Review Report

Embryo selection in equine in vitro embryo production: current practices, limitations and future directions

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Abstract

Equine in vitro embryo production has become widely commercialized due to refinements in ovum pick up, oocyte maturation and the reliability of intracytoplasmic sperm injection (ICSI). Although ICSI remains the primary fertilization method in horses, recent advances in sperm capacitation have enabled reliable in vitro fertilization with outcomes approaching those of ICSI, positioning in vitro fertilization as a complementary technology in equine embryo production. These techniques allow embryo production from subfertile mares or stallions with limited semen availability; however, accurate identification of embryos with predictable outcomes remains a limitation. In vitro-produced embryos exhibit higher rates of early pregnancy loss and monozygotic twins than in vivo-derived embryos, yet selection in many programs relies on traditional morphological assessment adapted from other species. Such assessments are weak predictors of foaling rates and lack standardization across laboratories, unlike systems established in cattle. In vitro-produced embryos display structural and nuclear deviations, including reduced cell numbers, poorly compacted inner cell mass, failure to form a glycoprotein capsule, and increased micronucleus formation. Consequently, clinical programs prioritize developmental rate during culture as a more reliable prognostic marker than morphology alone. Time lapse imaging further identifies abnormal first cleavage patterns as markers of poor outcome, supporting serial, dynamic assessment over subjective point-in-time evaluation. Current evidence supports a stepwise approach, prioritizing developmental timing, followed by equine-specific morphology and morphokinetic risk markers. Emerging technologies such as metabolic profiling, artificial intelligence and noninvasive preimplantation genetic testing require further validation against foaling outcomes before routine clinical adoption.

Keywords: Horses, embryo, blastocyst, morphokinetics, cleavage, morphology

Introduction

Assisted reproductive technologies have become integral to equine breeding, with ovum pick-up (OPU) followed by intracytoplasmic sperm injection (ICSI)-based in vitro production (IVP) of embryos now offered routinely by multiple commercial and academic programs worldwide.[1-4] Although conventional embryo transfer using in vivo-derived (IVD) embryos remains widely practiced, clinical use of IVP has expanded rapidly, particularly in sport horse breeding and in cases involving subfertile mares and stallions with limited semen availability.[1,3,5] The use of OPU-ICSI enables embryo production from genetically valuable subfertile mares and stallions while also allowing year-round, cycle-independent use of high-value donors, a more efficient use of stallions with limited availability and, in selected circumstances, facilitates postmortem oocyte recovery for genetic preservation.[5,6] Consequently, IVP embryos are now routinely produced and transferred into recipient mares in sporthorse and breeding populations across the world.[1,3]

A pivotal milestone in equine assisted reproduction was the report of the first pregnancy following ICSI in 1996, which demonstrated that micromanipulation could overcome the long-standing inability to achieve reliable fertilization in vitro in the horse due to failed sperm capacitation and zona pellucida penetration.[7] Despite this early success, widespread clinical implementation of equine IVP was delayed for many years by low efficiency, limited oocyte availability and poor repeatability across laboratories.[1,6,8-11] Refinement of ICSI methodology was therefore critical in transitioning equine IVP from an experimental approach to a reliable clinical procedure. Continued improvements in oocyte recovery, in vitro maturation, ICSI technique, and culture systems progressively increased blastocyst yield and pregnancy rates, enabling

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established programs to generate transferable embryos routinely and, in selected settings, to achieve foaling rates similar to those obtained in conventional ET.[1,3,12,13]

The expansion of equine IVP contrasts sharply with the scale of IVP in cattle. Global data published by the International Embryo Technology Society (IETS) indicate that > 2 million bovine embryos were produced in vitro worldwide in 2024, exceeding the number of IVD bovine embryos by ~ 7-fold. In contrast, only 15,867 transferable equine IVP embryos were reported in 2024.[14] Although reporting to IETS is voluntary and actual production numbers are likely higher, these figures underscore the industrial scale and maturity of bovine IVP systems and the extensive datasets available for validation of embryo selection strategies. Importantly, equine IVP programs typically produce only 1 or a few embryos per aspiration cycle,[1,15-18] limiting both flexibility and the development of large, outcome-validated selection models.

Despite substantial technical progress, equine IVP remains relatively inefficient and highly variable. Only a small proportion of recovered oocytes develop to the blastocyst stage,[19] not all transferred embryos establish or maintain a pregnancy,[1,20,21] and outcomes differ significantly among mares, stallions, semen batches, and laboratories.[12,22-24] This variability underscores the critical need for robust embryo selection strategies to improve foaling rates. Nevertheless, embryo selection in the horse still relies mostly on static morphological assessment at a single time point, a strategy increasingly recognized as insufficient for IVP embryos.[20,25-27] This limitation becomes particularly evident when comparing outcomes per embryo versus per donor mare. Fresh IVD embryos achieve higher initial pregnancy rates (86.4%) and lower early embryonic loss (4.4%) than individual IVP embryos (64.4% initial pregnancy and 13% loss).[18] However, OPU-ICSI can match the overall efficiency of embryo transfer at the level of the donor mare, as a single aspiration often yields 1.1-2.12 transferable blastocysts,[1,18] resulting in a similar mean number of day 45 pregnant recipients per procedure (0.53 for ET versus 0.50 for OPU-ICSI).[18] These contrasting per embryo and per donor outcomes highlight both the value of OPU-ICSI and the need to improve embryo-level selection to reduce losses and optimize foaling rates.

In species with more established assisted reproductive systems (e.g. cattle and humans), recognition of the limitations of morphology-based grading has driven the development of more objective, outcome-validated embryo selection approaches integrating morphokinetics, noninvasive metabolic assessment and in some programs, preimplantation genetic testing (PGT).[28-36] In human IVF, refinement of embryo selection enabled widespread adoption of elective single-embryo transfer while maintaining acceptable live-birth rates, demonstrating that strong selection pressure could reduce multiple pregnancies without compromising overall success.[37,38] Although this experience provides a useful conceptual benchmark, direct translation of such approaches to the horse is constrained by low embryo numbers, species-specific developmental features, and the clinical realities of equine IVP programs.[6,19,39]

OPU in horses introduces additional variability at the level of oocyte competence. Retrieved oocytes originate from follicles at various stages of development (i.e. growth, stasis and atresia) and may differ in cytoplasmic maturity and developmental competence even after in vitro maturation.[6,40,41] This variability is likely a major contributor to the wide range of outcomes observed among IVP embryos and complicates subsequent embryo selection, as embryos of similar morphology may arise from oocytes that differ in their ultimate developmental competence.[39]

The objective of this review is to synthesize current evidence relating to selection criteria and methods of IVP equine embryos, with an emphasis on clinically applicable, noninvasive or minimally invasive methods. Attention is focused on why embryo selection is uniquely challenging in horses, which embryo characteristics are most strongly associated with foaling rates, and how available information can be integrated into practical selection strategies for improving the predictive ability of embryo outcomes. After outlining advances in OPU-ICSI and IVF, the review outlines differences between IVP and IVD embryos, evaluates current selection methods, and proposes a stepwise framework for application.

Refining OPU-ICSI and pioneering IVF

For more than 2 decades, ICSI has been the only commercially reliable method for IVP in horses. Unlike cattle[42] and humans,[43] conventional IVF in horses was not commercially successful because reliable and repeatable sperm capacitation and penetration of the zona pellucida could not be achieved under standard laboratory conditions.[44,45] As a result, equine IVP systems have historically evolved around micromanipulation rather than conventional fertilization, fundamentally influencing both embryo biology and clinical practice.[44]

One of the most important advances in equine ICSI was the introduction of piezo-driven microinjection, first described for mouse oocytes in the mid-1990s.[46] This technique uses rapid, low-amplitude mechanical pulses to allow a blunt injection pipette to traverse the zona pellucida and oolemma with minimal cytoplasmic disruption.[47] Piezo-assisted injection proved particularly advantageous in the horse.[48] Compared to conventional sharp-pipette ICSI, piezo-driven ICSI achieved similar cleavage rates but resulted in blastocysts with higher total cell numbers, lower nuclear fragmentation, and more rapid sperm component remodelling and oocyte meiotic resumption after injection.[46,48-50] Incorporation of piezo-assisted ICSI into equine protocols markedly improved oocyte survival, cleavage and blastocyst development rates, thereby making ICSI sufficiently efficient and reproducible for routine IVP.[6,51,52] However, both conventional and piezo-assisted ICSI are currently used across equine IVP programs that remain concentrated within a relatively small number of specialized laboratories worldwide. ICSI stations have been established in at least 13 countries, including ~ 5 major centres in the United States.[53] Some laboratories achieve high blastocyst, pregnancy, and foaling rates using conventional or laser-assisted approaches without piezo.[54] For instance, the Veterinary Assisted Reproduction Laboratory at the University of California, Davis has reported no improvement in blastocyst rates with piezo-assisted ICSI, noting that success highly depends on the skills of embryologist performing ICSI.[54]

Another critical technical advance underpinning the clinical viability of equine IVP was the improvement in immature oocyte recovery using large-bore (12-gauge) double-lumen needles combined with repeated follicular flushing and active scraping.[4,6,55,56] This approach overcame the strong attachment of the equine cumulus-oocyte complex (COC) to the follicular wall[57] and transformed OPU from an experimental

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technique into a practical, repeatable clinical procedure.[58] Oocyte recovery increased from ~ 29% with aspiration only to ~ 64% when follicles were additionally flushed 10 times; furthermore, needle twisting for 1-2 seconds to scrape the follicular wall provided a further 10-15% gain without compromising COC morphology.[59] Therefore, performing brief needle twisting and using 10 follicular flushes appeared crucial for achieving high oocyte numbers. In established programs, OPU now yields a mean of 12-14 oocytes per session,[1,13,60] with recovery rates exceeding 50%.[61] However, this number varies among breeds, age of the mare, animal demographics, geographical locations, and aspiration teams.[1,2,55,61,62]

ICSI places exceptional emphasis on oocyte competence, as fertilization bypasses many natural selection barriers normally imposed by sperm-zona interactions.[40] As a result, cytoplasmic maturation, spindle integrity, and the oocyte's reprogramming capacity following sperm injection become major determinants of embryonic development.[40,63-66] Variability in oocyte quality arising from follicular origin, mare age, endocrine environment at the time of retrieval and in vitro maturation conditions contributes substantially to differences in cleavage patterns and blastocyst development among equine IVP embryos.[40]

Recent work has challenged the long-standing assumption that conventional IVF is not repeatable and commercially feasible in horses.[44,45] Prolonged preincubation of equine sperm in penicillamine, hypotaurine, and epinephrine medium substantially enhanced in vitro fertilization outcomes. This extended capacitation protocol proved effective across both fresh and frozen-thawed semen preparations, yielding fertilization and blastocyst development rates comparable to ICSI results.[67-70] Although this represented an important advance in understanding equine fertilization, clinical IVF currently requires substantially higher sperm numbers,[69] ~ 45,000-50,000 sperm per fertilization droplet,[68] compared to a single sperm per oocyte for ICSI, for which a single frozen semen straw can provide sufficient sperm for multiple injection sessions.[22,71] As a result, ICSI continues to be the prevailing method in equine IVP, fundamentally shaping the biology, variability, and developmental paths of produced embryos. Development of low-sperm-input IVF protocols is emerging as a priority research area to enhance the technique's commercial applicability.

Equally important for the clinical expansion of OPU-ICSI programs was recognition that immature equine oocytes retain developmental competence during overnight transport (~ 18-24 hours or even longer) at room temperature (~ 20 to 22-23 °C).[72-75] This observation facilitated the option for oocytes to be collected by clinicians in satellite practices, although for the most part, ICSI remains centralized within specialized laboratories. Additionally, exposure to overnight holding at room temperature may also function as a passive selection step, preferentially retaining oocytes with better inherent developmental potential.[76] The ability to separate oocyte recovery from on-site laboratory infrastructure has therefore been instrumental in scaling equine IVP and supporting more consistent embryo production and selection in routine clinical practice.

Why embryo selection is uniquely difficult in equine IVP

Embryo selection in equine IVP is uniquely challenging. Unlike cattle, where large numbers of embryos are routinely generated, equine IVP programs typically produce only a small cohort of embryos per aspiration cycle. According to the 2024 IETS report, bovine OPU-IVP programs yield an average of ~ 5.1 transferable embryos per donor, with 4.0 in dairy cattle (1,081,711 embryos from 267,841 donors) and 7.4 in beef cattle (929,474 embryos from 125,474 donors).[14] By contrast, equine OPU-ICSI programs report a mean of only ~ 2.12 embryos per aspiration session in established clinical settings.[1] Consequently, both clinical decision-making and scientific validation of embryo selection criteria are constrained by limited sample sizes. When only 1 or 2 embryos are available, discarding an embryo of uncertain viability may mean losing any chance of pregnancy outcome out of that aspiration cycle. These low embryo numbers also slow down the development and validation of reliable selection algorithms that typically require large datasets to establish predictive value. Moreover, the application of expensive or invasive selection technologies, such as PGT or advanced imaging platforms may be difficult to justify economically in the context of such limited embryo yield. As a result, clinical decision-making in equine IVP must carefully balance the pursuit of improved selection precision against the inherent risk of rejecting embryos that may still be capable of establishing and maintaining a pregnancy.

Equine embryo development itself is variable. Even under controlled laboratory conditions, equine embryos display wide variation in cleavage timing, developmental tempo, and structural organization.[25,77,78] This variability reflects unique characteristics of equine oocytes, which undergo a substantial portion of their cytoplasmic maturation in vitro.[79] Consequently, embryos that reach similar morphological endpoints may differ remarkably in underlying biological competence.[39,80] Because ICSI bypasses natural sperm selection processes,[22] embryo development appears to be substantially influenced by the quality of the oocyte[81] and the injected sperm.[71] Subtle defects in spindle integrity, chromatin remodelling, or cytoplasmic factor availability may not prevent blastocyst formation but can compromise later developmental stability.[82] These defects are rarely apparent on routine morphological assessment.[83] Finally, equine embryos undergo species-specific periimplantation events, including capsule formation, that are not replicated in vitro and occur only after transfer.[39,72,77] Many determinants of pregnancy maintenance therefore remain latent at the time embryos are selected, limiting the predictive value of static in vitro assessment and explaining why embryo selection strategies effective in other species translate poorly to horses.

The bovine IVP industry has been able to develop and validate somewhat advanced embryo selection models, incorporating morphokinetic parameters, metabolic profiling, and, in some programs, PGT.[36,84,85] Direct translation of bovine embryo selection strategies to equine practice is not necessarily seamless. Bovine embryos are structurally more resilient and less affected by the cytoplasmic dysmotility and fragmentation that complicate assessment of equine embryos.[80,86] In addition, bovine production systems can tolerate greater selection scrutinization, enabling higher embryo discard rates due to relatively higher embryo availability; however, this approach is generally not feasible in equine programs because of lower embryo numbers. Finally, the market economics of bovine embryo production support investment in advanced and expensive selection technologies that are not yet economically viable in equine IVP. Conversely, human IVF programs appear to have achieved high selection precision,[32,87] including

widespread adoption of elective single-embryo transfer without compromising live-birth rates.[88,89] This success has been linked to combining morphokinetic assessment, PGT, and measures of endometrial receptivity.[32,87] However, recent large, randomized evidence suggests these tools may not all contribute as much as assumed. In the largest adequately powered trial to date (n = 1,575), time lapse imaging, whether used for undisturbed culture alone or alongside morphokinetic-based embryo selection, did not improve overall live-birth rates compared to standard incubation and morphology-based selection.[30] Together, these findings suggest that in modern human IVF, much of the benefit in embryo selection may come from the added effect of extended culture to the blastocyst stage (days 5-6), which acts as a natural biological filter, combined with systematic morphological assessment.[30] Although morphokinetic information can offer additional insight in some settings, its added value over conventional morphology is still not entirely clear. Accordingly, the direct translation of human IVF selection strategies to equine IVP is constrained, not only by species-specific biological, ethical and practical considerations, but also by uncertainty regarding the added value of morphokinetic parameters beyond established selection criteria. In addition, human embryo research permits extensive biopsy, nontransfer analyses, and long-term outcome validation, facilitating rapid refinement of selection tools. Cost-benefit calculations also differ fundamentally; ~ 1 in 6 couples experience infertility requiring fertility advice,[90] creating widespread clinical demand for IVF and supporting routine integration of PGT into standard care. Finally, equine reproductive physiology, including endocrine dynamics, peri-implantation events, and the uterine environment differs from that of humans,[79] further limiting cross-species comparison. Recognition of these species-specific constraints is essential for the development of realistic and clinically meaningful equine selection approaches. Rather than adopting bovine or human methodologies completely, equine IVP programs must prioritize pragmatic, outcome-driven strategies that reflect the realities of clinical practice. These include limited embryo numbers per OPU session[1] and the distinctive structural features of equine IVP embryos[91,92] that may not match morphological ideals established in other species. Selection frameworks must also operate within the economic constraints of equine breeding, where the routine use of advanced and costly technologies may not be justified despite their success elsewhere.

Differences between IVP versus IVD equine embryos

Equine IVP embryos exhibit several morphological and structural features that distinguish them from IVD embryos and could therefore complicate objective selection (Table).[92] Compared to IVD embryos, IVP embryos commonly display irregular morphology, variable cell numbers, indistinct inner cell mass (ICM) organization, and cytoplasmic fragmentation following ICSI that can be difficult to interpret.[25,77,92] In addition, IVP embryos selected for transfer at day 7 are characteristically compact, with little to no blastocoele cavity, in contrast to the large, expanding blastocoele typical of IVD blastocysts.[78] This pattern is consistent with altered developmental programming rather than delayed maturation. Equine IVP embryos exhibit a developmental delay of ~ 24-48 hours compared to in IVD embryos.[93]

A days 7-8 IVP blastocyst is morphologically and developmentally equivalent to a days 5-6 IVD embryo.[21] This delay reflects slower cleavage rates, later blastocyst formation (days 6-9 in culture versus day 5.5-6 in vivo[94]), reduced cell numbers (317 versus 486 cells at day 7[78]), dispersed ICM organization, and absence of the blastocyst capsule that forms in vivo but only develops after IVP embryo transfer into a recipient.[40] Importantly, blastocyst formation alone does not ensure the capacity to establish and maintain pregnancy.[25] Even when genetically capable of development, IVP blastocysts appear to exhibit physiological limitations that maintain pregnancies after transfer. Despite these deviations, IVP embryos should not be assumed to lack developmental competence. Exposure to the uterine environment induces rapid morphological and developmental normalization. Within 48 hours after transfer, IVP embryos increase in diameter and cell number, demonstrate improved ICM compaction, and initiate capsule formation. This uterine 'rescue effect' highlights the importance of maternal-embryo interactions and adds complexity to embryo selection, given that embryos appearing suboptimal under in vitro evaluation may retain considerable developmental capacity once transferred and housed within the uterine environment.[77,93,95]

In that context, recipient synchronization and uterine environment are critical determinants of outcome in equine IVP and must be considered alongside embryo quality. Even well-ranked embryos may fail when transferred into suboptimal recipients, underscoring the need for close coordination between laboratory and clinical teams throughout the IVP process. For instance, IVP embryos require far tighter recipient synchrony than IVD embryos.[59] In a retrospective study of 264 IVP embryo transfers, recipients receiving an embryo after day 4 of ovulation achieved the highest ongoing pregnancy rates (69%), compared to day 3 (53.2%), day 5 (41.3%), or day 6 (23.1%). In contrast, IVD embryos tolerate a broader range of recipient asynchrony, typically from days 4-9 after ovulation, without significant reductions in pregnancy rates.[94] From a clinical standpoint, days 6-8 IVP blastocysts are therefore best transferred into recipients 4 days after ovulation. Transfer to recipients beyond day 4 after ovulation is associated with a progressive decline in ongoing pregnancy rates and should be avoided where possible. Early pregnancy losses are frequently associated with smaller embryonic vesicles at first detection (day 7 after transfer) compared to ongoing pregnancies, suggesting compromises in after-transfer development and early-pregnancy maintenance.[94]

Cellular composition is also linked to developmental tempo. IVD embryos recovered from the uterus on days 7-9 after ovulation consistently contain substantially more cells than age-matched IVP embryos.[77,92] Although some IVP embryos reach the blastocyst stage by days 7 or 8 after ICSI, others require extended culture to day 9 or later.[13,94] Furthermore, embryos that are cultured for additional days in IVP systems may have less favourable outcomes after transfer.[20,83] At evaluation, later-developing embryos may appear morphologically similar to earlier-developing equivalents, masking important differences in developmental history that are not captured by static morphology alone.[96]

One of the most pronounced differences between IVP and IVD embryos lies in ICM organization.[92] IVD embryos typically exhibit a compact, well-defined ICM with early lineage segregation, having a well-organised epiblast and a primitive endoderm layer along the blastocoele. In contrast, IVP embryos commonly display diffuse, poorly compacted ICMs with epiblast and primitive endoderm cells arranged in a dispersed 'salt-and-pepper' pattern.[77,97] In IVP embryos, epiblast

cells are more loosely arranged, most notably in slower-developing embryos, indicating differences in how cell lineages form in vitro.[77] Equine embryos are surrounded by specialized outer layers that develop differently under in vivo and in vitro conditions. In the uterus, IVD embryos form a thick glycoprotein capsule beginning around days 6-7 after ovulation, providing mechanical protection and enabling extensive uterine migration. In contrast, IVP embryos fail to form a complete capsule during in vitro culture, although they may secrete capsular glycoproteins that do not organize into a defined structure outside the uterine environment.[91-93,98] Zona pellucida dynamics further vary between systems; although the zona thins markedly during in vivo expansion, IVP embryos have limited thinning and retain a physical breach created during ICSI.[39,92] Trophectoderm cells or cellular extrusions frequently herniate through this opening during blastocyst expansion,[98] further complicating morphological interpretation.

At the nuclear level, IVP equine embryos exhibit a markedly higher incidence of abnormalities. Micronuclei (small extranuclear DNA fragments that arise from missegregated chromosomes during meiosis or early mitosis), are observed in ~ 10% of cells in IVP embryos compared to ~ 1% in IVD embryos. Nuclear fragmentation is also more common.[80,83,92] These features are consistent with increased chromosomal instability and presumptive aneuploidy and are thought to contribute to the high rates of early pregnancy loss[83] and the disproportionate incidence of monozygotic twinning reported after transfer of a single IVP blastocyst.[99]

In a large clinical series, monozygotic multiple pregnancy occurred in 1.6% (4/254) of IVP blastocyst transfers and was not observed following transfer of IVD embryos.[99] Monozygotic twins arising from IVP embryos are typically monochorionic and cannot be reliably identified during routine early pregnancy examinations performed 7-10 days after transfer, when only 1 embryonic vesicle is present. Multiple pregnancy generally becomes evident only after development of the embryo proper with a detectable heartbeat, making a single early ultrasound examination insufficient following IVP embryo transfer. Therefore, repeat ultrasonographic examination between 20 and 30 days after transfer is essential.[62] Outcomes of monozygotic twin pregnancies are uniformly poor, with documented cases resulting in early embryonic loss or late-term abortion.[99]

As a result of the previously described intrinsic differences between IVD and IVP embryos, cryopreservation strategies in equine IVP have been adapted to the distinctive features of IVP embryos. Cryopreservation of larger embryos (> 300 µm) have historically had poor survival following conventional cryopreservation, with pregnancy rates often < 40%.[100] This reduced tolerance is attributed to the presence of a large fluid-filled blastocoele and an unfavourable surface-to-volume ratio, which increase susceptibility to cryoinjury.[100-102] Introduction of blastocoele collapse prior to vitrification has substantially improved outcomes for large blastocysts, with reported pregnancy rates of up to ~ 70%[103] following warming and transfer of embryos approaching 600 µm in diameter. Pregnancy rates increase with the degree of blastocoele fluid removal,[104] supporting blastocoele collapse as a key determinant of successful vitrification. Accordingly, vitrification with blastocoele collapse is now standard practice for large equine blastocysts. Small IVD equine embryos (< 300 µm) tolerate cryopreservation well and can be preserved using either slow freezing or vitrification, with reported pregnancy rates of ~ 50-70% after transfer.[105-107] In equine IVP embryos, pregnancy and foaling rates following slow freezing or vitrification are comparable to those achieved after fresh IVP embryo transfer with reported pregnancy rates of up to 69% per embryo transferred and ~ 83% of established pregnancies resulting in a live foal. In contrast, cloned embryos consistently had reduced cryotolerance and poorer transfer outcomes (11% pregnancy rate and a foaling rate of 23%).[6] Following warming, reexpansion dynamics offer a useful functional indicator of embryo viability: rapid and complete reexpansion supports prioritization for transfer, whereas delayed or incomplete reexpansion warrants more cautious use when alternatives exist.[108-110] Embryos with poor developmental history or severe abnormalities are generally excluded from cryopreservation.[101]

Current methods for embryo selection in equine IVP

Morphology

Simple morphological grading by a human remains one of the most common methods of embryo selection in equine IVP programs. Most laboratories rely on grading systems originally developed for IVD embryos and later adapted from bovine and human IVF. Embryos are classified by developmental stage (e.g. morula, early blastocyst, expanded blastocyst) and assigned a quality grade based on overall symmetry, cellular integrity, and the presence or absence of debris.[84] Many programs also incorporate subjective assessment of inner cell mass (ICM) and trophectoderm (TE) appearance, commonly using variations of the IETS grading system or locally modified scales.[111,112] Assessment is typically performed at a fixed time point by an embryologist, most often days 7 or 8 (up to day 10) of development.[17,112]

In practice, equine IVP embryos are often difficult to evaluate using these criteria. Dark, lipid-rich cytoplasm, prominent perivitelline debris, and zona pellucida irregularities frequently obscure internal structures. In vitro culture also produces irregular blastocoel expansion, localized vacuolization, and partial zona thinning, features that are not readily accommodated by grading systems developed for more uniform embryos in other species.[39,78,91] Consequently, morphology-based scoring in IVP embryos is subjective and has limited agreement even among experienced embryologists, leading to inconsistent embryo ranking within and between laboratories.[20,85,113] This limitation is particularly pronounced in horses. When 4 experienced observers independently evaluated 316 equine IVP embryos, agreement on assigned grade code was only 37.3%, and agreement on developmental stage reached just 44.6%. Agreement was lowest for subjective predictions of foaling outcome, at 34.2%.[20]

To address issues of consistency, a simplified grading system linked to pregnancy outcome was proposed.[112] For instance, this system has a debris-based grading approach for vitrified day 8 equine blastocysts, in which the extent of extruded debris is estimated using a 'clock-face' reference.[112] Using this system, embryos with minimal debris are associated with higher pregnancy rates, whereas embryos with extensive debris rarely establish pregnancy.[112] These approaches aim to reduce descriptive complexity in favour of features that are easily recognized and more consistently assessed. Although they do not capture all biologically relevant variation, they represent a pragmatic refinement of morphology-based selection in clinical practice.

Table Comparison of IVD and IVP embryos

Feature	IVD	IVP
Developmental timing	Reach blastocyst stage by days 5.5-6 after ovulation in the uterus[94]	Reach blastocyst stage days 6-9 after ICSI[94] Around 24-48 hours delayed versus IVD[93]
Stage of development	Days 7-8 IVD (Figure 1)- more advanced morphology and expansion[92]	Days 7-8 IVP (Figure 2) = days 5-6 IVD embryos in morphology/ development[92]
Total number of cells on day 7	Higher, around 486 cells[77]	Lower, ~ 317 cells[77]
ICM	Compact, well-defined ICM with clear epiblast and primitive endoderm layers[77]	Diffuse, poorly compacted ICM with 'salt-and-pepper' pattern mixing of epiblast and primitive endoderm, especially in slower embryos[77]
Trophectoderm and zona	Uniform zona thinning with expansion, no ICSI breach[39]	Limited zona thinning, persistent ICSI breach with frequent herniation/ extrusion of trophectoderm cells[39]
Capsule formation	Thick glycoprotein capsule forming around days 6-7 in utero, enabling embryo migration[91,92]	Fail to complete capsule in culture, capsule only organizes after transfer to the uterus[92]
Cytoplasm and morphology	More regular morphology, clearer ICM, less debris[39]	Dark, lipid-rich cytoplasm, more perivitelline debris and irregular blastocoel expansion[39,78]
Nuclear abnormalities	Micronuclei in ~ 1% of cells, less nuclear fragmentation[83]	Micronuclei in ~ 10% cells, more nuclear fragmentation, consistent with higher chromosomal instability[83]
Monozygotic twins	less common in IVD embryos[99]	~ 1.6% after single IVP blastocyst transfer[99]
Recipient synchrony	Wider synchrony (days 4 and 9 after ovulation)[94]	Tighter synchrony (optimal day 4 after ovulation)[94]

Figure 1. A day 8 IVD expanded blastocyst

A unique finding in the horse is that unfertilized or degenerate oocytes can closely mimic blastocyst morphology by undergoing blastocoel-like expansion despite the absence of fertilization or cleavage.[39,96] Only 7 of 15 structures classified morphologically as blastocysts were validated as viable embryos by nuclear staining, with the remaining 8 embryos identified as degenerate or expanded oocytes.[95] These structures undergo cytoplasmic expansion and zona pellucida thinning similar to viable embryos, likely driven by a 'cytoplasmic developmental clock mechanism'[114] that proceeds independently of fertilization or cleavage. As a result, reliance on morphology alone substantially increases the risk of misclassification.[96]

Despite adaptations, static morphological assessment remains a weak predictor of pregnancy outcome in IVP embryos. This reflects a fundamental limitation of morphology: appearance at a single time point does not capture developmental kinetics, nuclear integrity, or other determinants of pregnancy competence.[113,115] Substantial variation in oocyte quality, nuclear abnormalities that are not detectable by light microscopy, differences in developmental timing between embryos that look similar, and species-specific features of equine IVP embryos such as reduced cell numbers, poor compaction, and increased micronuclei formation,[39,83,91,92] all contribute to the limited predictive value of morphology alone.

Developmental timing

One of the most reliable and reproducible findings in equine IVP research is the strong association between the day of blastocyst development and foaling outcome. In a large clinical series involving > 300 IVP blastocysts with known pregnancy and foaling outcomes, embryos were independently evaluated by multiple observers for day of development, developmental stage, quality grade, and specific morphological features.[20] Across these analyses, conventional morphological parameters (including blastocyst stage and many routinely used

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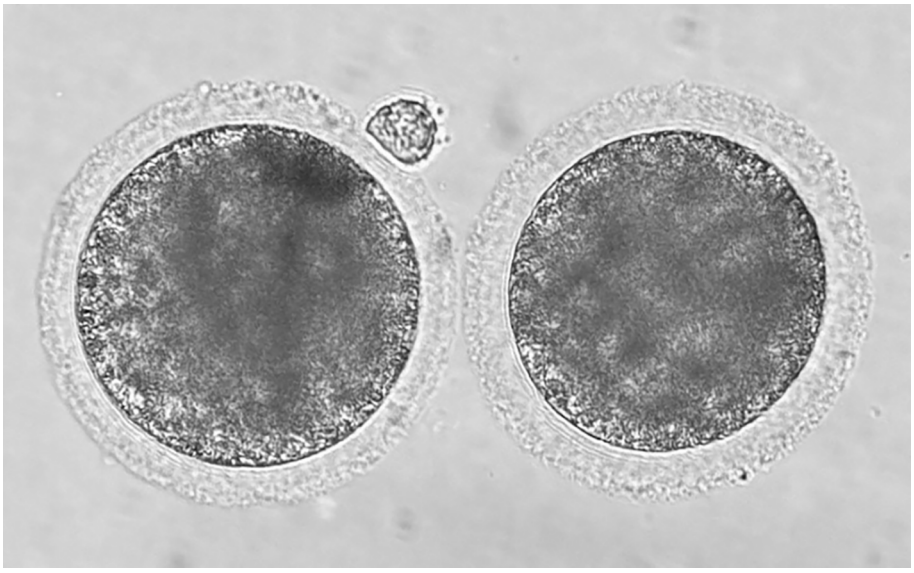

Figure 2. A day 8 somatic cell nuclear transfer blastocyst

grading criteria) had limited predictive value, whereas the day on which the embryo reached the blastocyst stage was strongly associated with subsequent foaling rate. Across 316 embryos, overall pregnancy and foaling rates were 76.9 and 56.3%, respectively. Blastocysts forming on day 7 had significantly higher foaling rates (71.7%) than those forming on days 8 or 9 (53.3 and 38.5% respectively), regardless of morphological grade. When compared within the same developmental day, blastocyst stage (early versus expanded) did not influence outcome, suggesting that apparent morphological 'catch-up' does not compensate for delayed development.[20] Morphological grade influenced outcome only in delayed embryos (day 9) and had little to no predictive value for earlier-developing embryos. Importantly, morphological descriptors commonly applied in cattle were not predictive in the horse. Compact inner cell mass morphology and tightly organised trophectoderm did not correlate with equine embryo viability, underscoring the need for stage-specific, species-appropriate selection criteria rather than direct application of bovine grading systems.[20]

Embryos that reach the blastocyst stage only after prolonged culture may exhibit subtle abnormalities in spindle assembly, chromosomal segregation, or metabolic regulation. Although such defects may not prevent blastocyst formation, they can compromise later developmental stability and pregnancy maintenance. Accordingly, delayed development or abnormally patterned cleavage timing is indicative of compromised developmental competence and an increased risk of early pregnancy loss or failure.[20,21,25,83,116]

From a clinical perspective, these findings support prioritization of developmental tempo over visual stage or degree of blastocyst expansion. Extending culture solely to obtain a more expanded or hatching blastocyst does not improve foaling outcome[20] and may, in some cases, mask underlying developmental delay rather than reflect superior embryo quality.

An additional and clinically relevant observation is that developmental speed in equine IVP embryos is sex dependent.[116] Faster-developing blastocysts are significantly more likely to be male.[116] In a large clinical series of 390 vitrified-warmed IVP blastocysts, the filly-to-colt ratio was 29-71% among day 7 blastocysts, compared to 46 to 54% among day-8 blastocysts. Overall, the sex ratio of IVP foals was shifted toward males (61% colts versus 39% fillies), with the odds of obtaining a colt being 2.28-fold higher following transfer of day 7 compared to day 8 embryos.[116] This phenomenon mirrors observations in bovine IVP, where male embryos also tend to develop more rapidly in vitro,[117] and suggests that developmental tempo reflects not only embryo quality but also intrinsic sex-linked differences in early gene expression, metabolism, and growth dynamics.[20,116]

Timelapse imaging and morphokinetics

Timelapse imaging (TLI) systems allow continuous, noninvasive monitoring of embryo development by capturing images at predefined intervals, typically every 5-20 minutes, without removing embryos from controlled culture conditions.[118] Originally developed for human IVF, TLI has been adapted for equine IVP with minimal modification to existing culture systems. This approach enables detailed documentation of cleavage events, blastocoel formation, and dynamic morphological changes that are not apparent during conventional static assessment.[25,28,80,119,120] Although timelapse imaging provides detailed morphokinetic data, its routine application in equine IVP remains largely experimental.

In equine IVP, TLI is particularly valuable because it provides insights into the developmental history of an embryo rather than a single end-point observation. Given the substantial variability inherent to equine embryos, this longitudinal perspective is especially useful for distinguishing embryos that reach the blastocyst stage through orderly, uninterrupted development from those that do so following delayed or abnormal cleavage.[25,54,120]

Timelapse imaging studies further support the view that blastocyst formation alone is insufficient to predict developmental competence. Incidence of abnormal cleavage patterns was similar in early-stage embryos that arrested and those that progressed to the blastocyst stage, indicating that abnormal cleavage does not necessarily prevent blastocyst formation in the horse.[25] Similar findings have been reported in bovine embryos, where ~ 36% of embryos that reach the blastocyst

stage exhibit abnormal cleavage patterns.[121] However, equine embryos exhibiting abnormal cleavage patterns had a significantly higher early pregnancy loss rate (53.3%) than those with normal cleavage (22.6%), suggesting that chromosomal defects persist into the blastocyst stage and then subsequently prove lethal after transfer.[25]

Initial morphokinetic studies in equine IVP demonstrated that embryos forming blastocysts exhibited earlier first cleavage[78,80,119] compared to those that failed to continue development during culture. Even though the timing differences were small, their consistency across datasets suggests that early mitotic events are biologically relevant, not erratic. Decision-tree models that incorporated the timing of the first 3 mitotic divisions had strong predictive accuracy for blastocyst formation. In particular, a second-to-third division interval of < ~ 4.75 hours combined with a first-to-second interval of < 10 hours predicted blastocyst development with accuracy exceeding 85%. Taken together, these findings suggest that orderly and timely progression through early cleavage divisions may be indicative of developmental competence.[80]

Importantly, the timing of blastocyst formation itself also correlates with pregnancy outcome. Embryos that cavitate and expand earlier are more likely to establish and maintain pregnancy, whereas embryos that reach the blastocyst stage only after prolonged culture had reduced foaling rates.[20,120] Timelapse imaging refines this observation by allowing discrimination between embryos that reach blastocyst stage promptly and those that do so only after periods of developmental arrest or erratic cleavage.[120] Several morphokinetic parameters therefore appear to have potential clinical utility in equine IVP. Earlier blastocyst formation (days 6-7 versus 8-9) is associated with improved pregnancy maintenance.[20,21] Abnormal, asynchronous, or delayed first cleavage is a consistent marker of compromised developmental competence.[25]

In cattle, morphokinetic selection had added predictive value beyond conventional morphological grading. Bovine IVP embryos that were selected using 3 morphokinetic criteria, namely early first cleavage (≤ 27 hours), normal 2-cell division, and ≥ 6 blastomeres at the onset of the lag phase, achieved a higher day-30 conception rate (59.3%) than embryos selected using standard IETS grading (31.8%).[122]

In humans, the degree of blastocyst expansion is a strong and consistent predictor of clinical outcome. Expanded blastocysts are associated with significantly higher implantation and live birth rates, approximating 45%, compared to early or unexpanded blastocysts, which achieve live birth rates closer to 27%.[123] In cattle, the timing of blastocoel expansion similarly correlates with underlying gene expression patterns and developmental competence.[124] However, recent 3-dimensional imaging studies indicate that simple volumetric measures alone, such as blastocyst size or degree of expansion, do not always reliably distinguish competent from incompetent embryos without more advanced clustering or dynamic analyses.[125]

Expansion is also not a static process. In human, bovine, and equine embryos, blastocyst expansion occurs through rhythmic cycles of contraction and re-expansion (pulsation). This dynamic behaviour is generally considered a positive indicator of viability, reflecting intact cell-cell junctions, active fluid transport, and functional trophectoderm physiology.[126,127] Whereas expansion is positively associated with viability in vivo, equine IVP embryos appear to represent an important exception. In contrast to human IVF, equine blastocysts that fully expand during in vitro culture prior to transfer have been associated with increased early pregnancy loss. Reported loss rates of ~ 44% for expanded IVP blastocysts, compared to ~ 10% for nonexpanded counterparts, suggest that premature or exaggerated expansion in vitro may reflect dysregulated trophectoderm function rather than enhanced developmental competence.[83] These findings highlight that, in the horse, blastocyst expansion should be interpreted within the specific context of IVP biology and culture environment, rather than extrapolated directly from human or bovine models. Beyond timing, TLI has revealed a high prevalence of abnormal cleavage patterns in equine IVP embryos, which when combined with other viability parameters, could be included in an overall predictive model.[25,119] These include direct cleavage from 1-4 blastomeres, multipolar division, reverse cleavage resulting from blastomere fusion, and fragmentation. Such patterns reflect possible disturbances in spindle formation, chromosome segregation, and cytokinesis.[25,128]

Although, of note, recent clinical data demonstrate that abnormal cleavage patterns are more strongly associated with early pregnancy outcomes rather than failing to reach a transferrable blastocyst.[34,36,122] Embryos exhibiting abnormal first cleavage, particularly asynchronous or multinucleate cleavage patterns at the 2-cell stage, may develop into morphologically acceptable blastocysts, yet have approximately double the rate of early pregnancy loss compared to embryos undergoing normal, synchronous bipolar division.[25] Together, these findings emphasise a central concept in equine IVP: good developmental progression in vitro does not always fully predict a maintained pregnancy.

Wider clinical adoption of morphokinetic assessment is currently limited by cost, infrastructure, and the lack of large, outcome-validated datasets. When TLI data are available, morphokinetics can be layered onto conventional selection criteria, or included into a multiparametric assessment augmented by algorithmic pattern recognition to aid objective selection. According to the revised literature, a pragmatic approach is to rank embryos first by day of blastocyst development and gross morphology and then use cleavage timing and pattern to refine decisions among embryos of similar rank. For example, between two day 7 blastocysts of comparable appearance, priority may be given to the embryo that cleaved earlier and exhibited a normal bipolar first division.

Timelapse imaging also offers value as a quality-control and training tool. Retrospective review of embryos with known outcomes allows laboratories to calibrate grading practices and identify culture conditions associated with delayed or abnormal development. Morphokinetic assessment offers a powerful tool for identifying embryos with abnormal cleavage patterns. Advances in machine learning and artificial intelligence are likely to further refine these applications; this will be addressed in a later section. TLI currently remains an addition rather than a replacement for conventional assessment in most equine IVP programs.

Structural, nuclear, chromosomal and genetic assessment

Morphological evaluation by light microscopy often fails to accurately predict the nuclear status of equine embryos.

Unlike human embryos, in which multinucleation is often appreciated during culture due to cytoplasmic transparency,[129] equine embryos have a dark, lipid-rich cytoplasm[130] that obscures nuclei. As a result, fixation and fluorescence staining are generally required to reliably detect nuclear abnormalities and quantify cell number. Consistent with this limitation, advanced imaging and cytogenetic approaches have demonstrated structural and nuclear abnormalities that are not apparent during routine assessment. These abnormalities occur at substantially higher frequency in IVP embryos than in those IVD and are increasingly suspected to contribute to early pregnancy loss and developmental instability.[80,83,92]

Fluorescence nuclear staining using Hoechst 33258,[55] Hoechst 33342,[83] or DAPI[102] is used in embryo research to assess embryo viability and estimate cell number. Complementary evaluation of DNA integrity and apoptosis can further help differentiate viable embryos from compromised ones.[80] The TUNEL assay, which detects DNA fragmentation associated with apoptosis, had higher levels of apoptotic cells in IVP embryos compared to IVD embryos in both equine and human systems.[92,131] Immunostaining for γ-H2AX, a marker of DNA double-strand breaks, and Lamin B1, a nuclear envelope protein, can also be used to identify DNA damage and micronuclei in equine embryos,[80] although, these staining techniques are not always practically applicable for routine assessment of embryos prior to transfer.

Among structural and nuclear abnormalities, formation of micronuclei is particularly prominent. These nuclear abnormalities are largely invisible to routine morphological grading and therefore represent a form of cryptic embryo compromise.[34,39] Consequently, IVP blastocysts that appear morphologically acceptable may still have reduced pregnancy rates, further reinforcing limitations of static or single-parameter visual assessments.

Chromosomal abnormalities, particularly aneuploidy, are a major cause of early pregnancy loss and developmental arrest across species.[83] In equine embryos, however, the prevalence and clinical impacts of chromosomal aberrations have historically been difficult to define, largely due to limited embryo availability for large-scale genetic analysis. Recent studies using high-density single nucleotide polymorphism arrays and haplarithmisis have identified genome-wide chromosomal errors, including triploidy and monosomy, revealing that arrested equine embryos exhibit high rates of chromosomal abnormalities (~ 83%) compared to transferable blastocysts (~ 14%).[132] These findings underscore the biological relevance of aneuploidy in equine embryonic development and further illustrate why progression to the blastocyst stage alone does not reliably predict posttransfer viability. The development of rapid, noninvasive approaches for identifying aneuploid embryos prior to transfer has the potential to provide substantial benefits to the equine industry.[133]

Preimplantation genetic testing, mostly applied as aneuploidy screening (PGT-A) in humans, provides an objective means of identifying chromosomally normal embryos prior to transfer.[134] At the blastocyst stage, laser-assisted trophectoderm biopsy is highly standardised in human IVF and is generally associated with minimal embryo compromise.[87]

Consequently, PGT-A has been routinely incorporated into many clinical programs to improve selection precision and reduce the transfer of embryos with low likelihood of establishing and maintaining pregnancy.[87,134] However, this currently still requires micromanipulation. These limitations have driven interest in less invasive and noninvasive alternatives. One promising approach is the analysis of cell-free DNA (cfDNA) released by embryo into the culture medium.[135] In humans, cfDNA from blastocyst culture media can be used to estimate chromosomal content, with compliance with trophectoderm biopsy for aneuploidy detection.[136] Recent studies have reported compliance rates of approximately 78% (866/1108 embryos), suggesting that noninvasive chromosomal screening may eventually have clinical utility.[137]

Analysis of cfDNA in spent embryo culture medium (SECM) has recently emerged as a novel, non-invasive alternative for preimplantation genetic testing in horses.[138] Although nuclear DNA has been detected in blastocoel fluid and culture media, current accuracy is insufficient for routine clinical application and remains experimental in horses.[138] Available studies in humans indicate that further research is needed to validate analytical methodologies, clarify the biological origin of cell-free nucleic acids, and establish clinical reliability.[137] In cattle, trophectoderm biopsy at the blastocyst stage permits reliable sex determination and genotyping without measurable impairment of subsequent development[139-142] and is now applied commercially, with nearly 25,000 embryos sexed or genotyped in 2024,[14] although still small in comparison to worldwide total embryo production. In contrast, routine application of PGT in equine practice remains limited.[132,143] Interpretation of genetic variants is complicated by the structural complexity and heterozygosity of the equine genome, and cost-benefit considerations differ substantially from human and bovine systems, particularly in programs producing only 1-3 embryos per cycle.[132] As a result, PGT currently serves as a supplementary function rather than as a primary selection tool in most equine IVP programs.

In horses, biopsy of IVD embryos is technically challenging due to formation of a thick glycoprotein capsule ~ days 6-7 after ovulation.[91] Breaching this structure may adversely affect embryo viability.[104] In contrast, IVP embryos are smaller, less expanded, and lack a fully developed capsule, making them more amenable to biopsy.[77,92,104] Based on these characteristics, Avantea (a leading European laboratory specializing in advanced technologies for animal reproduction and biotechnological research) has developed a minimally invasive biopsy technique for equine embryos that exploits trophoblast cells spontaneously herniating through the zona pellucida opening created during ICSI. This approach allows collection of extruded cells from days 7-9 embryos without laser or microblade assistance. Using this method, sex determination has been achieved with reported success rates of 92.8%, alongside reliable screening for monogenic disorders and coat-color genotyping. Importantly, pregnancy rates following transfer of biopsied embryos are comparable to those of nonbiopsied controls, indicating preservation of blastocyst integrity.[133]

Transcriptomic and proteomic approaches

Transcriptomic profiling provides insight into embryonic developmental status and the capacity to establish and maintain pregnancy.[144-147] In equine embryos, differential expression of genes involved in stress response, metabolism, cytoskeletal regulation, and pathways associated with the establishment of the pregnancy has been reported, with some studies suggesting improved prediction of pregnancy outcome

compared to morphology alone.[41,93] Current transcriptomic approaches require either embryo biopsy or complete cell lysis for RNA extraction. Although embryo biopsy is compatible with clinical practice, complete lysis compromises embryo viability, limiting its use in commercial ET programs.

Equine embryo biopsy is increasingly integrated into clinical programs; however, its use remains largely confined to PGT[133] rather than transcriptomic evaluation of embryo viability. Consequently, transcriptomic profiling remains predominantly a research tool and has not been widely adopted for clinical decision-making.

Proteomic analysis of spent culture medium represents an additional noninvasive approach, as developing embryos release proteins that may reflect developmental competence or chromosomal integrity.[148] Although protein biomarkers associated with implantation failure and aneuploidy have been described in human[149] and bovine[150] IVF systems, equine-specific markers have yet to be identified and validated. At present, transcriptomic and proteomic profiling in equine IVP remains largely research based.

Metabolic profiling and functional biomarkers

Embryonic metabolic rate, assessed through the consumption of glucose and amino acids and the production of lactate and pyruvate, has been shown to correlate with developmental competence and implantation success, providing a non-invasive approach to embryo selection that may be applicable to equine IVP.[151,152] By quantifying nutrient uptake and metabolite release into the culture medium, metabolic profiling offers an indirect, although informative window into embryonic physiology without physically disturbing the embryo.[153,154]

Substrate utilization in early embryos has been well characterized across species, with zygotes and early cleavage-stage embryos preferentially using pyruvate and lactate as their primary energy source while exhibiting minimal glucose uptake.[155-157] As development proceeds toward compaction, glycolytic activity increases, accompanied by a progressive rise in glucose consumption.[158,159]

In human and bovine IVP systems, multiple metabolic parameters have been identified as correlates of embryo viability and implantation potential. These include rates of glucose,[160,161] pyruvate,[152,155,162] and amino acid consumption,[163,164] lactate production as an indicator of anaerobic metabolism,[165] and analysis of spent culture media to assess overall metabolic activity.[166] More recently, emerging technologies have expanded metabolic assessment to include real-time metabolic imaging using fluorescent biosensors[167] and label-free optical methods[125,168] capable of estimating metabolic rate in living embryos, offering increasingly refined noninvasive tools for embryo selection.

In contrast, systematic evaluation of metabolic profiling in equine IVP has historically been limited. However, recent studies have begun to identify metabolomic features associated with developmental competence. For instance, noninvasive analysis of day 4 culture media from equine ICSI embryos has revealed distinct metabolic profiles between embryos that progress to the blastocyst stage and those that arrest. That includes increased consumption of dihydroxyphenylalanine and altered secretion of amino acid derivatives, lipids, and steroid-related metabolites in viable embryos.[169] These findings suggest that metabolomic profiling may enable early identification of competent embryos, potentially allowing embryo selection prior to blastocyst formation and reducing culture duration.[169] Despite these advances, these biomarkers remain in an exploratory phase. Most of the evidence supporting metabolic selection comes from human and bovine IVF systems; therefore, their direct translation to equine IVP remains uncertain. Species-specific differences in embryo physiology and metabolism require cautious interpretation when applying these findings to the horse, particularly in the absence of validation in larger prospective studies. Progress has been constrained by technical challenges related to analysing small numbers of embryos and by the need for large, prospective studies to establish meaningful associations with pregnancy maintenance and foaling outcomes. Consequently, metabolomic markers that reliably predict foaling success in the horse have not yet been defined. Several practical considerations further limit the clinical application of metabolic assessment in routine equine IVP programs. Metabolic measurements are highly sensitive to culture conditions, temperature, and timing of analysis, complicating standardisation across laboratories.

Artificial intelligence and emerging selection technologies

Artificial intelligence (AI) and machine-learning approaches are increasingly applied to embryo assessment in human and bovine IVF and are beginning to be explored in equine systems. Automated image-analysis models trained on large datasets in bovine can achieve agreement rates of ~ 85% with expert embryologists while maintaining perfect repeatability.[85] In equine IVP, such systems may offer value as consistency tools, reducing interobserver variability rather than replacing clinical judgement.

Current AI models employ a range of neural network architectures, including spatial attention mechanisms that focus analysis on biologically relevant regions such as the inner cell mass and trophectoderm, and deep convolutional networks that weight morphological features differently across developmental stages.[170,171] Effective deployment of these systems in horses will require large, well-annotated equine-specific datasets, which remain a limiting factor.

Other noninvasive technologies under investigation include electrical impedance spectroscopy, which measures changes in electrical properties associated with cell mass expansion, and microfluidic platforms designed to assess embryo elasticity[172] or stiffness.[168] These physical properties vary dynamically during development and may reflect cytoskeletal organisation and cellular integrity.[172] Although conceptually attractive, these approaches remain far from routine clinical application in horses.

From evidence to practice: embryo selection in equine IVP

Within the current constraints of equine IVP, i.e. low embryo yield per cycle, substantial oocyte variability, and species-specific patterns of development,[1] embryo selection must balance biological evidence with practical feasibility. A tiered, systematic stepwise approach provides a workable framework for prioritizing embryos in a way that maximises foaling rates without compromising viability and remains practical and compatible with routine clinical practice.

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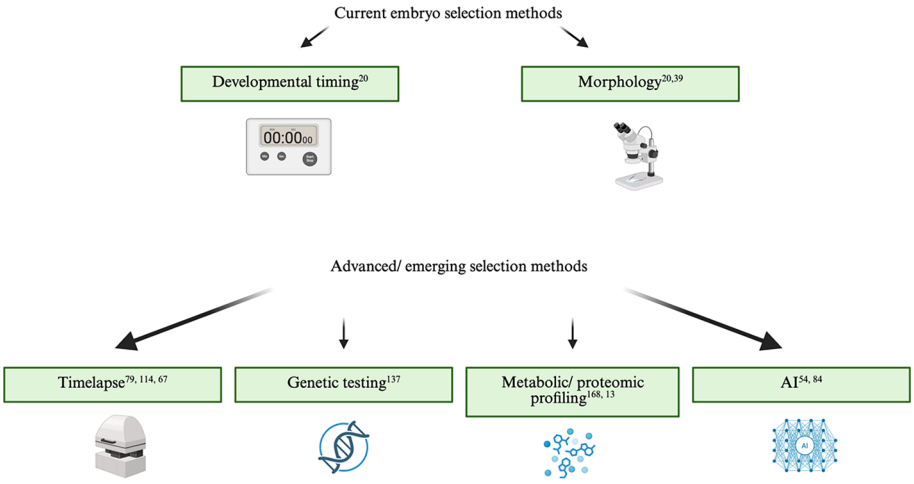

Figure 3. Current embryo selection methods

Across available datasets, developmental timing provides the most consistent and outcome-linked basis for embryo selection. Embryos that reach the blastocyst stage by days 6 or 7 of culture have higher rates of pregnancy establishment and, more importantly, pregnancy maintenance than embryos that develop later.[116] This association is supported by extensive clinical experience across multiple programs and has the advantage of being objective, reproducible, and easily recorded.[1,20,21] From a clinical standpoint, embryos that reach the blastocyst stage by day 7 should be prioritized for transfer. Furthermore, embryos that remain at the morula or early blastocyst stage on days 8 or 9 may still be transferred when no earlier-developing alternatives are available, but expectations regarding pregnancy outcome should be adjusted accordingly.[20,94,98] Where immediate transfer is not possible, such embryos may be considered for cryopreservation rather than automatically discarded.[21]

Once embryos have been prioritized based on developmental timing, morphological assessment could provide a secondary layer of discrimination within the available cohort. However, conventional grading systems require interpretation in the context of equine-specific developmental features. Morphological evaluation is most useful when focused on broad indicators of organisation rather than strict adherence to grading scales developed for other species. In this context, blastocoel expansion remains informative, with larger and more uniformly expanded blastocysts generally preferred, although embryos with more modest expansion may still be viable.[20,39,83] A continuous, cohesive trophectoderm layer is desirable, with focal disruptions or loosely arranged cells indicating reduced quality rather than absolute exclusion.[20,39] A distinct, compact ICM is preferred, whereas diffuse or poorly organised ICMs are associated with reduced viability but are not incompatible with pregnancy.[77] Overall contour, cell uniformity, and absence of marked cytoplasmic inclusions remain useful indicators.[20] In practical terms, when choosing between embryos, a morphologically modest days 6-7 blastocyst is generally a better transfer candidate than a morphologically excellent day 8 morula. Morphology should refine, not override, prioritization based on developmental tempo.

In programs with access to timelapse imaging, metabolic profiling, or other specialized analyses, these tools can provide additional information to refine selection among embryos of similar developmental stage and morphology. Additionally, these tools could be combined to support a multiparametric approach. However, these techniques are probably best considered complementary rather than primary embryo selection criteria, given limited outcome validation in equine systems and the logistical complexity involved. Timelapse imaging can identify embryos with normal, synchronous first cleavage and orderly subsequent divisions, which are associated with improved pregnancy maintenance. Conversely, embryos having abnormal first cleavage or irregular division patterns carry a higher risk of early pregnancy loss.[25] Where metabolic or proteomic assessment is available and validated locally, embryos demonstrating higher metabolic activity or favourable biomarker profiles may be preferentially selected, although robust prospective validation remains essential. Genetic information obtained through PGT, such as sex or targeted genotype, may influence selection in specific cases and breeds (Figure 3).

Limitations

Despite substantial progress, embryo selection in equine IVP remains constrained by several important limitations. Most available studies are retrospective and derived from single laboratories using specific culture systems, limiting broader applicability. Sample sizes with complete foaling outcomes remain modest, particularly for embryos evaluated using advanced tools such as timelapse imaging, metabolic profiling, or molecular assays.

Interlaboratory variability in oocyte maturation protocols, culture media, oxygen tension, and grading practices further complicates comparison between studies. Moreover, many investigations focus on blastocyst formation or early pregnancy establishment rather than pregnancy maintenance and foaling, potentially overstating the clinical value of certain selection criteria. A day-8 blastocyst that establishes pregnancy but fails at day 45 or later during pregnancy represents a crucially different outcome from one that progresses to term.

Systematic integration of outcome data generated within a single laboratory or clinical program represents one of the most effective strategies for improving embryo selection. Routine recording of embryo characteristics alongside pregnancy and foaling outcomes allows programs to validate selection markers within their own populations. Even modest datasets accumulated over 1 or 2 breeding seasons can reveal consistent mare, stallion, or laboratory-specific patterns that are not apparent from morphology alone.

Several emerging embryo selection technologies require well-supported prospective validation before routine clinical adoption. For timelapse imaging, further studies are needed to identify morphokinetic parameters predictive of foaling rather than blastocyst formation alone. For preimplantation genetic testing, large cohorts with documented foaling outcomes are necessary to better define the clinical relevance of aneuploidy in horses. Similarly, metabolic and proteomic biomarkers require further standardisation and outcome validation before they can be meaningfully integrated into routine practice.

Conclusion

Equine in vitro embryo production has rapidly progressed from research setting to a commercially viable advanced breeding technique. Despite this progress, objective and accurate embryo selection remains a formidable challenge in equine IVP. This reflects the unique features of equine embryos[25,39,77,92] and the practical constraints of IVP systems[6,48,52,56,63] that limit the direct translation of selection strategies developed for bovine or human IVF and underscore the need for equine-specific, outcome-driven approaches.

Across the body of evidence reviewed, developmental timing, most notably the day of blastocyst formation, consistently emerges as the strongest predictor of foaling outcome.[20,21] Morphological assessment retains clinical value but is most effective as a secondary tool when applied using criteria adapted to equine embryos. Where available, morphokinetic data can further refine selection by identifying embryos that reach acceptable stages, despite slower or abnormal development.

Clinically, a stepwise selection strategy that prioritizes developmental timing, refines ranking using equine-adapted morphological criteria, and incorporates morphokinetic information when available provides the best balance between biological insight and practical feasibility. Importantly, this approach emphasises relative prioritization rather than categorical exclusion, reducing premature embryo discards while making optimal use of limited embryo numbers.

Further progress will likely depend on integrating traditional assessment methods with molecular and genetic profiling tools that can be applied in clinical practice. Timelapse and PGT may expand in selected clinical settings, particularly for high-value embryos. Noninvasive approaches and AI have promise; however, it is still too early to know how useful these tools will be in practice, and additional large, coordinated studies will be needed before they are widely adopted.

Authors' contribution statement and agreement

PS: conceptualization, literature review, preparation of the original draft and manuscript revision; **RN:** conceptualization, reviewing and editing; and **AR**: conceptualization, reviewing and editing. Authors have read and approved final submission.

Conflict of interest

None to report.

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