41.6% for fresh and 31.2% for frozen semen respectively. Pregnancy rates in the same mares when treated with buserelin averaged 44.8%, with 48.4% for fresh and 41.8% for frozen semen. The 10.3% overall increase in the pregnancy rate in treated cycles meant an actual increase of nearly 30% in the total number of pregnancies.

It has been shown [83] that a GnRH antagonist given on Day 8 could have an adverse effect on pregnancy apparently by causing a sharp drop in progesterone concentrations while in three other reports, a GnRH agonist did not cause any P4 elevation [80-82].

Conclusions

There is therefore much clinical evidence that the administration of a single subcutaneous injection of 40 or 20 μg of aqueous buserelin to mares, 9 to 10 days after the detection of ovulation produces a highly significant improvement in pregnancy rate [77], including an increase in embryo numbers in multiple ovulating mares. This is evident by the time of early ultrasonic detection of the embryonic vesicle at 12 to 13 days after ovulation, before the time of luteolysis and only 2 to
4 days after treatment. This improvement in pregnancy rate has been demonstrated by various other studies in horses, cows, sheep and pigs, while in the latter two species, litter size may also be enhanced. Improvements in both pregnancy rate and litter size suggest a reduction in embryo loss between the time of buserelin administration and the time of pregnancy detection. Although there is no ultrasonic evidence that pregnancy rates are already elevated before the time of luteolysis, the mechanism by which post-luteolysis pregnancy rate is improved in dairy cows, is reasonably assumed to be by delaying the endogenous luteolytic drive; embryos with delayed development can gain sufficient maturity to block luteolysis. Increased progesterone concentrations may also boost conceptus development. Blocking luteolysis may also be the mechanism in pigs and sheep. However it would not account for any increase in litter size in those species since luteolysis is all or nothing. It is possible that there is differential sensitivity between embryos to progesterone concentrations.

Buserelin may act in two ways by both delaying luteolysis in pregnant ruminants (although it does not in either the horse or in the non-pregnant ruminant) whilst also reducing embryo loss in multiple pregnancies. Mechanisms for multiple embryo reduction occur in many species most notably the pig. The South American rodent, the Plains Viscacha ovulates 400-800 oocytes of which just 8 to 10 are fertilised, but normally produces only one or two young [84]. One might speculate that during evolution, from the tiny rodent-like mammals of the cretaceous period, which had evolved to produce large litters in order to counteract the effects of predation, (like most small modern rodents), to the large monotocous mammals of today like the elephant, the bovidae and the equidae, that a mechanism for embryo reduction, has been conserved in modern (normally) monovular species. If so, then it could be responsible for twin pregnancy reduction and even some reduction of single embryos.

High progesterone concentrations may well have a role in increased embryo survival in farm animals and other species. However in the mare there is neither evidence of any effect of buserelin on progesterone concentrations nor indeed any evidence that pregnancy is dependent on continuously elevated concentrations of progesterone. On the contrary, a pregnancy can survive, and develop at the normal rate as judged by growth rate of the early embryonic vesicle, in the presence of continuous prostaglandin suppressed progesterone concentrations, or even for short periods during non-luteal (<1 ng/ml) progesterone concentrations with the mare in oestrus [64,83,85].

If a mechanism to delay the luteolytic drive were to exist in the mare then the increase in pregnancy rate would not become apparent until after the time of luteolysis (after Day 15 or 16). The various studies show that the increase in mares occurs before the time of luteolysis. In any case, spontaneous luteolysis in the pregnant mare is at best unusual whilst embryonic loss which occurs after about Day 12 is rarely followed by luteolysis [58]. Unlike the cow, the CL persists after embryonic death since it is not dependent on embryonic support.

So if the embry support mechanism induced by buserelin treatment in the mare is independent of CL function or progesterone secretion per se, what other factors could be involved? That exogenous GnRH stimulates LH and FSH secretion both during oestrus and dioestrus is well established. Is it possible that GnRH stimulated LH and FSH release during dioestrus may have some effect independent of the ovary?

The role of oestradiol-17β in early equine pregnancy is unclear but as in the cow, buserelin may well influence oestradiol-17β secretion. Reduction of oestradiol-17β concentrations is important in the delayed development of oxytocin receptors and luteostasis in the cow. Exogenous oestradiol-17β significantly reduced progesterone concentration but did not affect embryonic development [62]. It has been argued [86] that increased follicular oestradiol-17β production is not necessary for luteolysis in mares. Since this steroid has not been investigated in buserelin treated mares, it is possible that its influence is on either the endometrium or directly on the embryo. Even if there is an interferon produced at this time, either by the embryo or by the endometrium, how could this be influenced directly or indirectly by GnRH?

GnRH analogues have been shown to have various sites of action other than the pituitary, most notably on the urinary tract of bitches [87], while GnRH receptors have been recognised in various other tissues [88] including the placenta [89]. Binding sites for LH / hCG are found in the uterus of several species with maximum expression in the luteal phase [90]. Maximum expression of the GnRH receptor in the human uterus also occurs during the luteal phase [90]. That author in reviewing the actions of gonadotrophins on the uterus, concluded that "The presence of gonadotrophin and GnRH receptors with a dynamic pattern in the endometrium, myometrium, oviduct and cervix of different species provides evidence that gonadotrophins and GnRH play a substantial role as molecular autocrine-paracrine regulators of the oestrous cycle and implantation" [90]. The author is suggesting that GnRH could have a direct role at the level of the reproductive tract that does not involve the ovary. Therefore there could be a direct effect of exogenous GnRH (buserelin) on the relationship between the embryo and the endometrium. Irrespective of the mechanism, it is evident that buserelin acts independently of the CL in the mare, by limiting the effect of any embryo reduction process operating between Days 9 to 10 and 13 to 14 of pregnancy.

References


