Uterine influences on embryogenesis and early placentation in the horse revealed by transfer of day 10 embryos to day 3 recipient mares

Sandra Wilsher and W R Allen

The Paul Mellon Laboratory of Equine Reproduction[†], 3 Tower Stables, Cheveley Park, Newmarket, Suffolk CB8 9DE, UK

Correspondence should be addressed to S Wilsher; Email: paulmellonlab@btconnect.com

[†]Formerly The Equine Fertility Unit, Mertoun Paddocks, Newmarket, UK

Abstract

Eight day 10 horse embryos were transferred non-surgically to recipient mares that had ovulated 7 days after the donors. The embryonic vesicle was seen ultrasonographically in all eight recipients, and three out of eight (38%) of the vesicles developed an embryo proper with a beating heart. Conceptus expansion was initially slower than that in control mares but continued until day 22 (recipient day 15). Time of fixation of the vesicle was related to its diameter, rather than uterine stage. Although the embryo proper first appeared ultrasonographically on day 22, as normal, it grew more slowly and the allantois expanded more slowly than that in control mares with normal pregnancies. The development of endometrial cups and their secretion of equine chorionic gonadotropin in the two mares allowed to remain pregnant to >50 days occurred at a conceptus age ~7 days later than that in the control mares. The results demonstrated the uniqueness of the horse conceptus in being able to overcome a 7-day asynchrony with the uterus, and also highlighted the overriding influence of the uterine environment on conceptus development in the mare.

Reproduction (2009) **137** 583–593

Introduction

Equids stand out among the large domestic animal species by having an embryo that takes as long as 6 days to traverse the oviduct and enter the uterus (Battut *et al.* 1997), a conceptus that remains spherical due to its envelopment by a tough glycoprotein capsule between days 6.5 and 23 after ovulation (Betteridge 1989, Oriol *et al.* 1993a, 1993b) and a conceptus that propels itself continually throughout the uterine lumen between days 6 and 17 after ovulation (Ginther 1983a) by means of its own release of prostaglandins F_{2α} and E₂ (Stout & Allen 2001). Subsequently, a discrete, annulate band of specialized invasive trophoblast cells forms the chorionic girdle that peels off the fetal membranes at around day 36 and adheres to the overlying endometrium, thereby enabling the binucleate trophoblast cells to vigorously invade the endometrium (Allen *et al.* 1973, Stewart *et al.* 1995). There the girdle cells enlarge greatly to form the endometrial cups and begin to secrete the unusual gonadotropic hormone, equine chorionic gonadotropin (eCG).

The mechanisms that control the rate of development and differentiation of the equine conceptus, and both the

rate and degree of development of the invasive chorionic girdle, have long aroused scientific interest due to the lack of stable attachment of the chorion to the endometrium until as late as days 40–42 after ovulation (Samuel *et al.* 1974, 1975). There is strong evidence that the uterine environment, perhaps acting via endometrial growth factor production, exerts an overriding influence on the development and subsequent secretory functions of the chorionic girdle (Allen *et al.* 1993).

The importance of the uterine environment to embryonic development was first highlighted by Nicholas (1933), who showed that altering the degree of synchrony between donor and recipient rats influenced the success of embryo transfers. Further work in other species highlighted the importance of uterine–embryo synchrony and the effects of the early uterine environment on the subsequent development of the embryo (see Pope 1988 for a review). For example, ovine embryos increase their rate of cell division when exposed to an advanced uterus (Wilmot *et al.* 1985) or slow down their development after transfer to a less advanced uterus (Wilmot & Sales 1981, Lawson *et al.* 1983). Furthermore, although ovine embryonic survival following asynchronous transfer is only slightly less than maximum when

the interval between donor and recipient differs by 2 days, pregnancy failure is almost inevitable when the level of asynchrony is increased to 3 days (Moore & Shelton 1964, Rowson & Moor 1966).

In this paper, successful transfer of horse embryos to recipient mares that ovulated 7 days later than the donors lends strong support to the concept of dominant uterine control over embryogenesis and fetal membrane development in equids.

Results

Embryo survival

All nine control pregnancies showed echogenically normal development. An embryo proper with a heartbeat was observed ultrasonographically in these animals between days 20 and 22 of gestation. Out of the eight day 10 embryos transferred to the day 3 recipient mares, three showed delayed, but otherwise echogenically normal, development. Two others failed to develop an embryo with a discernable heartbeat and the vesicles degenerated and eventually disappeared at days 33 and 35 respectively (recipient days 26 and 28). In both of these mares, a tiny embryo was detected ultrasonographically on day 20 (recipient day 13); in one mare, it was not seen again (Fig. 1.1–1.4a), and in the other, despite an initial enlargement of the allantois around day 28 (recipient day 21), the embryo never exhibited a detectable heartbeat (Fig. 1.1–1.4b). One further transferred embryo developed into an anembryonic trophoblast vesicle that degenerated at around embryo day 34 (recipient day 27; Fig. 1.1–1.4c) and the remaining two transferred embryos that appeared normal in the recipient uteri 2 days after transfer (Fig. 1.1–1.2d), had disappeared completely by the next scan 2 days later when an injection of 250 µg of the prostaglandin F2α analogue, cloprostenol (Estrumate; Schering-Plough, Middlesex, UK) was administered to induce luteolysis and a return to estrus.

The size of the embryo at the time of transfer was a significant factor in determining its ability to survive to the heartbeat stage in the recipient's uterus. The mean $\pm$ S.E.M. diameter of the three surviving embryos was 2.54 ± 0.23 mm, compared with 4.55 ± 0.26 mm for the five embryos that did not survive ($P=0.002$). However, despite this initial distinction, no further differences in vesicle diameter were observed between surviving versus non-surviving embryos at later stages of development between 12 and 20 days ($P>0.05$ in all cases). Therefore, mean $\pm$ S.E.M. embryo expansion over the initial 2 days post-transfer was greater in surviving versus non-surviving embryos (142.6 ± 14.8 vs $87.5 \pm 14.6\%$ increase in diameter respectively; $P=0.049$). Figure 2 shows the mean embryonic vesicle diameters of the six transferred embryos that survived past day 14 compared with those in nine control pregnancies in mares

inseminated normally. Mean vesicle diameter of the transferred embryos was significantly lower than that of the controls on days 12, 14, and 16 ($P \leq 0.01$, in all cases), showed no difference on days 18 and 20 ($P>0.05$), and was significantly greater on day 22 ($P=0.005$).

In the three mares that remained pregnant, one was given an injection of 250 µg cloprostenol on recipient day 29 to induce luteolysis and expulsion of the conceptus, and the pregnancies were allowed to continue in the other two mares. One of these (CB) was subsequently killed on recipient day 62 and her uterus removed intact for the recovery of biopsies of endometrial cups and the allantochorion–endometrium implantation interface. The second mare (RL) underwent an intra-uterine videoendoscopic examination on recipient day 62 to view the fetus, allantochorion, and endometrial cups before therapeutic abortion.

Conceptus development

In the three asynchronous recipient mares that maintained their pregnancies to the heartbeat stage, expansion of the embryonic vesicle was initially slower than that of a conventional pregnancy. However, the expansion rate increased markedly once the recipient reached day 7 so that, by the time the transferred embryo reached day 18, the vesicle was equivalent in size to a conventional day 18 conceptus, despite being in a day 11 uterus (Fig. 3). The embryonic vesicle remained spherical in these recipient mares until as late as embryo days 20–22 (recipient days 13–15), after which it became increasingly irregular due to disproportionate thickening of the dorsal (mesometrial) endometrial folds (Fig. 4.2b–d and 4.3b–d). Fixation of the embryo had occurred at embryo days 16–18 (recipient days 9–11). Although a very small echogenic spot, presumably representing an embryo proper, was visible in the ventral quadrant of the still spherical vesicle on embryo day 22 (recipient day 15), this was not seen again clearly until a much bigger embryo with a demonstrably beating heart became visible ultrasonographically at embryo days 26–28 (recipient days 19–21; Fig. 4.2b–d and 4.4b–d). In two out of the three ongoing pregnancies, at conceptus days 33–35 (recipient days 26–28), the development of the fetal membranes was such that half the volume of the spherical conceptus was occupied by allantois and the other half by yolk sac; hence, they were ultrasonographically more similar in appearance to a day 28 horse conceptus than the 33- to 35-day conceptuses they were chronologically supposed to be (Fig. 5.2a–d and 5.3a–d); the third conceptus lagged behind these two in terms of expansion of the allantois.

The two pregnancies that were allowed to proceed past conceptus day 33 (recipient day 28) showed further expansion of the allantoic cavity and regression of the yolk sac, resulting in the eventual coming together of

Figure 1 Development of the five transferred day 10 embryos that failed to progress to the stage of an embryonic heartbeat. The two mares in which an embryo (E) was present prior to pregnancy failure are shown in the first two columns (1.1–4a–b). Note the enlarging allantoic cavity (AC) and persisting yolk sac cavity (YSC) in 1.2–3b, despite the lack of a discernable heartbeat in the embryo. An increase in endometrial tone, with associated hypertrophy of the endometrial folds (asterisk) was evident by conceptus days 26–28 (recipient days 19–21). The collapse of the fetal membranes in these failing pregnancies is shown in 1.4a–b. The third column shows the vesicle that never developed an embryo proper (1.1–4c). The two embryos that were present at the first post-transfer scan but subsequently disappeared are shown in 1.1–2d. P, embryo age; Ov, days post-ovulation of recipient. Scale bar, 1 cm.

the membranes and associated vessels to form the umbilical cord (Fig. 5.4b–c). The cord, in both cases, was attached at the correct dorsal orientation. Further development of these two pregnancies, up to the time of termination at recipient day 62, appeared normal as judged ultrasonographically and grossly, either videonendoscopically or at post-mortem.

Endocrinological changes and endometrial cup development

In all six recipient mares in which the transferred embryos survived beyond recipient day 14, luteolysis did not occur and peripheral progesterone levels remained elevated, even in the absence of an embryo

proper with a heartbeat (Fig. 6). Progesterone profiles for the two mares in which the embryo was no longer visible at recipient day 7 are also shown in Fig. 6. No difference existed in the progesterone profiles of the recipient mares in which the embryos survived more than 4 days versus those in which they failed ($P > 0.05$).

As shown in Fig. 7a, in the two mares in which pregnancy continued beyond formation of the endometrial cups, eCG first became detectable in the serum on conceptus day 42 or 43 (recipient days 35–36) and, hence, 6–7 days later, in terms of conceptus age, than the time of detection in the blood of nine control mares monitored similarly while carrying their own normal horse conceptuses conceived by conventional artificial insemination (Fig. 7b). A sharp rise in

Figure 2 The mean ($\pm$ S.E.M.) diameter of vesicles in pregnancies resulting from artificial insemination (days 10–22; $n=9$; closed circles) and following transfer of day 10 embryos to day 3 recipients (embryo days 10–24; $n=6$; open circles). Individual vesicle diameters of the transferred embryos are shown for those that developed an embryo with a heartbeat (triangles) and those that did not (squares).

serum progesterone concentrations, indicating secondary ovulation, occurred in the two mares carrying the asynchronous pregnancies, but only after eCG had been detected in maternal serum. Rises in serum progesterone concentrations also occurred in the control mares after the appearance of eCG in the serum. However, individual variation between the control mares masked this rise on the mean profile. Serum progesterone and eCG concentrations in the control and recipient mares were within normal levels for pregnant mares.

Discussion

In the horse, previous studies have shown maximum pregnancy rates to be achieved when the embryos are transferred to the recipients that have ovulated between 1 day before and 3 days after the donor (Allen 1982, Squires *et al.* 1982, Stout 2003, 2006). That pregnancies resulted despite 7 days asynchrony between donor and recipient mares in three instances in the present experiment is quite remarkable in comparison with other species. For example, Moore & Shelton (1964) and Lawson *et al.* (1975) both established convincingly that a negative asynchrony (i.e., recipient ovulates after the donor) of 2 days is the maximum achievable in the cow and sheep, while Polge & Dziuk (1970) similarly established a limit of positive or negative asynchrony of 2 days in the pig.

The spherical shape of the horse conceptus, contrasting with the elongated conceptuses of the sheep, cow, and pig between 10 and 16 days after ovulation, combined with the very rapid post-ovulation rise in

maternal serum progesterone concentrations in the mare (Allen & Sanderson 1987) compared with the other domestic species (Wathes & Lamming 1995), and the very late stage for implantation and stable placental attachment in the mare (Amoroso 1952), may all contribute to widening the level of embryo–uterus asynchrony tolerated by the mare. The sharp rise in progesterone secretion after ovulation is likely to be the most significant of these factors by its rapid stimulation of histotroph secretion by the endometrial glands by the time the embryo enters the uterus on day 6.

In the present experiment, no significant differences were seen in the progesterone profiles of the recipient mares in which the transferred embryo survived versus those in which it either failed to develop a beating heart or survived for only a few days. Evidence from other species suggests that progesterone promotes changes in the uterine environment, which act to alter the course of embryo development. For example, sheep embryos from day 4 donors were retarded when placed in uteri 2 or 3 days behind and they failed to advance beyond the blastocyst stage (Lawson *et al.* 1983). However, day 10 sheep embryos could be transferred successfully to day 6 recipients if the latter received daily injections of progesterone from the day of estrus to advance the uterine environment (Lawson & Cahill 1983). Likewise, in cattle, higher systemic progesterone in the immediate post-conception period is associated with an increase in embryonic growth rate, interferon- τ production, and pregnancy rate (Garrett *et al.* 1988, Carter *et al.* 2008). In the horse, embryonic size and/or survival are not affected by administered progesterone (Weithenauer *et al.* 1989, Ball *et al.* 1992) or endogenous progesterone levels (Stout *et al.* 2004). Nevertheless, Zavy *et al.* (1979a, 1979b, 1982) demonstrated that changes in uterine protein secretions occur over time in relation to the number of days the uterus is under the dominance of endogenous progesterone, and Hinrichs *et al.* (1989) showed likewise with exogenous progesterone. Hence, in the horse, the duration, rather than the systemic level, of progesterone is the overriding influence on the uterine secretion of histotroph, which would explain the lack of any differences in progesterone profiles between the surviving and non-surviving transferred embryos in the present experiment.

The stage-specific changes in the uterine environment under the influence of progesterone are highlighted by the growth rate of the day 10 embryos transferred to the day 3 uteri. In normal pregnancy, once the embryo enters the uterus on day 6, its growth is exponential from day 6 until approximately day 15 (Ginther 1983a, Leith & Ginther 1984). Ultrasonographically, the embryonic vesicle can be seen to expand rapidly between days 11 and 15 of gestation (Ginther *et al.* 1985, Ginther 1986, Romagnano *et al.* 1989) after which expansion slows and then plateaus at around day 18. The delayed rapid expansion phase noted in the

Figure 3 Embryonic development between days 12 and 18 of gestation in a synchronous control pregnancy (3.1–4a) compared with that of the day 10 embryos that developed an embryo proper with a beating heart following transfer to asynchronous uteri (3.1–4b–d). Note the slower initial growth of the transferred embryos. At day 18 of gestation, the control pregnancy is changing to a triangular shape as uterine tone increases and the dorsal (mesometrial) uterine wall thickens (3.4a). By contrast, the transferred embryos remain spherical (3.4b–d) at this stage. P, embryo age; Ov, days post-ovulation of recipient or control mare. Scale bar, 1 cm.

transferred day 10 embryos, corresponding to recipient days 7–13, continued to a later stage than would normally be the case. Hence, vesicle diameter eventually surpassed that of similar stage synchronous, control embryos. Because a marked increase in myometrial tone in the pregnant equine uterus between days 17–26 has been suggested as one factor in the plateau in normal conceptus enlargement that occurs during this time period (Ginther 1998), the continued increase in vesicle diameter of the transferred embryos after day 18 (recipient day 11) may, in part, be explained by an absence of an increase in uterine tone.

Conceivably, the embryo transfer procedure *per se*, rather than the degree of asynchrony between donor and recipient, could have influenced the post-transfer expansion and development of the transferred versus the

control embryos in the present study. This is, however, unlikely in view of the results of a recent study that showed no significant difference in the diameters of embryonic vesicles derived from synchronous embryo transfer versus those from normal matings (Newcombe & Cuervo-Arango 2008).

Although the exact method of embryonic expansion remains unclear in the horse (Enders *et al.* 1993, Waelchli *et al.* 1997, Crews *et al.* 2007, Budik *et al.* 2008), the day 10 embryo certainly has the capacity to expand. Thus, its initial reduced expansion, compared with the controls, when placed in the day 3 uterus, could be a reflection of the uterine milieu. Therefore, as already implied, it is likely that uterine factors conducive to embryonic expansion are stage specific and are not expressed until the uterus has been under the influence

Figure 4 Conceptus development between days 20 and 26 of gestation in a synchronous control pregnancy (4.1-4a) compared with that of day 10 embryos that developed a beating heart following transfer to asynchronous uteri (4.1-4b-d). At day 20 of gestation, the embryo proper (E) and the hypertrophy of the dorsal endometrial folds (asterisk) can be seen clearly in the control pregnancy (4.1a) compared with the still spherical vesicle devoid of an embryo proper in the transferred embryos (4.1b-d). The embryo proper (E) becomes just visible in the transferred pregnancies on day 22 (recipient day 15; 4.2 b-d). By conceptus day 26, the allantoic cavity (AC) is clearly visible in the control conceptus (4.4a) but is still absent from the transferred embryos (4.4b-d). P, embryo age; Ov, days post-ovulation of recipient or control mare. Scale bar, 1 cm.

of progesterone for a set interval post-ovulation. Although others have noted that the equine embryo can continue to expand normally for a time in the absence of maternal progesterone after prostaglandin administration (Ginther 1985, Kastelic *et al.* 1987, Chu *et al.* 1997), and can develop up to the heartbeat stage following ovariectomy on day 12 (Kastelic *et al.* 1987), this is after the initial rise in progesterone concentrations following ovulation.

Although the cessation of embryo mobility was judged only on the basis of conceptus location between alternate day scans, conceptus size, rather than uterine stage, appeared to be the determining factor in conceptus fixation. Hence, this particular aspect of development appears to be predominantly influenced

by the conceptus. This would concur with Gastal *et al.* (1996), who noted that fixation occurred earlier for larger embryos. Furthermore, Bergfelt & Ginther's (1996) reported that similar sized vesicles fixed a day earlier in ponies than horses due to the smaller uterine horn diameter of the former, and suggests that relative size of the conceptus and uterus is important in determining the time of fixation. Other factors have been implicated in influencing fixation of the conceptus, namely the increase in uterine tone and associated decrease in uterine diameter (Ginther 1983b, Griffin & Ginther 1991, Bonafas *et al.* 1994, Bergfelt & Ginther 1996) and desialylation of the capsule (Betteridge 2000). The aforementioned increase in uterine tone around day 17 after ovulation still occurred in the recipient mares in the

Figure 5 Conceptus development between days 30 and 40 of gestation in a synchronous control pregnancy (5.1-4a) compared with that of the day 10 embryos that developed an embryo proper with a beating heart following transfer to asynchronous uteri (5.1-4b-d). At day 30 of gestation, the control conceptus shows enlargement of the allantoic cavity (AC) and regression of the yolk sac cavity (YSC) such that the each represents approximately one-half of the conceptus (5.1a). Although the conceptus in asynchronous mare CB shows slight enlargement of the allantoic cavity (AC; 5.1b) at the same age, this is not apparent at all in the other two conceptuses until day 33 (5.1c-d). By day 40 of gestation, the umbilical cord (UC) is clearly visible in the control pregnancy (5.4a), while in the recipient mares carrying the asynchronous transferred embryos the yolk sac cavity (YSC) is still visible (5.4b-c). P, embryo age; Ov, days post-ovulation of recipient or control mare. Scale bar, 1 cm.

present experiment, even in the presence of the now stationary 24-day conceptus, as indicated by the change in shape of the embryonic vesicle associated with the increased uterine turgidity and thickening of the mesometrial uterine wall.

In the present experiment, regardless of its developmental competence, the presence of a day 10 transferred embryo suppressed luteolysis, despite the fact that (i) movement of the vesicle had ceased by recipient days 9–11, and (ii) at the time when the suppression of luteolysis is assumed to occur (i.e., days 10–16), the conceptus was already 17–23 days old, and in the normal course of events, maternal recognition of pregnancy (MRP) would have already occurred. Hence, it may be concluded that either the day 10

horse embryo can act to suppress luteolysis for at least a further 7 days or that the MRP signal is very long acting. Rivera del Alamo *et al.* (2008) found that inserting a 20 mm water-filled plastic ball into the uterus of mares at 2–4 days after ovulation induced a prolongation of luteal function in 9 out of 12 treated mares, with concomitant decreases in PGF_{2α} metabolites in their peripheral plasma between days 11 and 16. This result argues against an obligation for either movement or the release of a MRP signal to achieve luteostasis.

Little is known about the processes of cell proliferation and morphogenesis in the equine conceptus, particularly the embryo proper. In the present experiment, development of the embryo proper was slower than in a normal synchronous pregnancy. This may have been due

Figure 6 The mean ($\pm$ S.E.M.) progesterone profiles of the eight recipient mares that received day 10 embryos 3 days post-ovulation. No significant differences exist in the progesterone levels measured in mares in which the transferred embryo developed a heartbeat ($n=3$, open circles), those in which the embryo did not develop a heartbeat ($n=3$, closed circles) and those in which the embryo had disappeared by recipient day 7 ($n=2$, squares).

to modifications of the availability and/or binding of growth factors such as insulin-like growth factor 1 (IGF1; see Simmen *et al.* 1995 for a review). IGF1 is known to be present in the yolk sac fluid of the conceptus, in uterine flush fluid, and in the endometrium of mares between days 12 and 18 of pregnancy (Salute & Tucker 1992, Walters *et al.* 2001) and the equine conceptus is known to bind IGFs at least from day 10 after ovulation (Herrler *et al.* 2000). The equine conceptus also produces significant quantities of estrogen from day 12 (Flood *et al.* 1979, Heap *et al.* 1982, Raeside *et al.* 2004). In the pig, endometrial IGF1 gene expression increases in early pregnancy and peaks on days 12–13, coincidentally with the secretion of estrogens by the elongating conceptus (Simmen *et al.* 1990, 1992). These embryonic estrogens upregulate fibroblast-like growth factor 7 (FGF7), which, in turn, stimulates proliferation and differentiation of trophoblast cells (Ka *et al.* 2000, 2001). Premature exposure of the pig's endometrium to estrogen prior to the normal period of its secretion by the conceptus alters gene expression patterns in the endometrium (Giesert *et al.* 2006) and premature proteolysis of IGF-binding proteins within the uterus on day 10 and a premature decline in uterine luminal IGF1 content results in embryonic death (Ashworth *et al.* 2005). Although Walters *et al.* (2001) failed to modulate IGF1 production in equine endometrial explants *in vitro* with estrogen, the possibility, nonetheless, exists that asynchronous embryo transfer may expose the uterus to conceptus estrogens at an inappropriate time which, in turn, may modulate the production of specific growth factors, thereby leading to alterations in growth and development of the embryo.

Figure 7 (a) Serum progesterone and eCG profiles in the two asynchronous recipient mares (CB progesterone, closed circles; CB eCG, open circles; RL progesterone, closed squares; RL eCG, open squares) in which the pregnancies continued to conceptus day >50 (recipient day >43). (b) Mean serum ($\pm$ S.E.M.) progesterone (closed squares) and eCG (open squares) concentrations in control mares carrying synchronous pregnancies conceived by artificial insemination ($n=9$).

The results of this experiment strongly suggest that uterine environment exerts an influence upon the rate of development of the fetal membranes, especially the invasive component of the trophoblast, the chorionic girdle. In both recipient mares in which the pregnancy was allowed to proceed to beyond day 50, the chorionic girdle cells did not invade the endometrium and begin to secrete eCG into the maternal circulation until conceptus day 42 or 43. This equated to the 35–36 days post-ovulation when chorionic girdle invasion would have been expected to have occurred in normal horse pregnancies (Allen & Moor 1972). Since it was impossible ultrasonographically to determine when girdle formation occurred in the asynchronous embryos, it could be argued that the delayed girdle invasiveness resulted from the delayed uterine receptivity rather than the delayed girdle cell development. However, the highly invasive nature of mature, day 33 chorionic girdle cells *in vitro*, and their ability to invade both diestrous and estrous endometrial explants and a variety of other equine tissue types make this unlikely (Meadows *et al.* 1995, Meadows 1996).

We showed previously that the transfer of mule embryos to the uteri of jenny donkeys results in the formation of much broader and more eCG productive endometrial cups than would be the case if the mule embryo was left in its natural horse mother (Allen *et al.* 1993). Thus, the mule embryo develops and behaves like a hinny embryo when placed in a donkey uterus, thereby reflecting the overriding influence of uterine environment on development of at least the invasive component of the fetal membranes. Similarly, in the present experiment, placing the horse embryos asynchronously into less advanced uteri (in terms of the length of exposure to the elevated progesterone levels of diestrus) caused a delay in the rate of development of the conceptus, as evidenced both by the ultrasonographically observed delay in the replacement of the yolk sac by the allantois during days 23–35 of gestation and the delay in the invasion of the chorionic girdle to form the eCG-secreting endometrial cups and the rise in serum progesterone concentrations from secondary corpora lutea.

When considering the possible intra-uterine mechanism that might cause such delay in chorionic girdle maturation and endometrial cup development, the mitogenic growth factor, epidermal growth factor (EGF), is one likely candidate. Gerstenberg *et al.* (1999) showed that *EGF* mRNA is upregulated in the epithelial cells lining the apical portions of the endometrial glands at 33–35 days of gestation. Furthermore, this sudden upregulation of the EGF message in the endometrium can be mimicked precisely in anestrus or ovariectomized mares by the daily administration of exogenous progesterone, but only after 35 days of continuous treatment; fewer than 33 days continuous administration was not accompanied by any detectable *EGF* mRNA in the endometrial tissues (Gerstenberg *et al.* 1999). This being so, it is interesting to speculate that endometrial EGF, in addition to allantoic hepatocyte growth factor-scatter factor (HGF-SF; Stewart *et al.* 1995), may be important in stimulating maturation of the chorionic girdle and its ability to secrete eCG; previous experiments involving the *in vitro* culture of chorionic girdle tissue recovered at selected stages of gestation between 28 and 35 days after ovulation revealed that the girdle cells mature and progressively acquire the ability to secrete eCG between days 30 and 35 of gestation (RM Moor, DF Antczak and WR Allen, unpublished observations). Thus, it is entirely conceivable that, in the two mares CB and RL in the present study, EGF secretion by the endometrial glands and HGF-SF secretion by the allantoic mesoderm were both controlled by the length of time that the uterus had been under the influence of progesterone. That is, uterine stage rather than conceptus age.

In the equine fetus and its extraembryonic membranes, specific patterns of development clearly depend upon ordered processes of cell proliferation, migration and differentiation, and upon a complex, stage-specific interaction between the conceptus and

the endometrium. The experiment described here has highlighted the remarkably large window of uterine asynchrony that a day 10 horse embryo will tolerate. This model will provide a means of studying early maternal–embryonic interactions in the mare.

Materials and Methods

Mare management and embryo recovery

Follicular development and ovulation in control, donor, and recipient Thoroughbred experimental mares were monitored by daily ultrasound scanning of their ovaries during estrus, combined with daily measurements of serum progesterone concentrations (Allen & Sanderson 1987). Control and donor mares were inseminated once with 300×10^6 to 500×10^6 freshly collected, extended stallion spermatozoa when they exhibited a dominant ovarian follicle of ≥ 35 mm diameter. All mares received a single i.m. injection of 0.75 mg of the GnRH analogue, deslorelin (BET Pharm Laboratories, Kentucky, KY, USA), to induce ovulation of the mature follicle 40–42 h later (Fleury *et al.* 2004).

On day 10 after ovulation (day 0), the uterus of each donor mare was flushed twice with 500–1000 ml of a commercial embryo transfer flushing medium (Emcare Complete; ICPbio Ltd, Auckland, New Zealand) using a standard two-way silicone 28 French embryo flushing catheter (Stallion Foley Catheter; SurgiVet, Waukesha, WI, USA). The flushing medium was recovered by gravity and the flow was halted as soon as the embryo appeared in the embryo filter. After microscopic assessment and measurement of its diameter, the embryo was washed with a commercial holding and transfer medium (Emcare Hold; ICPbio Ltd) and transferred non-surgically to the designated recipient mare.

Embryo transfer

Eight day 10 embryos, with a mean ($\pm$ S.E.M.) diameter of 3.80 ± 0.41 mm, were transferred each in 2.5–3.0 ml of embryo transfer medium, using the equine embryo transfer method described by Wilsher & Allen (2004), to the uteri of recipient mares that had ovulated only 3 days previously, thereby creating a donor–recipient asynchrony of 7 days.

Conceptus growth and endocrinological monitoring of control and recipient mares

Beginning 2 days after embryo transfer, or 12 days after ovulation, the uteri of the recipient or control mares respectively were scanned on alternate days to record the position, diameter, and ultrasonographic features of the conceptus. The diameter of the vesicle was measured ultrasonographically six times to obtain a mean measurement. Peripheral blood samples were recovered on alternate days by jugular venipuncture. The serum was decanted after centrifugation and subsequently assayed for progestagen concentrations, using the amplified enzyme-linked immunoassay (AELIA) described by Allen & Sanderson (1987), and for eCG concentrations using the AELIA described by Allen *et al.* (2002).

Statistical analysis

All statistical analyses were performed using a computer software package (SigmaStat v. 2.03; SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by the Tukey test was used to determine differences in the embryonic vesicle growth at different stages of development. Statistical significance was set at $P \leq 0.05$. In addition, one-way RM ANOVA was used to determine whether the levels of progesterone in the recipient mares' blood affected the likelihood of a transferred embryo surviving.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was kindly supported by the Horseracing Betting Levy Board and the Childwick Trust.

Acknowledgements

The authors would like to thank Miss Amber Clutton-Brock for her expert assistance with the embryo transfers, and Dr Jenny Ousey and Mrs Michelle Wright for their technical assistance with the management of the mares.

References

- Allen WR 1982 Embryo transfer in the horse. In *Mammalian Egg Transfer*, pp 33–48. Ed. CE Adams. Boca Raton: CRC Press.
- Allen WR & Moor RM 1972 The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. *Journal of Reproduction and Fertility* **29** 313–316.
- Allen WR & Sanderson MW 1987 The value of a rapid progesterone assay (AELIA) in equine stud veterinary medicine and management. In *Proceedings of the 9th Bain-Fallon Memorial Lectures*, pp 75–82. Sydney: AEVA.
- Allen WR, Hamilton DW & Moor RM 1973 The origin of the equine endometrial cups. II. Invasion of the endometrium by trophoblast. *Anatomical Record* **177** 485–502.
- Allen WR, Skidmore JA, Stewart F & Antczak DF 1993 Effects of fetal genotype and uterine environment on placental development in equids. *Journal of Reproduction and Fertility* **97** 55–60.
- Allen WR, Wilsher S, Stewart F, Ousey J & Fowden A 2002 The influence of maternal size on placental, fetal and postnatal growth in the horse. II. Endocrinology of pregnancy. *Journal of Endocrinology* **172** 237–246.
- Amoroso EC 1952 Placentation. In *Marshall's Physiology of Reproduction*, pp 127–311. Ed. AS Parkes. London: Longmans, Green and Co Ltd.
- Ashworth MD, Ross JW, Stein DR, Allen DT, Spicer LJ & Geisert RD 2005 Endocrine disruption of uterine insulin-like growth expression in the gilt. *Reproduction* **130** 545–551.
- Ball BA, Miller PG & Daels PF 1992 Effect of exogenous progesterone on early embryonic development in the mare. *Proceedings of the 12th International Congress on Animal Reproduction*, The Hague, The Netherlands, pp 1843–1845.
- Battut I, Colchen S, Fieni F, Tainturier D & Bruyas JF 1997 Success rates when attempting to non surgically collect equine embryos at 144, 156 or 168 hours after ovulation. *Equine Veterinary Journal* **25** 60–62.
- Bergfelt DR & Ginther OJ 1996 Ovarian, uterine and embryo dynamics in horses versus ponies. *Journal of Equine Veterinary Science* **16** 66–72.
- Betteridge KJ 1989 The structure and function of the equine capsule in relation to embryo manipulation and transfer. *Equine Veterinary Journal* **8** 92–100.
- Betteridge KJ 2000 Comparative aspects of equine embryonic development. *Animal Reproduction Science* **60–61** 691–702.
- Bonafas LD, Carnevale EM, Smith CA & Ginther OJ 1994 Development of uterine tone in nonbred and pregnant mares. *Theriogenology* **42** 1247–1255.
- Budik S, Walter I, Tschulenck W, Helmreich M, Deichsel K, Pittner F & Aurich C 2008 Significance of aquaporins and sodium postassium ATPase subunits for expansion of the early equine conceptus. *Reproduction* **135** 497–508.
- Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF & Lonergan P 2008 Effect of increasing progesterone concentration from day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reproduction, Fertility, and Development* **20** 368–375.
- Chu JKW, Sharom FJ, Oriol JG, Betteridge KJ, Cleaver DB & Sharp DC 1997 Biochemical changes in the equine capsule following prostaglandin-induced pregnancy failure. *Molecular Reproduction and Development* **46** 286–295.
- Crews LJ, Waelchli RO, Huang CX, Canny MJ, McCully ME & Betteridge KJ 2007 Electrolyte distribution and yolk sac morphology in frozen hydrated conceptuses during the second week of pregnancy. *Reproduction, Fertility, and Development* **19** 804–814.
- Enders AC, Schlafke S, Lantz KC & Liu IKM 1993 Endoderm cells of the equine yolk sac from day 7 until formation of the definitive yolk sac placenta. *Equine Veterinary Journal* **15** 3–9.
- Fleury PDC, Alonso MA, Alvarenga MA & Douglas RH 2004 Intervals to ovulation after treatment oestradiol cypionate (ECP) or BioRelease Deslorelin (BRT-DES). 6th International Symposium of Equine Embryo Transfer. In *Havemeyer Foundation Monograph Series No 14*, p 89. Eds M Alvarenga & JF Wade. Newmarket, UK: R&W Communications.
- Flood PF, Betteridge KJ & Irvine DS 1979 Oestrogens and androgens in blastocoelic fluid and cultures of cells from equine conceptuses of 10–22 days gestation. *Journal of Reproduction and Fertility* **27** 413–420.
- Garrett JE, Giesert RD, Zavy MT & Morgan GL 1988 Evidence for maternal regulation of early conceptus growth and development in beef cattle. *Journal of Reproduction and Fertility* **84** 437–446.
- Gastal MO, Gastal EL, Kot K & Ginther OJ 1996 Factors related to the time of fixation of the conceptus in mares. *Theriogenology* **46** 1171–1180.
- Geisert RD, Ross JW, Ashworth MD, White FJ, Johnson GA & DeSilva U 2006 Maternal recognition of pregnancy signal or endocrine disruptor: the two faces of oestrogen during establishment of pregnancy in the pig. *Society of Reproduction and Fertility Supplement* **62** 131–145.
- Gerstenberg C, Allen WR & Stewart F 1999 Factors controlling epidermal growth factor (EGF) gene expression in the endometrium of the mare. *Molecular Reproduction and Development* **53** 255–265.
- Ginther OJ 1983a Mobility of the early equine conceptus. *Theriogenology* **19** 603–611.
- Ginther OJ 1983b Fixation and orientation of the early equine conceptus. *Theriogenology* **19** 613–623.
- Ginther OJ 1985 Embryonic loss in mares: nature of loss after experimental induction by ovariectomy or prostaglandin $F_{2\alpha}$. *Theriogenology* **24** 87–98.
- Ginther OJ 1986 *Ultrasonic Imaging and Reproductive Events in the Mare*. Cross Plains, WI: Equiservices.
- Ginther OJ 1998 Equine pregnancy: physical interactions between the uterus and conceptus. *Proceedings of the American Association of Equine Practitioners* **44** 73–104.
- Ginther OJ, Bergfield DR, Leith GS & Scraba ST 1985 Early embryonic loss in mares: incidence and ultrasonic morphology. *Theriogenology* **24** 73–86.
- Griffin PG & Ginther OJ 1991 Dynamics of uterine diameter and endometrial morphology during the estrous cycle and early pregnancy. *Animal Reproduction Science* **25** 133–142.
- Heap RB, Hamon M & Allen WR 1982 Studies on oestrogen synthesis by the perimplantation conceptus. *Journal of Reproduction and Fertility* **32** 343–352.
- Herrler A, Pell JM, Allen WR, Beier HM & Stewart F 2000 Horse conceptuses secrete insulin-like growth factor-binding protein 3. *Biology of Reproduction* **62** 1804–1811.

- Hinrichs K, Kenney RM & Sharp DC 1989 Differences in protein content of uterine fluid related to duration of progesterone treatment in ovariectomised mares used as embryo transfer recipients. *Equine Veterinary Journal* **8** 49–55.
- Ka H, Spencer TE, Johnson GA & Bazer FW 2000 Keratinocyte growth factor: expression by endometrial epithelia of the porcine uterus. *Biology of Reproduction* **62** 1772–1778.
- Ka H, Jaeger LA, Johnson GA, Spencer TE & Bazer FW 2001 Keratinocyte growth factor is up-regulated by oestrogen in the porcine endometrium and functions in trophoblastic cell proliferation and differentiation. *Endocrinology* **143** 2303–2310.
- Kastelic JP, Adams GP & Ginther OJ 1987 The role of progesterone in mobility, fixation, orientation and survival of the equine embryonic vesicle. *Theriogenology* **27** 655–662.
- Lawson RAS & Cahill LP 1983 Modification of the embryo–maternal relationship in ewes by progesterone treatment early in the oestrous cycle. *Journal of Reproduction and Fertility* **67** 473–475.
- Lawson RAS, Rowson LE, Moor RM & Tervit HR 1975 Experiments on egg transfer in the cow and ewe: dependence of conception rate on the transfer procedure and stage of the oestrous cycle. *Journal of Reproduction and Fertility* **45** 101–107.
- Lawson RAS, Parr RA & Cahill LP 1983 Evidence for maternal control of blastocyst growth after asynchronous transfer of embryos to the uterus of the ewe. *Journal of Reproduction and Fertility* **67** 477–483.
- Leith GS & Ginther OJ 1984 Characterization of intrauterine mobility of the early conceptus. *Theriogenology* **22** 401–408.
- Meadows SJ 1996 The development and differentiation of the chorionic girdle in equids. *PhD Thesis*. University of Cambridge.
- Meadows SJ, Allen WR & Antczak DF 1995 Factors affecting the maturation and invasiveness *in vitro* of chorionic girdle cells of different genotypes. *Biology of Reproduction* **1** 171–180.
- Moore NW & Shelton JN 1964 Egg transfer in sheep. Effect of degree of synchronization between donor and recipient, age of egg, and site of transfer on the survival of transferred eggs. *Journal of Reproduction and Fertility* **7** 145–152.
- Newcombe JR & Cuervo-Arango J 2008 Does the process of embryo handling during embryo transfer induce a detrimental effect on early post-transfer embryo growth rate? *Proceedings of the 7th International Symposium on Equine Embryo Transfer*, Cambridge, UK, pp 48–49. Abstract.
- Nicholas JS 1933 Development of transplanted rate eggs. *Proceedings of the Society for Experimental Biology and Medicine* **30** 1111–1113.
- Oriol JG, Betteridge KJ, Clarke AJ & Sharom FJ 1993a Developmentally regulated changes of the equine embryonic capsule. *Molecular Reproduction and Development* **34** 255–265.
- Oriol JG, Sharom FJ & Betteridge KJ 1993b Developmentally regulated changes of the equine embryonic capsule. *Journal of Reproduction and Fertility* **99** 653–664.
- Polge C & Dzuik PJ 1970 Time of cessation of intrauterine migration of pig embryos. *Journal of Animal Science* **31** 565–566.
- Pope WF 1988 Uterine asynchrony: a cause of embryonic loss. *Biology of Reproduction* **39** 999–1003.
- Raeside JI, Christie HL, Renaud RL, Waelchli RO & Betteridge KJ 2004 Estrogen metabolism in relation to estrogen concentration in yolk-sac fluid. *Biology of Reproduction* **71** 1120–1127.
- Rivera del Alamo MM, Reilas T, Kindahl H & Katila T 2008 Mechanisms behind intrauterine device-induced luteal persistence in mares. *Animal Reproduction Science* **107** 94–106.
- Romagnano A, Betteridge KJ, Richer CL, Sirois J & King WA 1989 Growth rates of male and female equine embryos between Day 6 and 15 of pregnancy. *Equine Veterinary Journal* **8** 40–42.
- Rowson LEA & Moor RM 1966 Embryo transfer in the sheep: the significance of synchronizing oestrus in the donor and recipient animal. *Journal of Reproduction and Fertility* **11** 207–212.
- Salute ME & Tucker KE 1992 Insulin-like growth factor I (IGF-I) in yolk sac fluid, uterine flush and conceptus conditioned media during early pregnancy in mares. *Biology of Reproduction* **46** 68 (Abstract).
- Samuel CA, Allen WR & Steven DH 1974 Studies on the equine placenta I. Development of the microcotyledons. *Journal of Reproduction and Fertility* **41** 441–445.
- Samuel CA, Allen WR & Steven DH 1975 Ultra-structural development of the equine placenta. *Journal of Reproduction and Fertility* **23** 575–578.
- Simmen RCM, Simmen FA, Hofig A, Farmer SJ & Bazer FW 1990 Hormonal regulation of insulin-like growth factor gene expression in pig uterus. *Endocrinology* **127** 2166–2174.
- Simmen FA, Simmen RCM, Geisert RD, Martinat-Botte F, Bazer FW & Terqui M 1992 Differential expression during the oestrous cycle and pre- and post-implantation conceptus development, of messenger ribonucleic acids encoding components of the pig uterine insulin-like growth factor system. *Endocrinology* **130** 1547–1556.
- Simmen RCM, Green ML & Simmen FA 1995 IGF system in peri-implantation uterus and embryonic development. In *Molecular and Cellular Aspects of Peri-implantation Processes*, pp 185–204. Ed. SK Dey. New York: Springer-Verlag.
- Squires EL, Imel KL, Iuliano MF & Shidler RK 1982 Factors affecting reproductive efficiency in an embryo transfer programme. *Journal of Reproduction and Fertility* **32** 409–414.
- Stewart F, Lennard SN & Allen WR 1995 Mechanisms controlling formation of the equine chorionic girdle. *Biology of Reproduction Monograph Series* **1** 151–159.
- Stout TAE 2003 Selection and management of the embryo transfer donor mare. *Pferdeheilkunde* **19** 685–688.
- Stout TAE 2006 Equine embryo transfer: review of developing potential. *Equine Veterinary Journal* **38** 467–478.
- Stout TAE & Allen WR 2001 Role of prostaglandins in intrauterine migration of the equine conceptus. *Reproduction* **121** 771–775.
- Stout TAE, Tremoleda JL, Knapp J, Daels PF & Colenbrander B 2004 Does compromised luteal function contribute to failure to establish pregnancy after non-surgical transfer. In *Havemeyer Foundation Monograph Series No 14*, pp 8–9. Eds M Alvarenga & JF Wade. Newmarket, UK: R&W Communications.
- Waelchli RO, MacPhee DJ, Kidder GM & Betteridge KJ 1997 Evidence for the presence of sodium- and potassium-dependent adenosine triphosphate $\alpha 1$ and $\beta 1$ subunit isoforms and their probable role in blastocyst expansion in the preattachment horse conceptus. *Biology of Reproduction* **57** 630–640.
- Walters KW, Roser JF & Anderson GB 2001 Maternal-conceptus signalling during pregnancy in mares: oestrogen and insulin-like growth factor I. *Reproduction* **121** 331–338.
- Wathes DC & Lamming GE 1995 The oxytocin receptor, luteolysis and the maintenance of pregnancy. *Journal of Reproduction and Fertility* **49** 53–67.
- Weithenauer J, Sharp DC & Sherrin PC 1989 The effects of steroids on nutrients in yolk sac fluid of Day 18 pony conceptuses. *Equine Veterinary Journal* **8** 25–28.
- Wilmut I & Sales DI 1981 Effect of an asynchronous environment on embryonic development in sheep. *Journal of Reproduction and Fertility* **61** 179–184.
- Wilmut I, Sales DI & Ashworth CJ 1985 Physiological criteria for embryo mortality – is asynchrony between embryo and ewe a significant factor. In *The Genetics of Reproduction in Sheep*, pp 275–289. Eds RB Lard & D Robinson. London: Butterworths.
- Wilsher S & Allen WR 2004 An improved method for non-surgical embryo transfer in the mare. *Equine Veterinary Education* **16** 39–44.
- Zavy MT, Bazer FW, Sharp DC & Wilcox CJ 1979a Uterine luminal proteins in the mare. *Biology of Reproduction* **20** 689–698.
- Zavy MT, Mayer R, Vernon MW, Bazer FW & Sharp DC 1979b An investigation of the uterine luminal environment of non-pregnant and pregnant pony mares. *Journal of Reproduction and Fertility* **27** 403–411.
- Zavy MT, Sharp DC, Bazer FW, Fazleabas A, Sessions F & Roberts RM 1982 Identification of stage-specific and hormonally induced polypeptides in the uterine protein secretions of the mare during the oestrous cycle and pregnancy. *Journal of Reproduction and Fertility* **64** 199–207.

Received 3 August 2008

First decision 26 August 2008

Revised manuscript received 5 December 2008

Accepted 11 December 2008