

Review Report

What has embryo recovery and transfer taught us about equine reproductive physiology?*

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Abstract

Equine embryo transfer has been the foundation stone on which modern reproductive technologies and novel research in this species has been built. Nonsurgical embryo recovery and transfer methods developed from the original surgical methods, and the ease with which these procedures can be undertaken has enabled these techniques to play a continuing role in original research. We document how embryo recovery and/or transfer have increased our knowledge about many of the events involved in equine reproductive physiology. These include, when the equine embryo enters the uterus, the importance and development of the blastocyst capsule, secretory products of early embryos, early maternal-embryo interactions, effects of uterine asynchrony on early embryo development, the role of endometrial cups and maternal immunological tolerance of extraspecies embryo transfers, and the influence of uterine environment on fetal growth and development.

Keywords: Fetomaternal interactions, nonsurgical embryo transfer, surgical embryo transfer

Introduction

Our ability to collect an equine embryo and transfer it successfully to a recipient's uterus is considered a standard modern breeding technology, which was first successfully accomplished 50 years ago.¹ The first report detailed a surgical technique (midline laparotomy) for both recovery and transfer of embryos. These first transfers were undertaken solely for experimental purposes (to study the effect of interspecies transfers between donkeys and horses) and it was not uppermost in the researchers' minds to make this technique a routine veterinary procedure for the commercial production of horses. Since this first successful embryo transfer (ET), the technique of nonsurgical embryo recovery (ER) and transfer have been further developed (Figure 1) and can now be undertaken with relative ease, allowing their routine use in modern assisted reproductive techniques (ART).²⁻⁴

In addition to having a role in ART, both ER and ET have benefited the horse industry in general, even those sectors that do not allow embryo technologies to be used for breeding (e.g. Thoroughbred industry). This is because veterinary researchers have been able to utilize the techniques involved in ER and

ET to gain a clearer understanding of early embryonic development in the mare, maternal and fetal interactions required to establish ongoing pregnancy, and the influence of maternal uterine environment on fetal health and postnatal growth. Additionally, information gleaned from such studies will also pay dividends in unforeseen ways in the wider field of reproductive biology.

Some of the discoveries relating to equine reproductive physiology that would not have been possible without ER and/or ET are presented below. This is by no means a complete list and the authors have chosen to discuss some of those that are of personal interest.

Timing of embryo entry into uterus

From early attempts at flushing equine embryos or collection of early embryos from the oviduct either at slaughter or surgery,^{1,5,6} it became apparent that equine embryos were retained in the oviduct until 5 or 6 days after ovulation. However, determination of ovulation was not possible with any great precision as ultrasonography of the reproductive tract was not yet commonplace and it relied on teasing and transrectal palpation of the ovaries. With the advent of ultrasonography of the reproductive tract,^{7,8} a more accurate determination of ovulation was possible and when combined with nonsurgical ER techniques, experiments could be undertaken

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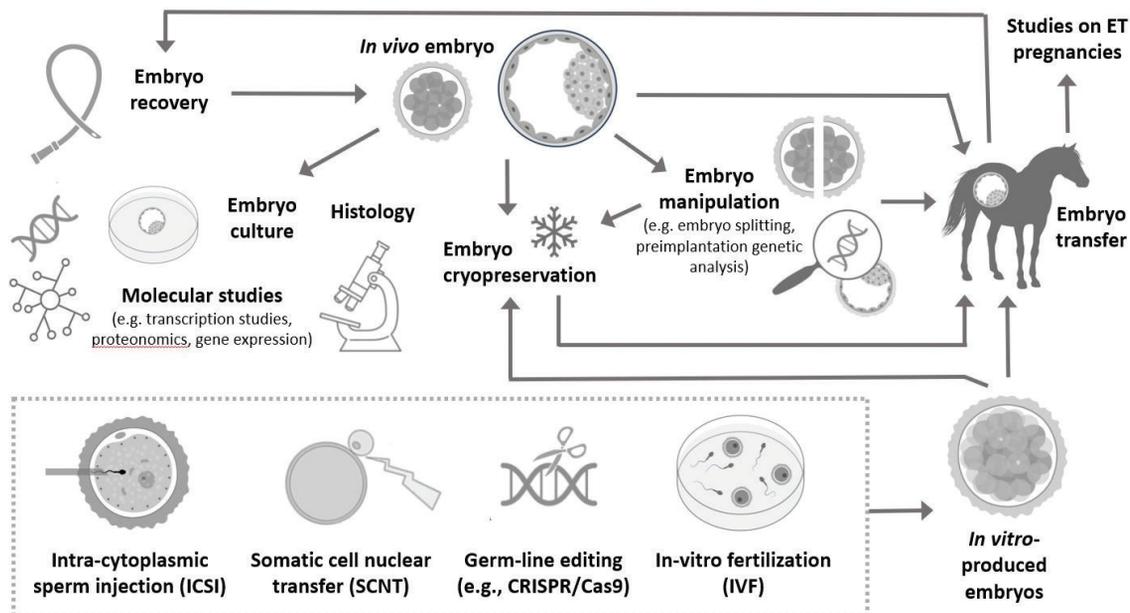


Figure 1. A diagrammatic representation of how embryo recovery and transfer fit into both clinical assisted reproductive technologies and research.

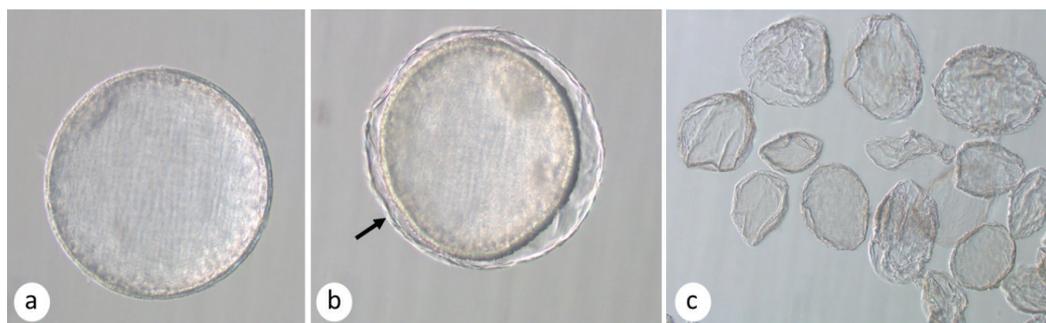


Figure 2. A 600-µm expanded blastocyst (a), loose capsule (arrow) around collapsed (via a fine acupuncture needle) blastocyst (b), and (c) capsular vesicles (100-300 µm), recovered from a recipient mare (via uterine flushing) 48 hours after transfer.

to try and pinpoint the precise timing of embryo entry into the uterus.^{9,10} Using hourly ultrasonography of the ovaries to determine the time of ovulation, mares were subsequently flushed once at 144 ± 0.5 , 156 ± 0.5 , or 168 ± 0.5 hours after ovulation. With this methodology, it was determined that embryos entered the uterus between 144-156 hours after ovulation.⁹ However, these researchers noted that the interval between ovulation and fertilization appeared to be inconsistent and/or embryonic development rate differed among individual embryos, as there was variation in the size and degree of development in embryos collected at similar time points.^{9,10} A subsequent study, using a similar protocol with successive uterine flushings at 3 or 6 hours intervals on the same mare until an embryo was obtained, tried to determine a more precise timing for transport of the embryo through the oviduct.¹¹ There was wide variation in the timing of entry of the embryo into the uterus, with some embryos collected from flushes as early as 144 ± 0.5 hours after ovulation and others as late as 150 ± 0.5 hours. A more recent study¹² undertook consecutive day flushing when attempting to recover embryos ≤ 300 microns for vitrification, with ER initially undertaken 156 ± 12 hours after ovulation for those bred with chilled semen or 168 hours after ovulation for those bred with frozen semen. If an embryo was not recovered at the first flushing, 2 more

flushings were tried at 24 hours intervals. In 7 of 35 mares (20%) an embryo was recovered on the third flush (204 ± 12 hours for chilled semen; 216 hours for frozen semen),¹² suggesting that the timing of embryo entry into the uterus may have a wider timespan than originally thought.

Despite the observed biological variation in the duration of oviductal transport, the knowledge that the majority of embryos will be present in the uterus at 156 hours (6.5 days) after ovulation has been instrumental in obtaining small embryos ≤ 300 microns that were suitable for successful freezing¹³ or vitrification¹⁴ in early cryopreservation protocols.

Importance and development of equine blastocyst capsule

Recovery of early embryos from the uterus has also allowed reproductive physiologists to discover more about the equine blastocyst capsule (Figure 2). This unique acellular layer develops underneath the zona pellucida (ZP) and envelops the equine embryo from \sim days 7 to 21 of pregnancy, once the ZP is shed¹⁵ The capsule is essential for the embryo's early survival in the uterus as demonstrated in an elegant experiment in

which day-7 embryos were flushed from the uterus and their capsules removed before being transferred to recipient mares.¹⁶ None of the 15 denuded embryos developed, whereas 4/6 (67%) control embryos, subjected to the same manipulations without removal of the capsule, developed. This clearly demonstrated that the presence of a capsule is essential for embryo survival.¹⁶

Analysis of capsules from in vivo-derived embryos has revealed they are composed of mucin-like glycoproteins that are produced primarily by the trophoblast^{17,18} with possible contributions from the endometrium. Macroscopic and molecular changes in its development over time have also been noted. For example, the weight of the capsule increased over time as it migrated throughout the uterus and, once the conceptus became immobile at the base of a uterine horn ~ day 16, the sialic acid content of the capsule markedly declined.¹⁸

During in vitro embryo production, the capsule failed to form properly, but following their transfer to recipient mares, such embryos formed a capsule within the uterus.^{6,19} Recent work has demonstrated that denuded embryos or trophoblast vesicles (TVs), when transferred to the uterus of a recipient mare, can produce copious amounts of capsular material that forms vesicles, but they fail to encapsulate the embryo and remain free within the uterus. The number of capsular vesicles that form when TVs are transferred is dependent on the stage of the recipient's cycle, with more prodigious formation between days 6 and 12, when an in vivo embryo would normally be losing the ZP and increasing the thickness of the capsule.^{20,21} Interestingly, equine trophoblast transplanted to immuno-deficient mice produced capsular material.²² Further efforts are required to elucidate the manner by which the trophoblast synthesizes capsular material in these atypical situations.

Secretory products of early embryos

Recovered embryos have also been used in various experiments to determine steroids, receptors, proteins, peptides, eicosanoids, and various other products of equine metabolism using a variety of techniques, including in vitro culture, immunological, and molecular methodologies.

Estrogen and androgen production by the early equine conceptus has been demonstrated from as early as the 1970s²³⁻²⁵ with variations in steroid production from endoderm and trophoblast.²⁶ More recent studies not only identified that embryonic steroid production is a feature of early pregnancy but mRNA for progesterone and estrogen receptors were also present on early conceptuses,²⁷ suggesting a likely role for these hormones in the early development of the conceptus.

In addition to steroids, eicosanoids are also produced by the early conceptus, with the production of both PGE₂ and PGF_{2α}.²⁸⁻³¹ It is believed that these 2 prostaglandins have a role in the transuterine migration of the equine blastocyst before days 16-17 of pregnancy³² but may also be involved in the unknown mechanisms that prevent the demise of the corpus luteum during maternal recognition of pregnancy in the mare.

From the 1990s, equine embryos have also been used to examine the array of proteins and peptides that they produce or to

characterise single ones.³³⁻³⁶ As technology has progressed, gene expression, transcriptome and proteomics studies have also been conducted on early conceptuses.³⁷⁻⁴⁶ Some of these studies have aimed to find clues towards the as yet unknown maternal recognition of pregnancy (MRP) signal in the mare and although, to date, a specific protein for this role remains elusive, a vast array of proteins that are up or down regulated and/or expressed has been demonstrated. Further work will help to elucidate how these proteins function in many key events during early pregnancy and embryo-maternal communication.

Early maternal-embryo interactions and asynchrony

The equine embryo takes ~ 6-6.5 days to traverse the oviduct and enter the uterus, suggesting that successful interaction between the embryo and the uterus may only be possible after this time. However, the high survival rate of day-4 embryos flushed from the oviduct of mares and subsequently transferred to recipients that had ovulated within 1 day of the donor proved otherwise.⁴⁷⁻⁴⁸ This was the first chronological evidence that equine embryos can tolerate considerable asynchrony with the maternal uterine environment.

Asynchronous ET has also demonstrated that the equine embryo can withstand a much wider degree of developmental asynchrony (Figure 3) with the uterus than the embryos of cattle, sheep, and pigs. For example, this is illustrated by the successful transfer of day-10 embryos to recipient mares that had ovulated between 7 days after to 2 days before the embryo donor.^{49,50} In addition to identifying considerable tolerance of equine embryos to asynchrony, these experiments also demonstrated the marked dependency of embryonic development on maternal uterine stage. This was best illustrated by the transfer of day-10 embryos to recipients with the greatest asynchrony (i.e. transfer to day-3 recipient mares equating to 7 days asynchrony). Only 3 of 8 embryos transferred with this degree of asynchrony survived past the heartbeat stage, but all 3 had a dramatic slowing of development of both the membranes and the embryo itself to match the stage of the negatively asynchronous uterus. This effect of embryonic development matching uterine rather than its own age was noted in all the asynchronous transfers.^{49,50}

Further studies have also demonstrated that, when placed in a negatively asynchronous uterine environment, the horse conceptus adjusts its transcriptome, resulting in many differentially expressed genes being up regulated, whereas the endometrial transcriptome is only modified slightly by the presence of an asynchronous embryo.^{51,52}

Despite embryonic development becoming aligned with uterine age, one event in early embryonic development does not appear to be governed entirely by the uterus. Namely, intrauterine mobility of the equine embryo up to days 16-17 of pregnancy. In recipient mares carrying asynchronous embryos, fixation of the conceptus at the base of a horn occurred when the conceptus was ~ 17 days of age, regardless of the degree of uterine asynchrony. This finding would appear to suggest that although an increase in uterine tone has also been associated with conceptus fixation,⁵³⁻⁵⁵ intraluminal impediment, with or without increased uterine tone, would appear to be the overriding influence on fixation time since mobility of the transferred embryos ceased at the same conceptus age, regardless of the stage of the uterus.

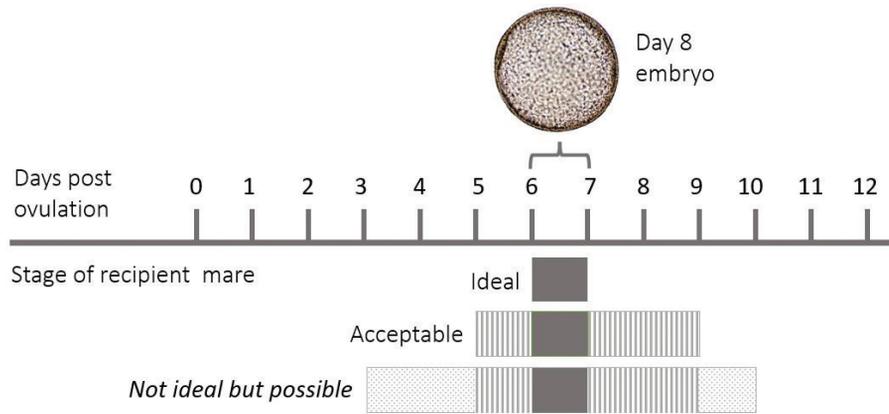


Figure 3. Compared to the embryos of other large domestic animal species the equine embryo has an amazing ability to tolerate a wide range of donor-recipient asynchrony. In most instances an embryo flushed from a donor's uterus on day 8 would be transferred to a days 6 or 7 recipient mare. However, equine embryos survive in recipients that ovulate up to 5 days after the donor or up to 2 days before the donor. Even though such asynchrony is possible, embryos transferred to asynchronous uteruses had developmental and transcriptome changes in keeping with uterine not embryonic age.^{49,50}

Another unexpected finding from the asynchronous transfer of day-10 embryos was the ability of such embryos to prevent luteolysis and survive when transferred to day-12 recipient mares. It had long been assumed that the equine blastocyst must transmit its antiluteolytic MRP signal to the mare before day 10, if it is to suppress the luteolytic cascade and hence, prevent luteolysis. However, the aforementioned experiment clearly demonstrated that day-10 embryos could provide the MRP signal as late as day 12 and suppress luteolysis of the primary corpus luteum.

Furthermore, it has been widely accepted that the continuing movement of the embryo until day 16 is essential to enable the MRP signal to be distributed throughout the uterine lumen.⁵⁶⁻⁵⁷ However, in some of the asynchronous recipient mares the conceptus ceased to move when the uterus was only 9 days after ovulation, yet luteostasis still occurred. Indeed, luteostasis occurred in all the recipients in which an embryonic vesicle was detected after transfer, regardless of its developmental competence, and including both anembryonic or trophoblastic vesicles and normal embryos suffering delayed development of the embryo proper and its membranes.^{49,50}

Recent experiments undertaken by one of the authors utilizing ET have also shed new light on the role of embryo migration in MRP signalling. Transfer of day-11 embryos to recipient mares on days 10 (n = 4), 11 (n = 6), 12 (n = 6), or 13 (n = 6) after ovulation followed by manual reduction of the embryo 24 hours later resulted in luteostasis in 100, 83, 10, and 83% of mares, respectively. Furthermore, when day-11 embryos were transferred to day-11 recipients (n = 5) and manually reduced just 12 hours later, all recipient mares became luteostatic. Hence, an intact embryo need only be present in the uterus for 12 hours to cause luteostasis.⁵⁸ An extension of this work demonstrated that when ruptured day-11 embryos were transferred to recipient mares on days 11 or 12 (n = 8/group) significantly more day-12 recipients entered a period of luteostasis compared to day-11 ones (75 versus 12.5%). Control mares that received only transfer medium without a ruptured embryo had an incidence of luteostasis of 12.5% in days 11 or 12 (n = 8/group) recipients. Hence, this study suggested that the presence of medium containing blastocoele fluid, a ruptured capsule and trophoblast in the uterine lumen prevented

luteolysis around the time of MRP (day 12).⁵⁹ This work led to the question of how long the MRP signal needs to be given to prevent luteolysis, and to what extent embryo mobility is involved in MRP.

Endometrial cup reaction and maternal immunological tolerance

Another unusual feature of equine pregnancy revealed by ET was the remarkable immunological tolerance of the mare's uterus to accept and carry to term a very 'foreign fetal occupant'. A series of elegant experiments conducted in the early 1980s transferred Przewalski's horse embryos (*Equus ferus przewalskii*; 2n = 66), domestic horse embryos (*E. caballus*; 2n = 64), donkey embryos (*E. asinus*; 2n = 62) and Grant's zebra embryos (*E. quagga*; 2n = 46) to horse, donkey, and mule (horse♀ x donkey♂, 2n = 63) recipients and obtained live viable offspring (Figure 4) from every type of extraspecies transfer.⁶⁰

Before the first equine ET was undertaken in the early 1970s, it had been noted that fetal genotype exerted a marked influence on placental development in equids, especially on the width and general development of the chorionic girdle, the progenitor tissue of the endometrial cups, which secrete equine chorionic gonadotrophin (eCG). Dramatic differences in the size, productivity, and lifespan of the endometrial cups in mares carrying horse or mule conceptuses versus donkeys carrying donkey or hinny (donkey ♀ x horse ♂; 2n=63) conceptuses were noted. Serum eCG concentrations were higher in mares and Jenny donkeys carrying fetuses with horse paternity (horse or hinny, respectively) than in donkeys and mares carrying fetuses with donkey paternity (donkey or mule, respectively). This appeared to indicate that, as has been demonstrated in mice, the paternal genome contributed preferentially to the placenta. However, in a subsequent experiment, demi mule embryos (created by bisecting a mule morula) were transferred to horse and donkey recipients and 2 intact mule blastocysts into 2 further donkey recipients. The results were noteworthy; the 3 donkeys carrying mule embryos produced large endometrial cups had very high eCG concentrations, whereas the mare carrying the other demi mule embryo had typical small endometrial cups and



Figure 4. Extraspecies transfers (a) a Grant's zebra (*E. quagga*; $2n = 46$) carried to term in a domestic horse (*E. caballus*; $2n = 64$) recipient, (b) a donkey (*E. asinus*; $2n = 62$) carried to term in a domestic horse recipient, and (c) a domestic horse carried to term in a donkey recipient.

correspondingly low eCG concentrations. Thus, contrary to the view that imprinted paternal genes-controlled development of the chorionic girdle, the extraspecies transfer experiment demonstrated clearly that uterine environment exerts an overriding influence on the whole process.^{60,61}

Extraspecies ETs also revealed the likely importance of the endometrial cup reaction in the development of the equine allantochorionic placenta and maternal immunological tolerance. Although the majority of equine extraspecific pregnancies are carried to term without issue, transfer of donkey embryos to horse mares (D-in-H) resulted in complete failure of the endometrial cup reaction and a consequential absence of eCG from maternal blood. Interestingly, the reverse occurred following transfer of horse embryos to Jenny donkeys (Hin-D) in which very large and active endometrial cups developed and secreted very high eCG concentrations into maternal blood. In the D-in-H model, development of the donkey progenitor chorionic girdle was retarded to such an extent in the recipient horse uterus that it completely failed to invade the maternal endometrium on days 36 to 38 in most cases. Nevertheless, the conceptus continued to develop normally to ~ 60 days of pregnancy, beyond which a clear division occurred in the outcome of the pregnancy. In approximately 70% of the D-in-H pregnancies the donkey allantochorion failed to interdigitate with recipient horse endometrium and the developing fetus starved to death ~ days 80-90 as the unattached allantochorion degenerated and was actively attacked by maternal immune cells accumulated in the underlying endometrium. In the remaining 30% of D-in-H pregnancies, however, implantation, interdigitation and placentation, albeit delayed and rather irregular, did occur during days 40-100 of pregnancy. Thereafter, the pregnancies continued to term with the birth of live foals that ranged from well-grown and healthy to dysmature, small and barely viable due to varying degrees of inadequacy of the placenta.^{60,62}

It is tempting to conclude that the lack of eCG secretion, and the consequential failure of plasma progesterone and estrogen concentrations to increase after day 40 as a result of the lack of secondary corpora lutea or resurgence of the primary corpus luteum, was responsible for pregnancy loss in those mares that aborted. However, subsequent supplementation of mares carrying donkey fetuses with altrenogest and/or eCG failed to prevent fetal death. Notwithstanding, immunological therapy in the form of either passive immunization using serum from mares carrying normal intraspecific pregnancies, or active immunization with donkey lymphocytes, appeared to increase the rate of a successful pregnancy, although it did not prevent fetal demise in all the treated recipient mares. Identifying the

exact cause of pregnancy loss in this model is difficult, especially since some form of immunological memory or tolerance seemed to be involved. Namely, the small percentage of horse mares that carry a transferred donkey fetus to term will normally do so again and again, whereas the mares that abort donkey fetuses do so repeatedly at earlier stages of pregnancy in successive D-in-H pregnancies.^{60,62} It is now some 40 years since these experiments were undertaken and suggested an important role for the specialized, invasive trophoblast cells of the chorionic girdle in initiating and driving attachment and interdigitation of the noninvasive placenta while at the same time modulating maternal immunological responses to enable survival of the foreign donkey fetus in the horse uterus. Revisiting such interspecies ET experiments may well prove timely in trying to unravel the persisting mysteries of the possible role(s) of the equine-unique endometrial cup reaction in promoting and maintaining pregnancy in this genus.

Fetal growth and development

The production of genetically identical horse twins either by transferring blastomeres from 4- to 8-cell embryos into empty pig zona pellucida and maturing them to blastocysts in the oviduct of a sheep for subsequent transfer to recipient mares,⁶³ or by bisecting a morula to produce demi embryos⁶⁴⁻⁶⁶ for transfer to 2 recipient mares, has demonstrated the role of maternal uterine environment on fetal growth and development. For example, from a phenotypical point of view, such genetically identical twin foals are very alike in general conformation and appearance, but they frequently had quite marked differences in the white markings on their face and legs; similar variability in white markings have been observed in cloned offsprings.⁶⁷ Hence, the degree of spread of melanocytes from the neural crest during fetal development to dictate the positioning of white markings must, to some part, be dictated by the uterine environment.

The size of the recipient mare and, correspondingly, uterine size can also influence markedly, the size and conformation of foals carried. This originates from uterine size controlling placental size and function by limiting the area of microscopic contact between fetal trophoblast and maternal endometrial epithelium at the placental interface. In 1938, before ET had been undertaken in equids, the effects of maternal size on foal birthweight (by crossing large Shire horses with much smaller Shetland ponies) was first demonstrated.⁶⁸ The latter hybrid offspring were much smaller at birth and the size disparity continued into adulthood. Subsequently, Konik pony embryos were transferred into larger draught-type recipient mares

before remating the Konik pony donors to the same Konik pony stallion as controls. In all of the 3 sex-matched pairs of full sibling foals produced in this manner, those from the larger recipient mares weighed considerably more and were taller than their full siblings born from the genetic Konik pony mothers; as with the Shire and Shetland crosses, these disparities in body size persisted in later life.^{69,70}

A similar experiment used reciprocal ET between large Thoroughbred and small Welsh pony mares to again highlight the marked influences of maternal uterine size on placental and fetal growth.⁷¹ Ponies in Thoroughbred recipients (P-in-Tb) were born ~ 15% bigger (weight and height) than their Pony-in-Pony (P-in-P) controls, whereas Thoroughbred embryos in Pony recipients (Tb-in-P) were born some 15% smaller than their Thoroughbred-in-Thoroughbred (Tb-in-Tb) counterparts. These considerable bodyweight differences correlated closely with differences in the weight and gross area of the respective allantochorions and with the total microscopic area of contact at the fetomaternal interface calculated stereologically. The birth size differences persisted to 3 years of age and although a degree of 'catch-up growth' occurred in the undersized Tb-in-P foals, they nevertheless remained appreciably smaller than their normal Tb-in-Tb controls.⁷²

In addition to changes in placental morphology, the function of the fetoplacental unit as an endocrine organ was also modified. For example, Pony mares carrying Thoroughbred foals had much higher maternal plasma concentrations of progestagens during the second half of pregnancy than the other 3 groups, presumably as a result of fetal stress initiating the production of pregnenolone, the precursor for progestagens, from the fetal adrenal glands.⁷³ In addition, both growth-restricted (Tb-in-P) and growth-enhanced intrauterine existence (P-in-Tb) in the above animals altered cardiovascular function and other metabolic systems in postnatal life.^{74,75}

More recently, the influence of both breed and maternal size on placental and fetal development in an ET model was reexamined; possible effects on the epigenome of equine embryo technologies were reviewed.⁷⁶

Conclusion

The experiments outlined above clearly demonstrated the great potential value of ER, horse-horse ET, and intra- and interspecies ET in dissecting out the stage-related occurrence and significance of early maternofetal interactions in driving the development and the physiological efficiency of the diffuse, epitheliochorial placenta in the horse and other equids. They have also highlighted the amazing, and apparently unique, mechanism of the equine uterus in its immunological tolerance towards a range of genetically diverse fetuses. Further, they have identified the long-lasting influences on the morphological and endocrinological functions of transferred embryos in their pre- and postnatal lives. The ease with which both ER and ET can be undertaken in equids has and remains a significant boon to research in equine reproduction and reproductive biology in general.

Conflict of interest

None to report.

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