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The growth of Assisted Reproductive Technologies in horse breeding

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Abstract

Over the last 50 years, several assisted reproductive technologies (ARTs) have been adopted in sport horse breeding, with the primary aim of increasing the speed of genetic selection, while also addressing various types of sub-fertility. For many horse registries, artificial insemination (AI) and embryo transfer (ET) have become standard practice, and improved protocols for cryopreservation have facilitated long-distance, international dissemination and/or collection of germ plasm outside the breeding season. Recently, in vitro embryo production (IVEP) via ovum pick-up and intracytoplasmic sperm injection (OPU-ICSI) has enabled more intense selection of both male and female lines, and made it possible to produce embryos from animals that are severely sub-fertile. In fact, in established OPU-ICSI programs the mean number of embryos produced per procedure (≥ 2) has reached a level at which some owners are choosing to perform all their commercial breeding via IVEP. On the other hand, in Europe the popularity of IVEP has slowed during the last year, partly because large numbers of stored embryos are still waiting to be transferred, but also because of questions from outside the ART industry about the ethics of performing an invasive procedure (OPU) electively, and the possible impact of OPU-ICSI on the gene pool.

Keywords*: assisted reproductive technology, artificial insemination, embryo transfer, ovum pick-up, intracytoplasmic sperm injection*

Introduction

In the 1970s nearly all sport horse foals were the product of natural covering. Over the last 50 years, a broad range of assisted reproductive technologies have been developed and refined for use in horses and are now used routinely to either increase the number of foals produced from selected stallions or mares, or to salvage genetic potential after loss of fertility or death (Allen, 2005; Hinrichs, 2018). Compared to the cattle industry, however, progress in the development and application of ARTs to horse breeding has been slow, for several reasons. Firstly, many studbooks and sporting organizations have taken a conservative approach to approving certain ARTs and/or have placed restrictions on the number of foals that may be registered per mare per year (Squires *et al*., 2003), citing concerns about the potential impact of ARTs on inbreeding coefficients, but also because of fears that making the most desirable stallions more accessible could impact the livelihood of the owners of other stallions. In addition, techniques that work well in cattle have not always transferred easily to horses and, because the equine sector is relatively small, it has sometimes taken many years to refine these techniques to the point where they are commercially viable. For example, although transvaginal ultrasound guided oocyte recovery (ovum pick-up: OPU) was first reported in the early 1990s (e.g. Brück *et al*., 1992), and the use of ICSI to produce horse pregnancies was documented a few years later (e.g. Cochran *et al*., 1998), because of low oocyte recovery (<25%) and poor blastocyst per injected oocyte production rates (<10%), it wasn't until 2007 that embryo per procedure production rates were reported (0.6: Galli *et al*., 2007) that compared favorably to conventional embryo flushing after AI. Similarly, while the production of a foal from a frozen-thawed embryo was first reported in 1982 (Yamamoto *et al*., 1982), it wasn't until 30 years later that a method was described for attaining acceptable post-cryopreservation pregnancy rates with expanded horse embryos (Choi *et al*., 2011). Nevertheless, over the last 10-15 years significant breakthroughs have been made in oocyte collection, *in vitro* embryo culture, embryo cryopreservation, and somatic cell nuclear transfer, and all are now implemented in commercial programs.

Of course, the emphasis when developing new techniques is on the technological and biological requirements for success, with less focus on the potential unintended consequences of implementing these techniques on a commercial scale (e.g. epigenetic signature of resultant offspring, narrowing of the gene pool). During the last 10 years, there has been an enormous increase in the uptake of OPU-ICSI in the Warmblood horse breeding industry, in particular for showjumpers. However, the number of OPUs

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performed has plateaued during the last year, in part because large numbers of embryos have been produced and cryopreserved but are yet to be transferred, but also because foal prices have dropped now that the novelty of being able to buy ICSI foals or embryos from stallions and/or mares that were otherwise not accessible has worn off. In addition, there have been questions from outside the equine ART world, as to whether it is justifiable to perform OPU on healthy, fertile mares just because the embryo production rates are higher. In short, while there is still technological progress to be made in producing or selecting embryos of the desired sex or with specific genotypic or phenotypic characteristics, and in improving the developmental competence of IVP embryos (higher pregnancy rates, lower embryonic loss rates), it will be equally important to demonstrate that the ARTs are currently being applied ethically and with attention to the health and welfare of the donor mares and resulting foals. This manuscript will focus on the breakthroughs and recent developments in equine ARTs.

Progress in artificial insemination (AI)

Artificial insemination is now a normal part of horse breeding in most commercial breeds, with the obvious exception of the racing Thoroughbred. Clearly, AI has great advantages with respect to the number of mares that can be mated per ejaculate, and in terms of biosecurity (reduced risk of disease transmission), animal welfare (transporting semen instead of animals, avoiding natural covering of nervous maiden mares), and international dissemination of genetic material. Techniques that have been developed to further enhance the advantages include low dose deep intrauterine AI (Morris & Allen, 2002); in this respect, it has become clear that by depositing the semen deep in the uterine horn ipsilateral to the dominant follicle or fresh ovulation, the number of sperm per AI dose can be reduced from the previously recommended 250-300 million morphologically normal, motile sperm to somewhere closer to 20-50 million; there are, however, significant variations between stallions, and low dose AI is rarely a solution to poor fertility. Nevertheless, it has become common to use a single straw of frozen semen per inseminated estrous cycle in mares, even when the semen was originally frozen with the intention of using 4-8 straws per dose. Moreover, AI within 6h after ovulation with a single straw of semen has been reported to yield acceptable pregnancy rates in practice (33-65%: Immonen & Cuervo-Arango, 2020; Pasch *et al*., 2024), although there are considerable differences between stallions and not all stallions are suitable for AI with low doses of frozen-thawed semen. In fact, low dose AI was initially developed to allow the use of semen sorted by flow cytometry for either X or Y chromosome-bearing sperm (sex-sorting) and, while sex-sorting has been used commercially, it is currently limited by poor results when flow-cytometric sorting is combined with cryopreservation (Gibb *et al*., 2017). To deal with poor quality of spermatozoa in general, both gradient density centrifugation techniques (Umair *et al*., 2021) and sperm motility selection devices (Vigolo *et al*., 2022) have been developed that allow selection of a sperm population enriched for motile, morphologically normal, DNA-intact spermatozoa. The relatively small numbers of spermatozoa selected are, however, typically suitable only for deep intrauterine insemination, *in vitro* fertilization or ICSI.

Progress in embryo transfer (ET)

Conventional embryo transfer has been well established since the 1980s (Squires *et al.*, 2003) and the major advances in recent years have been in cryopreservation of expanded blastocysts, biopsy of embryos for genetic testing (Hinrichs, 2018), and refining the selection of recipient mares. For a long time, it was accepted that to offer an acceptable chance of pregnancy, recipient mares should ovulate between 1 day before and 3 days after the donor (Squires *et al.*, 2003). More recent surveys from commercial programs have demonstrated that in fact, pregnancy rates differ very little between recipient mares that ovulate between 1 day before and up to 5 days after the donor (Cuervo-Arango *et al*., 2019a), although in recipient mares that are 5 days behind the donor, embryo development is retarded by approximately 2 days. One important aspect of this longer window of acceptable synchrony, is that pregnancy rates after transfer are better if the recipient exhibited at least 5 days of clear endometrial edema in the estrus immediately preceding ET. This means that, if sufficient recipient mares are available, it is generally better not to induce ovulation but rather to allow the mares to ovulate naturally. Similarly, if an acyclic hormone-treated recipient is to be used, attention should be paid to a long enough pretreatment period with estradiol before starting the progesterone (Silva *et al*., 2024). If induction of ovulation is required, for example to synchronize a single recipient with a given donor, induction can be delayed until 2-3 days after the donor mare ovulates rather than on the same day, thereby allowing up to three days of additional estrus to better prime the uterus. The other major breakthrough is in the cryopreservation of large expanded day 7-8 equine embryos. While it has been possible to achieve acceptable pregnancy rates (>60%) with small (<300 mm) embryos for some time, post-thaw survival of larger embryos was poor (<20%: see Stout, 2012). However, Choi *et al.* (2010) discovered serendipitously that vitrifying embryos after they had collapsed as a result of biopsy to collect cells for genetic testing, led to surprisingly high pregnancy rates. Subsequent studies established vitrification of embryos after puncture to aspirate the blastocoele fluid as the cryopreservation technique of choice for larger flushed horse embryos. Alternatively, recent studies have shown that by extending the time period during which the embryo is incubated in the first equilibration solution of commercial human embryo vitrification kits, pregnancy rates of >75% can be obtained for embryos as large as 480 mm without the need for puncture and aspiration (Kovacsy *et al*., 2024). These successive developments have made cryopreservation of flushed embryos a commercially viable prospect and have led to programs for cryopreserving embryos, both to allow embryo production outside the regular breeding season and/or to biopsy embryos for genetic selection, with the embryos being vitrified and stored while waiting for the results of the genetic tests (sex determination or testing for monogenetic diseases: Hinrichs, 2018).

Ovum pick-up and Intracytoplasmic sperm injection

Although ovum pick-up in mares was first reported in the 1990s, oocyte recovery rates from immature follicles were low (<30%) and, since the percentage of sperm-injected oocytes that developed to the blastocyst stage during *in vitro* culture was also low (<10%), it was difficult to collect enough oocytes for a reasonable chance of producing a transferrable embryo; for this reason, OPU-ICSI was not a commercially viable procedure in the early 2000s. This began to change when Galli *et al.* (2007) reported the use of a double lumen 12-gauge needle to repeatedly aspirate and flush mid-size follicles; when accompanied by rotating the needle to scrape the inner wall of the follicle, this improved oocyte recovery rates to >50%. Combined with refinements to the oocyte maturation, ICSI and embryo culture procedures, this enabled Galli *et al.* (2007) to generate blastocyst yields of around 0.6 per OPU, which was comparable to that being achieved in commercial embryo flushing programs in which mares were inseminated with cooled-transported or frozen-thawed semen (Panzani *et al*., 2014). The commercial viability of OPU-ICSI was further confirmed by Hinrichs *et al.*'s (2014) presentation of blastocyst yields of ~1 per OPU session, while the practicality and accessibility were further assisted by the discovery that immature horse oocytes tolerate overnight 'holding' at room temperature (Hinrichs, 2018). Overnight holding of oocytes allows for more convenient planning of the onset of *in vitro* oocyte maturation, to better schedule sperm injection 24-28 h later. More importantly, the ability of oocytes to withstand holding meant that they could be shipped overnight at 20ºC from a donor mare facility to a specialized ICSI laboratory, with little impact on blastocyst production rates (Galli *et al*., 2016). Furthermore, because equine IVP blastocysts are small and have no capsule, they survive freeze-thawing well and can either be slow-frozen or vitrified for shipping to an embryo transfer facility with no noticeable effect on pregnancy rates.

The rapid growth of OPU-ICSI programs has been paired with refinements in many aspects of the process such that results have continued to improve year-on-year (Lazzari *et al*., 2020). The fact that experience markedly improves results probably helps to explain why it can take new laboratories a long time (2-3 years) to reach commercially acceptable levels of embryo production and embryo quality. It has also become clear that some mares are better donors than others (higher blastocyst per oocyte yields) and that embryos that reach the blastocyst stage more quickly (6-7 days) are more likely to yield on-going pregnancies (Claes *et al*., 2020). Interestingly, it also transpires that while the vesicles resulting one week after transfer of ICSI embryos suggest that IVEP blastocysts are equivalent in developmental stage to day 5-6 *in vivo* embryos, the pregnancy rates are better when the embryos are transferred to recipient mares on day 3 or 4 after ovulation rather than days 5 or 6 (Claes *et al*., 2020: Stout, 2020).

Recovering immature oocytes has the advantage of allowing IVEP to be performed year-round (e.g. mares in transitional anestrus often have large numbers of small to medium sized follicles), while ICSI allows extremely efficient use of spermatozoa from stallions that are either dead or from which very few straws of semen are available. While there are still challenges related to higher rates of embryonic loss for OPU-ICSI (up to 15%: Cuervo-Arango *et al*., 2019b) compared with other breeding methods, and a surprisingly high incidence of mono-zygotic twins (1-2%: Dijkstra *et al*., 2020), the likelihood of generating embryos from mares that are unable to yield foals via other techniques because of age-related changes is surprisingly good, with no clear drop off in embryo production rates or likelihood of pregnancy after transfer of ICSI blastocysts until the oocyte donor mares exceed 20 years of age.

Conventional IVF

ICSI became widespread in equine IVEP because conventional IVF (i.e. incubating the oocyte with spermatozoa) was not successful, where the lack of success with conventional IVF appeared to be due primarily to an inability to adequately induce capacitation in stallion spermatozoa *in vitro* (Leemans *et al.*, 2019). Recently, Felix *et al*. (2022) solved this problem by incubating stallion spermatozoa for a prolonged period (22h) in a medium containing penicillamine, hypotaurine and epinephrine (PHE); this prolonged PHE incubation appears to allow the spermatozoa to maintain viability and motility while the sperm plasma membrane changes required for zona pellucida penetration take place. Moreover, the IVF protocol appears to yield very high blastocyst rates (>70% of cleaved zygotes), and was soon followed by the description of a shorter incubation (9 h) for frozen-thawed semen (Felix *et al*., 2025), that also yields high blastocyst production rates (>50%) and is better suited to the reality of clinical practice, where frozen semen is more commonly used than fresh or cooled-transported semen. While the development of these IVF protocols is a significant breakthrough, they are yet to be established in commercial practice, and it will be interesting to see whether IVF replaces ICSI in practice in the coming years. In human clinics, although conventional IVF has the advantage of not requiring micromanipulation and of being considerably less time-consuming than ICSI, many clinics prefer to go straight to ICSI because it is more reliable in producing pregnancies, especially when only very low numbers of viable sperm are available, and adequate sperm function cannot be guaranteed.

Cloning, and ethical and welfare aspects of ARTs

Of course, cloning by somatic cell nuclear transfer has also been developed (Hinrichs, 2018) and is now used in commercial programs, especially in south America. In Europe, cloning is not common due to a combination of regulatory restrictions and high up-front costs. Moreover, the principal reason that cloning was once desired in Europe was to produce clones of stallions that had died, become infertile or been gelded, so that the breeding potential of these desirable sires could be recovered or increased. Instead, the improvements in OPU-ICSI have enabled the production of foals from very small numbers of spermatozoa, even from poor quality semen, such that the major reason for cloning has disappeared. In addition, European breeders will often not buy semen from a clone unless they have seen him compete at a high level, even though the genetic material will be identical to that of the original stallion, whereas performance could be affected by numerous environmental and training factors.

While there are still improvements to be made to most of the existing ARTs, such as improved semen freezing protocols for poor-freezing stallions and sex-sorted semen, and reliable multiple ovulation protocols to improve output from conventional embryo flushing, the biggest challenge in the immediate future is likely to be convincing public opinion that OPU is ethically acceptable and that everything possible is being done to minimize pain, and to safeguard the welfare of donor mares, alongside demonstrating that there are no unwanted epigenetic consequences for the resulting offspring. In this respect, a recent survey indicates that a sizeable percentage of mares (up to 25%) do suffer mild post-OPU discomfort or temperature elevation (Hinrichs *et al*., 2025); it is important to modify OPU protocols to minimize these minor complications. Fortunately, although more serious complications such as ovarian abscesses, peritonitis, rectal tears or hemorrhage do occur, the estimated incidence is very low (about 0.1% of procedures: Hinrichs *et al*., 2025). Nevertheless, rather than waiting for regulations to be imposed on equine ARTs, it is important for the industry to be proactive in producing its own guidelines, and in designing and performing studies and surveys to show that the procedures are being performed responsibly and with attention to the welfare and well-being of the donor mares and resulting foals.

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