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EDITORIAL

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Clinical insights: Assisted reproductive techniques: More than a solution to subfertility?

1 | INTRODUCTION

Assisted reproductive techniques (ARTs) are an increasingly popular means of breeding sport horses. And while the main reason for developing the ARTs was their potential to accelerate genetic improvement (ie allowing more offspring to be produced from the best stallions and/or mares), early uptake in practice has been biased towards their capacity to salvage the breeding career of valuable horses suffering from intractable subfertility; when a new technique offers the only realistic route to producing a foal from an important mare or stallion, concerns about cost and success rate often take a backseat. This virtual issue brings together selected articles published recently in EVJ that have contributed to the advance of equine ARTs.

Various ARTs have now been proven to overcome, or reduce the impact of, specific causes of subfertility. Nevertheless, ARTs are not a panacea for fertility problems and can create issues of their own, not least because in vitro manipulation may introduce epigenetic changes with initially imperceptible, but potentially life-long effects on the health of the offspring. More obviously, ARTs involve additional interventions and, therefore, additional opportunities for (human) error. As a result, in the case of healthy fertile animals, the newer ARTs are sometime less, or only marginally more, efficient than more established approaches to breeding and may be considerably more invasive and expensive. Not surprisingly, there is also some way to go before some of the more complex techniques are optimised for clinical practice. That said, the need for ongoing research to optimise results also applies to more hands-off breeding systems, such as intensively managed natural mating programmes, since there is still much that we do not understand about common fertility-limiting conditions, such as persistent post-mating endometritis, declining endometrium and oocyte quality in older mares and other factors that predispose to fertilisation failure or early pregnancy loss. As a result, there is no consensus on the "best" approach to management, monitoring or treatment to maximise the likelihood of obtaining a healthy foal after mating or insemination, and many therapies are implemented on the premise that "they may help and probably don't harm". This highlights another advantage of the development of the ARTs; by separating the pathway from fertilisation to birth into its constituent parts (eg gamete quality, fertilisation, early embryonic development, pregnancy establishment and pregnancy maintenance), ARTs create opportunities to study processes critical to producing a healthy foal, both in an experimental and in a clinical setting. Better understanding of factors limiting successful pregnancy in turn leads to novel approaches or treatments to improve the likelihood of success in either an ART or a natural mating setting, and can be invaluable when giving advice about which approach is likely to offer the best balance between success, cost and invasiveness for a given mare or stallion. This collection brings together recent studies that have helped to identify underlying causes of subfertility in horses, and/or address the management, efficiency and success of the newer ARTs.

2 | ENSURING SPERMATOZOA AND OOCYTE MEET AT THE RIGHT TIME AND RIGHT PLACE

Ensuring that mating or artificial insemination (AI) take place close to the time of ovulation is a central part of successful breeding management. While proximity to ovulation is most critical when performing AI with frozen-thawed semen, trying to decide if and when a mare should be booked to a busy stallion for a single cover early in the breeding season, sometimes days in advance, can be at least as challenging. In both cases, inducing ovulation using either human chorionic gonadotrophin (hCG) or a gonadotrophin releasing hormone (GnRH) analogue is an important part of ensuring that ovulation takes place within a short and predictable timeframe. In recent years, long-acting GnRH analogue preparations have become more popular than hCG since they appear to more reliably induce ovulation within a narrower interval.[1] It is therefore interesting to note that in the case of barren mares pre-treated with the dopamine antagonist, sulpiride® (to accelerate the development of the first ovulatory follicle), hCG was considerably more reliable than a GnRH analogue in ensuring ovulation within 48 hours (90% vs 38%).[2] This is presumably because hCG works directly on the follicle (in a luteinizing hormone (LH)-like fashion), whereas a GnRH analogue must elicit LH release from the anterior pituitary. This further supports the supposition that the primary factor limiting ovulation during the spring transitional period is pituitary ability to generate an adequate LH surge, rather than inadequate follicular responsiveness to LH.

In mares that are already cycling, ovulation induction is used to "synchronise" oocyte maturation and ovulation for a range of applications in ARTs, although it is most commonly used to help optimise the timing of mating or AI. In this latter context, ovulation induction with a GnRH analogue was also recently shown to improve pregnancy rates in a donkey AI programme, despite reducing the frequency with which follicle development was monitored. In jennies, administering a GnRH analogue when

the pre-ovulatory follicle reached 31–35 mm appeared to be the optimal basis for fixed-time AI using either chilled semen (AI 8 hours after GnRH analogue administration) or low doses of frozen-thawed semen (AI 34 and 42 hours after GnRH)[3]; achieving pregnancy rates of ~30% per cycle with as few as 100 million motile frozen-thawed spermatozoa was particularly noteworthy given that previous studies have struggled to achieve commercially acceptable pregnancy rates using frozen-thawed donkey semen.

While the timing of ovulation with respect to breeding is clearly important, so is ensuring that a high-quality spermatozoon is able to reach the site of fertilisation, that is, the oviductal ampulla. One obstacle to spermatozoa reaching the oviduct is the uterine neutrophil release response to insemination. Uterine neutrophils are thought to be released primarily in response to the presence of the spermatozoa themselves and, as well as binding directly to sperm, have been reported to capture spermatozoa by extruding their DNA to produce "neutrophil extracellular traps".[4] Fortunately, stallion seminal plasma has been shown to contain proteins that inhibit the binding or capture of live sperm (eg CRISP3) and others that enhance the binding of dead sperm (eg lactoferrin) by neutrophils.[5] Surprisingly, it appears that in donkey semen the seminal plasma, rather than the spermatozoa, is the primary stimulator of neutrophil extracellular trap formation.[4] Clearly, there is a need for further research into the proteins present in seminal plasma, or that could be added to semen extenders or introduced into the uterus of the mare prior to breeding, that can protect high-quality spermatozoa from elimination by neutrophils as they pass through the uterus towards the oviduct.

Once in the oviduct, the sperm are thought to establish a temporary "sperm reservoir" by binding to epithelial cells in the isthmus; close to the time of ovulation, a small number of spermatozoa are induced by unknown signals to activate ("capacitate"), detach from their epithelial binding and ascend to the ampullary portion of the oviduct where they can fertilise the oocyte. A further impediment to sperm–oocyte interaction and, in particular, the subsequent descent of the fertilised embryo into the uterus, is occlusion of the ampulla by accumulations of inspissated follicular fluid, unfertilised oocytes or degenerate embryos which do not secrete the prostaglandin E2 required to initiate their descent into the uterus. Administering PGE2 onto the surface of the oviduct laparoscopically is one way to salvage fertility in mares presumed to be suffering from oviductal occlusion[6]; recently, endoscope-guided flushing of the oviducts via the uterus ("hysteroscopic hydrotubation") was shown to be a valid alternative treatment for re-establishing oviductal patency in mares, while avoiding the risks of laparoscopy.[7] Of course, oocyte recovery for oocyte transfer or fertilisation by intracytoplasmic sperm injection (ICSI) is also a way to salvage fertility in the case of oviductal occlusion.

3 | AN APPROPRIATE ENVIRONMENT FOR EARLY EMBRYO DEVELOPMENT

One of the major challenges to economic in vitro embryo production is recapitulating the oviductal environment that supports early embryo development; although media for culturing horse embryos from the zygote to the blastocyst stage have improved markedly over the last decade, in vitro produced (IVP) horse embryos are still inferior in quality to embryos flushed from the uterus of young fertile mares.[8] This, at least in part, explains the higher rate of pregnancy loss seen after the transfer of ICSI embryos, compared with flushed embryos.[9] Of course, pregnancy loss is also a significant, but poorly understood, cause of subfertility for more natural breeding techniques. Just as sub-optimal culture media can compromise the development of IVP embryos, a sub-optimal uterine environment, due for example to chronic endometrial degeneration or inadequate endocrine priming, can predispose to pregnancy failure or retarded fetal development. Chronic endometrial degeneration typically results in poor placental development, leading to intrauterine growth retardation and either late gestation abortion or the birth of a small foal after a prolonged gestation[10]; however, severe endometrial degeneration can also compromise early embryonic development. Indeed, the fact that endometrial degeneration is predominantly a mare age-related phenomenon, explains why the marked ageing-associated increase in the incidence of pregnancy loss seen in mares pregnant after natural mating or AI, is ameliorated when embryos are either flushed from the uterus of, or produced in vitro after harvesting oocytes from, old mares and transferred into the uterus of healthy young recipients.[11] On the other hand, embryo transfer (ET) does not reduce the importance of adequately priming the uterus to support pregnancy; indeed, it appears that the oestrus preceding transfer of an embryo to the uterus of a recipient mare must have a duration (characterised by endometrial oedema) of at least 6 days for an optimal likelihood of pregnancy.[12] Inadequate endometrial support may also contribute to the very early growth retardation and abnormal development of neural structures identified by a recent survey as two of the most common abnormalities in pregnancies that failed between days 22 and 46 in Thoroughbred mares.[13] However, these abnormalities could also have arisen from intrinsic embryo abnormalities, and a related study revealed aneuploidy (ie abnormal chromosome number) as the likely cause for 12 of 55 Thoroughbred pregnancies that failed between days 14 and 67.[14] This latter finding supports the hypothesis that aneuploidy arising either during oocyte maturation (ie during meiosis) or the early cell divisions of embryonic development is a significant contributor to early pregnancy loss. Indeed, an age-related reduction in oocyte quality is thought to be a major contributor to reduced embryo viability in older mares, most probably as a result of reduced ability of oocytes to correctly align their chromosomes on the meiotic spindle prior to segregation.[15]

4 | IN VITRO PRODUCTION OF HORSE EMBRYOS

While AI and transfer of flushed embryos are well-established in clinical practice, IVP of horse embryos is relatively new. Indeed, IVP has only become commercially viable in equine practice during the last 10 years.[16] Although it has the capacity to circumvent many different causes of subfertility, the application of IVP was initially

hampered by poor rates of oocyte recovery, failure of conventional in vitro fertilisation (ie fertilising a mature oocyte by simply incubating with capacitated sperm), and sub-optimal development of oocytes fertilised by ICSI into blastocysts suitable for transfer into the uterus of a recipient mare.[16] Nowadays, oocyte recovery rates in commercial ovum-pick up (OPU) programmes exceed 50% (yielding means of 5–13 immature oocytes), somewhere between 60% and 80% of these oocytes are successfully matured to the MII stage at which they can be fertilised by ICSI, and blastocyst production rates in a commercial programme can exceed 20% of injected oocytes and 1.5 blastocysts per OPU, with more than 65% of all OPUs yielding at least one transferrable blastocyst.[11] Most surprisingly, given the documented decrease in oocyte quality in aged mares, the ability of ICSI embryos to yield viable pregnancies does not appear to fall as dramatically with increasing donor mare age as it does for all other breeding techniques (ie natural mating, AI and conventional ET)[11]; this suggests that IVP may be the technique of choice for maximising the likelihood of foal production from sport horses mares beyond 18 years of age. In fact, the high success rates for OPU-ICSI make it a more efficient way of producing embryos and foals than conventional insemination and embryo flushing; add this to the fact that OPU-ICSI can be performed as a one-off, out-patient procedure at any time of the year and it becomes easy to understand why some breeders of sport horses now perform the bulk of their breeding via OPU-ICSI. This is not to say that the system is perfect; OPU is an invasive procedure and while complications are not common they can be serious (eg rectal tear, abdominal haemorrhage, peritonitis and ovarian abscess), and at least 10% of mares will suffer mild post-OPU discomfort. Moreover, pregnancy rates after transfer of ICSI embryos are still 15%–20% lower than those for flushed embryos, and pregnancy loss rates are slightly higher.[17] Part of the difference appears to derive from ICSI embryos tolerating only a very narrow window of recipient mare synchrony, with pregnancy rates significantly better when the embryos are transferred into a mare on day 4 as opposed to day 5 or 6 after ovulation.[9,17] The importance of monitoring ICSI pregnancies for possible failure is emphasised by the finding that more than 1% yield monozygotic twins or triplets (only detectable after day 21 when formation of more than one embryo proper within a single vesicle becomes apparent[18]) which will almost inevitably end in pregnancy failure or abortion as a result of placental insufficiency.

5 | FROZEN STORAGE OF EMBRYOS OR OOCYTES

One of the advantages of IVP is that the embryos are amenable to cryopreservation with no appreciable difference in pregnancy rates between freshly transferred and cryopreserved ICSI embryos.[9] The growth in the use of IVP embryos has therefore served to remind both breeders and veterinarians of the numerous practical advantages of frozen storage of embryos, for example, producing embryos outside the normal breeding season, only thawing an embryo when a suitable recipient is available and never needing to skip using a suitable recipient because of failure to collect an embryo. While the advantages of cryopreservation could also apply to embryos flushed from inseminated mares, uptake in practice has been inhibited by the very poor cryosurvival of horse and donkey embryos larger than 300 μm in diameter.[19] Since horse embryos only arrive in the uterus at approximately 6.5 days after ovulation, and often later in the case of older mares or after post-ovulation insemination with frozen-thawed semen, but expand very rapidly after reaching the uterus, it is very difficult to determine exactly when a mare should be flushed to ensure that the embryo has entered the uterus but is still under the 300 μm cut-off point, that is, flushing exactly 7 days after ovulation will be perfect for some mares/embryos, but either too early or too late for others. In recent years, however, a number of studies have demonstrated that reducing the volume of larger expanded blastocysts by puncture and aspiration of the blastocyst fluid significantly improves their ability to withstand cryopreservation; it now appears that simply puncturing embryos up to 550 μm in diameter to allow the fluid to escape and cryoprotectant to enter, results in very good pregnancy rates after vitrification-warming (>75%).[20] However, embryos larger than 550 μm need to be actively collapsed by aspirating the contained fluid if they are to survive vitrification-warming. In short, the requirements for cryopreserving flushed horse embryos are size-dependent: embryos ≤300 μm can be frozen without manipulation, embryos >300 μm but ≤550 μm need to be punctured and embryos ≥550 μm require both puncture and aspiration if they are to survive vitrification.

From the perspective of genetic storage, the downside of freezing embryos is that the choice of sire has already been made. Cryopreserving oocytes would allow postponement of sire choice and enable storage of genetic material from young mares with high genetic potential when they are young and oocyte quality is high, but waiting until the mare had proven itself in competition before deciding to proceed with fertilisation and embryo production. However, while the first foal from an equine oocyte vitrified in the immature state was recently reported, the percentage of vitrified oocytes that yielded a foal from the best protocol (1/179) was very low,[21] and it is clear that cryopreservation of equine oocytes is currently far behind that of human oocytes and not yet ready for clinical practice.

6 | CONCLUSIONS

ET and IVP have proven to be powerful techniques for resolving subfertility; for example, transferring embryos into the uterus of young recipient mares markedly reduces the risks of pregnancy loss due to age-related endometrial degeneration, while IVP allows production of embryos from mares or stallions that are unable to produce viable embryos via conventional insemination. Although the ARTs are still in development and have both known (reduced quality of IVP embryos) and unquantified (epigenetic perturbations) downsides, growth in the use of IVP has made it clear that, even for fertile animals, the ARTs can offer significant increases in overall efficiency

(more embryos per cycle than possible by conventional ET) and practicality (OPU can be performed as a one-off, out-patient procedure at any time of the year or stage of the oestrous cycle, and the embryos frozen until a suitable recipient is available during the breeding season). While some techniques are technically complex and not yet optimised, studies into factors limiting their success may not only lead to future improvements but also yield information useful to tackling specific causes of subfertility in mares or stallions used for natural mating or AI.

Tom A. E. Stout[1]
Huw Griffiths[2]

[1]*Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*
[2]*Liphook Equine Practice, Liphook, UK*

Correspondence
Tom A. E. Stout, Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584 CM Utrecht, the Netherlands.
Email: t.a.e.stout@uu.nl

ORCID

Tom A. E. Stout https://orcid.org/0000-0001-5321-8095

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