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Assisted reproduction techniques in horses

by

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Abstract

This thesis explores the diverse techniques of assisted reproduction in horses. It offers a comprehensive overview of established and emerging methodologies for overcoming fertility challenges, enhancing genetic diversity, and expanding breeding possibilities.
A comprehensive analysis of methods such as sperm collection, sex determination, artificial insemination, embryo transfer, oocyte recovery under ultrasound guidance, in vitro maturation and fertilisation, intracytoplasmic sperm injection (ICSI), gamete intrafallopian transfer (GIFT), cryopreservation, and cloning is presented.

Each technique is evaluated regarding procedure, clinical application, and success rates, focusing on its practical implications and transformative impact on equine reproduction. AI and ET have become basic techniques in the field. At the same time, advancements in OPU, IVM, and ICSI have provided innovative solutions for addressing infertility in mares and optimising the use of limited or compromised stallion semen. Cloning offers a unique pathway for preserving rare genetic lines and breeding elite equine athletes but raises ethical concerns and practical challenges.

The discussion also addresses the technical constraints imposed by species-specific reproductive physiology and the economic and regulatory barriers that limit the widespread adoption of certain technologies. Despite these challenges, recent advancements in laboratory protocols, embryo culture systems, and cryopreservation techniques have significantly improved the efficiency and reliability of assisted reproductive technologies (ART) in horses.

Ultimately, this thesis underscores the critical role of ART in revolutionising equine breeding, enabling the conservation of valuable genetic traits, and supporting the industry's sustainability. Building on these advances, the future of equine reproduction promises even greater integration of these technologies into standard breeding practices, enhancing their accessibility and success on a global scale.

Absztrakt

A szakdolgozat a lóvakban alkalmazott asszisztált reprodukciós technikákat mutatja be. Az ismertetett eljárások a következők: spermagyűjtés, ivardeterminált sperma előállítása, mesterséges termékenyítés, embrió transzfer, ultrahangos ellenőrzés mellett végzett petesejtgyűjtés, petesejtek in vitro érlelése, in vitro fertilizáció, spermium injektálás (ICSI), petesejt transzfer, a petesejt petevezetőbe történő transzferje (GIFT), embriók mélyhűtése, klónozás.

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List of abbreviations

Assisted Reproduction Techniques	ART
Artificial Insemination	AI
Embryo Transfer	ET
Oocyte Transfer	OT
In Vitro Fertilisation	IVF
Ovum Pick-Up	OPU
Intracytoplasmic Sperm Injection	ICSI
Gamete Intrafallopian Transfer	GIFT
Somatic Cell Nuclear Transfer	SCNT
Corpus Luteum	CL
Prostaglandin F2α	PGF2α
In Vitro Maturation	IVM
In Vitro Fertilisation	IVF
Transvaginal ultrasound-guided follicle aspiration	TVA
Dominant Stimulated Follicles	DSF
Cumulus-Oocyte Complex	COC
In Vitro-Produced	IVP
Hypothalamic-Pituitary-Gonadal-axis	HPG
Gonadotropin-Releasing Hormone	GnRH
Luteinising Hormone	LH
Follicle-Stimulating Hormone	FSH
Breeding Soundness Examination	BSE
Equine Viral Arteritis	EVA
Artificial Vaginas	AV
Computer-Assisted Sperm Analysis	CASA

Introduction

Assisted reproductive technologies (ART) have significantly advanced equine reproduction, offering solutions for fertility challenges and expanding breeding possibilities. Over the years, techniques such as artificial insemination (AI), embryo transfer (ET), intracytoplasmic sperm injection (ICSI), and nuclear transfer have gained traction in equine practice. Embryo transfer, a cornerstone of equine ART, allows for multiple foals from a single mare per season and has become widely accepted in the industry [1]. However, innovations such as ICSI and oocyte transfer have provided new methods to address infertility caused by issues in the mare's reproductive tract or low sperm quality [2].

Recent developments have included advancements in vitro oocyte maturation and embryo culture, enabling the successful production of blastocysts with comparable efficiencies to bovine IVF systems [3]. Additionally, techniques like somatic cell nuclear transfer (SCNT) have further expanded the scope of equine ART by enabling cloning to preserve unique genetic lines [4].

Despite these advancements, challenges persist, particularly in achieving routine application of some techniques due to species-specific reproductive physiology and technical constraints. As these technologies evolve, they continue to reshape equine breeding strategies globally, promising improved genetic preservation and enhanced reproductive outcomes [5].

Assisted Reproduction Techniques (ART) in stallion

Semen collection

Collecting semen from stallions is essential for assisted reproductive technology (ART). Elia Ivanoff, a Russian and Soviet scientist, pioneered animal AI in the early 1900s. He began his work by inserting a special sponge into a mare's vagina to collect stallion semen, using a special press to obtain it from the sponge. It was in the early 1930s when artificial vaginas (AV) were invented and made semen collection more effective [6]. Effective use of artificial insemination (AI) in equine breeding programs can significantly enhance operational efficiency and expand the availability of sires to the public [7].

A thorough breeding soundness examination (BSE) for the stallion should be carried out. This includes a medical history, a general physical check-up, an evaluation of both external and internal genitalia, culture swabs from the urethra and penis, assessment of libido and breeding capability, as well as collection and analysis of at least two ejaculates taken one hour apart [8, 9]. Numerous techniques and methodologies have been documented to collect stallion ejaculate properly [10].

Artificial vagina collection

Before the semen is collected, the stallion’s penis is washed with warm water to remove dirt. Blood sampling and swaps from the penis should be collected to be tested on infectious diseases such as equine viral arteritis (EVA), equine coital exanthema or dourine. Testing for EVA can be important as this virus even survives the freezing procedure. The stallion should be trained to mount a breeding phantom to reduce the risk of injuries; a sound mare can also be used. There are numerous numbers of artificial vaginas (AV) which can be chosen upon the stallion’s and operators’ preferences. The collection of the stallion’s semen should happen routinely to ensure a cleanout of the tracts [10, 11].

The AV must be adequately prepared before the stallion can mount the phantom. All components of the artificial vagina that will come into contact with the semen, including lubricant, sleeve, collection receptacle, rubber liner, and disposable liner, should be non-

spermicidal. The stallion is sensitive to temperature and pressure, so the AV is filled with warm water ranging from 45 to 55°C, depending on the specific AV used [7, 10].
The stallion can mount the phantom, and the artificial vagina must be positioned to emulate the posture and configuration of the mare's vagina. Significant movements of the artificial vagina are rarely necessary to facilitate ejaculation [10].
Following semen collection, the gel fraction is discarded while the gel-free ejaculate undergoes analysis for volume, concentration, motility, and total sperm count. Volume can be assessed using a graduated cylinder (in ml) or a scale (in grams) since 1 g of semen corresponds to 1 ml, providing the most accurate volume measurement. The concentration of raw semen is expressed in millions of sperm per ml, determined with a densimeter or a nucleocounter. At the same time, motility is evaluated using light microscopy or Computer Assisted Sperm Analysis (CASA) [7, 11].

Ground collection

For this technique, the stallion is teased with a mare till he is erected, and the penis is washed. The AV is placed on his penis and pushed towards the base of the penis to encourage thrusting. After 5-10 pelvic thrusts, stallions normally ejaculate; if not, the procedure is repeated until successful [7].
An alternative procedure can be done with the use of a plastic bag. The procedure involves positioning a plastic bag around the penis up to the mid-shaft level. Ejaculation may be induced by applying a warm towel compress, with a temperature ranging from 45 to 50 degrees Celsius, to both the glans and the base of the penis [7, 10].
Chemical ejaculation is typically utilised for stallions facing ejaculatory issues linked to neurologic or musculoskeletal disorders that prevent mounting [7].

Epididymal semen harvesting

Post-castration epididymal semen extraction is an innovative technique for semen collection that facilitates extraction where alternative methods may not be viable. This procedure involves the retrieval of semen from the epididymis of the testes and can be employed following routine castration, upon the death of the animal, or in cases where the testes have experienced

significant trauma or torsion; the latter unanticipated circumstances represent the most critical application of this method [12].

Various methods are delineated for recovering sperm from the cauda epididymis, including aspiration, flotation, and retrograde flushing. The latter two techniques are employed in castration or post-mortem collection from dead animals, whereas aspiration is applicable in living animals and requires anaesthetic administration [13].

Semen sorting

As AI became more influential, there was also a need to improve fertilisation and pregnancy rates. Various methods can be reviewed [14], but two techniques will be of greater importance. Density Gradient Centrifugation (DGC) and Swim-up (SU) may be the most used techniques due to their simplicity.

DGC functions by placing a single or double-density colloid gradient in a centrifuge tube filled with a mixed-quality semen sample. Density gradually increases from the top to the bottom of the gradient. The tube with the gradient and the ejaculation is then spun at a moderate g-force for 15–30 minutes to facilitate the passage through the gradient, allowing for the separation of high-quality and low-quality spermatozoa. The motile spermatozoa are concentrated at the bottom [14, 15].

The SU procedure is one of the oldest methods. It does not require a centrifuge and solely relies on the sperm's ability to swim upwards through a medium for 30 to 60 minutes. This technique shows promising results in humans and selects for highly motile, morphologically normal, DNA-intact sperms. Nonetheless, SU's recovery rates are low, so only highly concentrated samples should be used [14, 15].

Combining the two techniques above, SU and DGC, is also possible. DGC allows the sperm to pellet and then swim upwards through an overlying medium with the SU technique. The combination has shown better motility and morphology than SU alone, decreases abnormalities, and selects for better DNA than DGC alone. It has also been shown to be an efficient way to remove pathogens, such as the equine arteritis virus, from the semen. Thus,

this approach may offer considerable advantages for other equine diseases transmitted through ejaculate [14].

Semen sexing

Choosing sperm based on whether they carry an X or Y chromosome is highly relevant for numerous species. Sex selection is also significant in the equine and agricultural sectors, where either female or male traits may be more advantageous for sports or production results and align with owner preferences [14, 16].

Flow cytometry is currently the sole technique employed for sexing horses.

It has been successfully applied and demonstrates over 90% accuracy in determining the sex of live foals [14, 16]. However, the technique can severely damage spermatozoa, making it challenging to produce an adequate dose for artificial insemination[14].

Consequently, pregnancy rates vary between 10% to 50% [14, 16]. Fresh, sex-sorted semen can achieve rates of up to 52%, while frozen-thawed sorted semen results in rates as low as 12% [16].

In other species, a method known as zeta potential sex selection has been reported to be successful [14].

Assisted Reproduction Techniques (ART) in mare

Artificial Insemination (AI)

Artificial insemination (AI) is the process where semen, be that fresh (native), chilled extended, or frozen, is deposited in the reproductive tract not by natural mating but by artificial means. This technique reduces the risks associated with natural mating and lowers the likelihood of transmitting sexually transmitted diseases. It also allows the mare to be bred at a location separate to the stallion thereby minimising travel and hence stress of the mare (and her foal). Artificial insemination also allows breeders access to the bloodlines of stallions worldwide.
Although AI was likely undertaken, possibly centuries before, Sir Walter Heape, in the late 1800s, was one of the first to report on the successful collection of stallion semen and it subsequent insemination into a mare in Russia and China extended the use of AI between 1930 and 1960, and although they were also developing techniques for semen freezing, much of this early work was never published. However, in the late 1960s, Professor B.W. Pickett and his research team at Colorado State University published numerous papers extending knowledge on semen physiology, methods for collecting semen, its subsequent dilution, cooling and deep freezing. Artificial insemination gradually became accepted by veterinarians, breed societies and breeders, and it is now used routinely in the breeding of horses [17, 18].

Semen used for AI can take three forms: fresh (or native), cooled or chilled, and frozen [6].

Fresh semen

Inseminations can be undertaken with fresh semen that has not been diluted with extenders. The stallion and the mare need to be at the same stud or close by as un-diluted semen does not survive for long periods. Fresh semen can also be used for mares that show unfavourable reactions to extenders, and in such cases, the use of undiluted semen may improve conception rates [19, 20]. Certain papers refer to diluted semen as fresh semen. Consequently, the dilution ratio is 1:1, mixing semen with a commercial diluent, yet the semen is still administered directly to the mare. [6, 21, 22].

Cooled semen

In order for semen to be kept for any length of time it needs to be diluted to prevent the semen clumping together and also to ensure there are enough nutrients (such as sugars) to ensure the spermatozoa can survive for extended periods of time

Sometimes the seminal plasma is removed from the native semen prior to diluting and for some stallions this may improve the longevity and help to maintain the quality of the spermatozoa. Numerous commercial semen extenders are available, many of which contain milk products and sugars. By extending semen it is possible to cool it and ship it to another location or store it for later use.

To chill semen it is slowly cooled to 4-5°C. At this temperature it can remain viable for 48-72 hours [6, 17, 19, 23, 24], making shipping overnight [19] or longer possible [17].

Frozen semen

Cryopreserved semen is widely used nowadays. The ability to deep freeze semen and store it in liquid nitrogen in 0.25- 0.5-ml straws can preserve the genetics of important stallions, acting as an insurance policy should the stallion be injured or die and no longer be able to be collected. Frozen semen can be shipped at any time so that when the mare is ready to breed it is instantly available negating the need to worry about ordering chilled semen or considerations over semen collection days or semen availability of busy stallions [21]. Frozen semen can also be shipped over greater distances than chilled semen. Once it has arrived at its destination frozen semen can be stored indefinitely in liquid nitrogen tanks [19]. Moreover, it permits the use of semen from stallions involved in competitive sports, which typically restricts semen collection during their busy competition schedules [25]. Cryopreserved semen can be preserved indefinitely at -196°C as long as the supply of liquid nitrogen is maintained maintained [11].

Less favourable effects of the freezing procedure are the reduced viability and longevity of thawed frozen semen in the equine uterus compared to fresh or chilled semen. This leads to more intense ultrasonographic monitoring of the follicle close to ovulation to ensure the thawed frozen semen is inseminated close to the time of ovulation [17].

Freezing can be done using either a programmable freezer or a manual method that involves a Styrofoam box over liquid nitrogen vapour. As sperm cells are cooled from room temperature to -196°C, the cooling rate must be slow enough to allow cellular dehydration but fast enough to prevent prolonged exposure to hyper-concentrated conditions as the water freezes.

Freezing occurs in two steps: the first step cools the semen from room temperature to 5°C at a rate of 3–5°C/min, with the optimal duration dependent on the specific extender used. The second step cools the semen from 5°C to −196°C at a quicker rate of 20–50°C/min. If a programmable freezer is available, it can be programmed to follow the desired freezing curve. In a manual method, the rack of straws is placed in a refrigerator for 20 minutes, then held 3 cm above the liquid nitrogen vapour for another 20 minutes before being submerged in liquid nitrogen.

Before freezing, the straws are printed and collected; each straw is labelled with the stallion's name, date of birth, breed, registration number, and any additional information from the owner or breed registry requires [11]

Typically, frozen semen is thawed at around 38°C for 10 seconds [23], although protocols may vary.

Artificial insemination procedure

Before insemination, certain steps are undertaken to ensure the procedure is performed correctly. Prior to insemination, the contents of the rectum are removed, and the reproductive tract assessed by palpation and ultrasongraphy. If it is determined that the mares is ready to breed her tail is secured, and the vulva and perineal area are meticulously cleaned to reduce the risk of contamination during insemiantion. A plastic sleeve is put over the operator's hand, which is then lubricated using a sterile, non-spermicide lubricant. The sterile catheter or insemination pipette is gently inserted through the mare's cervix, guided by the operator's index finger, and into the uterine lumen. The semen is gradually deposited into the uterus, and a small amount of air may be introduced to ensure that all the semen is expelled from the insemination pipette [26].

Although the method described above is a standard approach for inseminating mares, according to [6], an alternative technique has been developed for deep intrauterine insemination in situations where sperm viability is low or of poor quality. This technique involves guiding the placement of the semen using palpation or ultrasound transrectally to the tip of the uterine horn

ipsilateral to the dominant follicle. According to [27], favourable outcomes have been reported with this insemination technique, particularly when using low-quality semen with reduced spermatozoa counts.

Timing of insemination

Insemination is done around the time of ovulation hence it is important to know the reproductive state of the mare. How close to actual ovulation depends on the type of semen and its longevity. For fresh semen, insemination is undertaken up to 48 h prior to ovulation and for frozen semen ±12 h of ovulation, with general recommendations that 300–500 million motile sperm are deposited in the mare's uterus [28]. With transported cooled semen AI has been reported from 36 h pre-ovulation up to 16 h post-ovulation [24]. However, in general the time of insemination with cooled semen is usually undertaken 0–24 h pre-ovulation. Thus, a common practice is to treat mares in oestrus with a follicle of ≥35 mm with an ovulatory drug, such as human chorionic gonadotrophin (hCG) or a GnRH agonist, which induces ovulation after 36 – 40 h, 1 day before the semen's expected arrival time so that the spermatozoa are deposited in the mare's uterus 12–24 h before ovulation [24]. Artificial insemination within 6 to 12 h post-ovulation also results in an acceptable pregnancy rate [22].

Success rates

As detailed in [24] pregnancy rates following AI depends on the semen used (fresh, cooled or frozen), sperm numbers, its viability, the insemination protocol and also mare factors such as age. Insemination with cooled semen st 36 to 24 hours before the ovulation had shown lower pregnancy rates than insemination performed 24 hours before to 16 hours after ovulation [24]. The number of progressively motile sperm capable of fertilising is the overriding factor in determining pregnancy rates. For example, a study demonstrated that large insemination volumes with low sperm concentrations resulted in fewer embryos than smaller doses with higher sperm counts [29].

As mentioned earlier, pregnancy rates per cycle using frozen-thawed semen are usually lower than those for cooled or fresh semen [11, 30].

Embryo Transfer (ET)

Embryo Transfer (ET) is a well-established assisted reproduction technique (ART) in horses, which was first described in the 1970s. Although the use of ET in horses came relatively late compared to cattle, this may be due to the inability to induce superovulation in a mare and breed registration authorities' resistance to registering offspring produced with ARTs [1, 17].

This procedure involves the recovery of an embryo from a donor mare's uterus and its subsequent transfer to a recipient mare. One of the significant advantages of ET is its ability to allow breeders to produce multiple foals from a single donor mare during the breeding season [1, 31]. It is particularly beneficial when the donor mare holds significant value, and there is a desire to avoid potential pregnancy complications or health issues, such as lameness, pelvic damage, or disruptions to the mare's competitive career. Mares which are too old, have reproductive problems such as repeated embryonic death, or if they can not carry or produce a healthy foal due to injury, ET may be considered as well [16, 31]. Embryos can also be obtained from two-year-old fillies, which are too immature to carry a foal to term themselves [16, 31, 32].

Nevertheless, the success of this technique in horse breeding depends on several factors, such as the fertility of the donor mare, the quality of the semen used, and the quality and synchrony of the recipient mare.

Early in the history of ET, surgical recovery and transfer of embryos were used. However, non-surgical techniques were developed; nowadays, these are routinely used in ET [17, 32, 33].

It was in 2002 when the American Quarter Horse Association, one of the largest breed organisations in the United States, allowed unlimited registrations of foals from ET. Many other breed societies followed suit, which has elevated ET's use globally [1, 16, 31].

Procedure

For ET to be successfully accomplished, the reproductive cycle of both donor and recipient must be carefully monitored using transrectal ultrasonography. In an ideal scenario, the recipient mares should ovulate on the same day, one day before or up to 3 days after the donor mare ovulates [17, 34]. The recipient mare is as important as the donor; although her genetics will not influence the foal in her uterus, she has to carry and nurture the foal to term and then provide milk and maternal care to the foal once born. Ideally, the recipient mare should be of a

similar height and width as the donor, aged (3-10 years), ideally not a maiden mare but one that has successfully carried a foal to term and had good mammary development [34].

If no synchronised recipient mare is available, other options are to ship the embryo to a centre that maintains large numbers of recipient mares, easing the selection of a synchronised recipient, or the embryo can be cryopreserved and stored in liquid nitrogen for transfer at a later date. If a recipient mare is available, then typically, providing she has a follicle ≥35 mm and uterine oedema, she would be administered human chorionic gonadotropin (hCG) or a gonadotropin-releasing hormone (GnRH) analogue at the time the donor ovulates, so ovulation occurs within 48 h [1, 30, 31, 35, 36]. Using both prostaglandins to lyse the corpus luteum and control the timing of oestrus and ovulatory drugs to control the time of ovulation are essential tools in managing the cycles and ensuring the synchronisation of donor and recipient [30].

Any semen can be used for donor mares and is carried out as described earlier. As the equine embryo does not enter the uterus until around six and a half days after ovulation, it is usual to flush the donor mare's uterus 7 or 8 days after ovulation; day 0 is considered the day of ovulation. Mares inseminated with frozen semen, especially post-ovulation, are usually flushed on Day 8 rather than Day 7. Embryo recovery is commonly done by flushing the mare's uterus with between 750 – 2000 ml of a physiological solution such as Ringer's Lactate, the volume dependent on the size of the mare's uterus. Commercial flushing solutions are also available, and these frequently contain low levels of antibiotics and a surfactant to prevent small embryos from sticking to the tubing. The uterus is filled until distended. The operator will usually manipulate the uterus per rectum to check it is completely filled and to ensure the fluid is agitated to aid embryo recovery. The fluid is then recovered by gravity and passes through an on-line filter; this is usually repeated three times [1, 17, 31, 32]. Oxytocin is often administered at the end of the last flush to help ensure all the fluid is recovered [17].

After recovery, the embryo is washed several times in a Holding medium [35]. The embryo is usually graded [37, 38] and, in some cases, measured before transferring into a synchronised recipient mare [17, 34, 35]. Alternatively, the embryo can be cryopreserved and transferred at a later date [16, 32].

Before the transfer, the embryo has to be loaded from the washing medium into a straw. Usually, a 0.5 ml straw is used [38]. The straw contains three columns of fluid, with air spaces in between. The middle column houses the embryo, while the first column is designated for lubrication, and the third column is for flushing out the embryo [38].

The transfer of a recovered embryo was done using surgical approaches; nowadays, they are not used anymore due to the invasiveness and the invention of more straightforward non-surgical procedures [17, 38]. Some countries even banned the surgical approach due to welfare concerns [38]. Two non-surgical ways are well-described and widely used [17, 38]. One is the transcervical transfer, where the transfer pipette is introduced into the uterus without contamination. For this, the pipette is covered in a sterile plastic sheath and introduced into the cervix; before entering the uterus, the sheath is pulled slowly but firmly backwards. Then, the pipette can be advanced into the uterus, and the embryo can be 'squirted' out of the straw [38]. The alternative technique developed by Wilsher and Allen in 2004 involves a duck-billed speculum to visualise the cervix, along with Wilsher's forceps to grasp the cervix and facilitate the passage of the transfer pipette [17, 36, 38].

The recipient mare is checked for pregnancy 6 – 7 days after the transfer, then early development of the conceptus is checked at regular intervals, e.g. on day 30 and 50 of pregnancy [36].

Success rates

We have to differentiate between the success of recovery and the success of an embryo transfer. For the recovery, the timing of insemination and the fertility of the mare and stallion play a primary role [38]. In a young fertile mare inseminated with fresh semen from a fertile stallion, recovery rates over 70% are suspected [16, 31, 38]. However, this number drops when mares over 14 years of age are used, a history of subfertility is known, and chilled or frozen-thawed semen is used [38].

Successful embryo transfer and subsequent pregnancy depend on several factors, including embryo quality, transfer technique, synchrony between the donor and recipient, and other aspects of recipient suitability. The recipient must be in good overall health and ideally aged between 3 to 12 years. Additionally, she should exhibit normal cyclicity and show no indications of reproductive tract issues [35, 38]. With a suitably well-selected recipient mare, the pregnancy rate is approximately 75% [1, 31, 35].

Oocyte Recovery / Ovum Pick-Up (OPU)

The oocyte recovery procedure was first developed in humans in the early 1980s to recover oocytes for in vitro fertilisation. In the late 1980s, invasive approaches like laparotomy,

colpotomy, and blind flank approach through the paralumbar fossa were initially developed in horses to obtain mature or immature oocytes. Today, oocytes are harvested using transvaginal ultrasound-guided ovum pick-up (OPU) sometimes referred to as transvaginal ultrasound-guided follicle aspiration (TVA) [3, 31, 39, 40].

OPU can be used for mares who suffer from subfertility or infertility and cannot get pregnant or provide an embryo. This may result from abnormalities or disease in the reproductive tract (cervix, uterus, oviduct) [1, 31]. Examples of such issues include an inability to ovulate, especially in older mares, pathological changes within the oviduct, uterine pathologies, cervical or uterine adhesions or urine pooling. In such cases, OPU can sometimes provide a viable solution for obtaining offspring from these mares [41].

In addition, some semen, typically in short supply or from stallions that are deceased is only available for use via ICSI, making OPU the only option if an owner wishes to use that stallion's genetics [4, 42].

Recovered oocytes can be transported to a laboratory and inseminated there via ICSI or other procedures, which leads to a higher yield of embryos which can be transferred later on [3, 31, 42].

Oocytes can be harvested *in vivo* via the aspiration of dominant, stimulated follicles or immature follicles. Pre-ovulatory or mature oocytes can be useful for procedures such as GIFT. However, it is more common to aspirate numerous immature follicles using OPU and then mature the collected oocytes. This method allows for the collection of more oocytes, providing a greater chance of obtaining a pregnancy. The procedure can be repeated (in some cases, biweekly) as new follicle waves occur [1, 31].

Ovaries obtained via post-mortem can be dissected, and the follicles scraped and flushed extensively to cause detachment of the cumulus-oocyte complexes (COCs), which in the horse, unlike the cow, are tightly bound to the follicle wall [3, 31]. Recovery of oocytes from post-mortem ovaries allows genetic salvage from mares that have to be euthanised or die unexpectedly.

Oocytes retrieved from both OPU or at post-mortem are matured in vitro and then used for intracytoplasmic sperm injection (ICSI). In vitro fertilisation (IVF) has been achieved in the horse but the technique is currently not as reliable as ICSI, at the moment it is being offered by a handful of laboratories worldwide [43].

Procedure

Prior to the OPU, the mare is checked with an ultrasound to assess the follicular development in her ovaries. For ICSI, large numbers of smaller follicles yield the best results [31, 32, 36]. The standard OPU procedure is done with the mare restrained in stocks under sedation and, depending on the preference of the collector, with epidural anaesthesia [36, 44]. Antispasmodics (e.g. hyoscine butylbromide) are frequently given as well to relax the uterus and allow better manipulation of the reproductive tract [45].

The OPU system consists of three primary components: the ultrasound scanner, an aspiration pump, and a needle guidance system linked to a specialized collecting tube for oocytes. This ultrasound setup requires a specific transducer unit that incorporates the ultrasound probe and the needle guide. This feature allows the operator to visualize the needle on the ultrasound monitor and improves needle manipulation accuracy. A vacuum pump connects to the needle via silicone or Teflon tubing, with a collection device positioned in between to capture the oocytes and follicular fluid, typically an embryo filter [45, 46]. Follicles are typically punctured with a 12G double-lumen needle [1, 3, 31, 36, 45]. The double lumen needle's introduction in 1998 significantly enhanced the procedure, made it available for horses and boosted the recovery rate of immature follicles to over 50% [39, 45]. The inner stylet connects to a vacuum pump, with the collecting vessel situated between them. The outer needle serves to introduce the flushing medium after the initial follicular fluid has been extracted. This double lumen design significantly lowers the risk of an oocyte being left in the needle’s dead space, which could otherwise lead to it being repeatedly flushed back into the follicle[45].

Typically, three professionals are needed to handle the system. The first person needs to manipulate the ovaries through the rectum to position them more caudally over the broad ligament, close to the vaginal wall against the ultrasound probe [31, 46]. The ovarian ligament is placed between the handler’s fingers to prevent the needle from hitting important vessels or intestinal segments. One of the other two personnel handles the needle, piercing through the vaginal wall and ovary to access the follicle, as well as rotating the needle to release the COC from the wall of the aspirated follicle. The third operator is responsible for flushing the aspirated follicles, with each follicle flushed and scraped 8-10 times, using flushing media containing 5 IU/mL of heparin to inhibit clotting of the follicular fluid and blood [46, 47]. It is usual to record the number of aspirated follicle on each ovary [31, 32, 36, 46].

The recovered fluid, with the potential oocytes, is usually bloody from the scraping and is filtered with an embryo filter and searched for oocytes [36, 46]. Any recovered oocytes are washed, typically in a commercial media (e.g. Hepes Synthetic Oviductal Fluid), and when ready to start maturation they are moved into maturation media and placed in an incubator [36]. After the procedure anti-inflammatory drugs to lessen the discomfort and antibiotics to prevent bacterial infection are usually administered. Monitoring every two hours on the day of the OPU procedure and twice a day the following days, at least three, is important. The ideal for a mare would be two days of rest and at least 7 to 10 days for sport mares [46].

In some instances, a pre-ovulatory follicle, sometimes called a dominant stimulated follicle (DSF) is aspirated. If using OPU their aspiration offers several advantages and disadvantages compared to immature follicles. Their larger size makes the follicle easier to aspirate for inexperienced practitioners and the oocyte recovery rate is higher as the cumulus-oocyte complex (COC) from the follicle wall is more loosely attached to the follicle wall as the oocyte approaches maturity via gonadotropin stimulation. However, precise timing following hCG/GnRH treatment is required for undertaking the OPU procedure to ensure the oocyte is reaching maturity but has not yet ovulated. In addition, for subsequent utilization of the recovered oocyte, gonadotropin stimulation must be aligned with the processing laboratory, as this process initiates meiotic resumption [31, 48]. Oocytes recovered from pre-ovulatory follicles are also much more sensitive to temperature fluctuations than immature ones and, hence, must be handled with great care [1]. Perhaps the major disadvantage is that it will usually only be one follicle, and therefore one oocyte recovered at each OPU session as mares do not respond well to superovulation protocols [1, 43].

In contrast, the aspiration of immature follicles can be done biweekly without checking the ovarian activity between aspirations [48]. Alternatively, the donor mare can be observed via ultrasound to identify the best time for aspiration, ideally when there are enough small follicles present and no large preovulatory follicle exists. Follicle sizes should be below 1 cm in diameter, and greater number of follicles on the ovaries usually result in a higher number of oocytes recovered.Most practitioners aim for 5-12 immature follicles to be present on the ovaries [31, 32, 36]. This procedure can be conducted throughout the year, although follicles generally tend to be smaller during the non-breeding season [31, 36].

The benefits of immature follicles include their ability to be stored at room temperature and maintained overnight in a basic holding medium, allowing for flexibility in scheduling tasks.
The disadvantages include increased difficulty puncturing and aspirating the follicles due to their small size and the non-expanded cumulus around the oocytes, which causes the COCs to still be tightly adhered to the follicle wall. As a result, it requires scraping the wall and repeated filling and emptying of the follicle. Since the oocytes are immature, they must undergo in vitro maturation (IVM). Not all of these oocytes will mature, resulting in a lower number of fertilizable oocytes [31].

Success rates

In 2021, Claes and Stout conducted 515 OPU sessions, puncturing an average of 25.9 antral follicles. Each mare yielded an average of 13.8 oocytes, leading to an average oocyte recovery of 53%. The donor's age is shown to be an essential indicator of the collection's success [49]. Similar numbers can be reviewed in [50, 51].

In Vitro Maturation (IVM)

If we recover oocytes from immature follicles, we consequently need to mature them for further use.
If the oocytes are recovered at a different location to the laboratory they need to shipped. Immature oocytes are shipped in a H-SOF medium in a container which can maintain the temperature from 18-22°C [36], according to [32] temperatures from 15-18°C are enough for shipping. However when collected from dominant follicles the temperature needs to be higher at around 37°C to avoid damage of the oocyte [32].
For the successful fertilisation the oocyte needs to reach metaphase II [32].

Procedure

The maturation is done within incubators, maintaining a temperature of 38°C and a controlled atmosphere featuring 5-6% CO2 and 5% O2 to minimise superoxide radical formation. After the oocyte culture period, the cumulus-oocyte complex is denuded through repeated pipetting

with enzymes that facilitate the breakdown of the dense intracellular matrix, making the polar body visible and enabling ICSI to be performed [31, 36, 46, 52].
The duration required to reach MII varies among cumulus types. Oocytes with compact cumuli that do mature take about 30–36 hours to reach MII, while those with expanded cumuli need a shorter in vitro maturation time of 22–24 hours [31, 32, 39, 46, 53]. When pre-ovulatory oocytes are extracted via OPU 24 hours post-gonadotropin treatment, most of the maturation process is already completed, requiring only approximately 12 hours of culture for the oocytes to mature before ICSI, with 91% achieving metaphase II [39, 46, 53].

Success rates

Primary factors influencing the developmental potential of equine oocytes include mare-specific aspects like age, stage of follicle development, oocyte storage conditions, and the customisation of the in vitro maturation protocol to align with these criteria. Nuclear maturation rates of 60–80% are anticipated [31, 39, 49].

In Vitro Fertilisation (IVF)

This technique describes the co-incubation of the female and male gametes (incubation of oocyte with sperm for fertilisation) [1, 47].
The use of in vitro fertilisation (IVF) is very successful in a lot of other species, especially cattle and humans. Through recent improvements, better outcomes of IVF can be seen, but only if fresh semen is used. Therefore, the clinical practical use is limited. There are still processes not fully understood, such as sperm capacitation in horses. Prolonged culture in capacitation media before putting in the COC shows success [25]. The sperm can not penetrate the zona pellucida of the in vitro matured oocyte easily due to the hardening of zona pellucida; this withdrawal, either partial or total, of the zona pellucida results in polyspermy [54].

Procedure

An oocyte has to be recovered via OPU or from aviaries post-mortem. The oocytes need to be matured, and when mature, a droplet of sperm is added for sperm-oocyte co-incubation [55].

A key barrier to creating an effective IVF protocol for horses is the limited knowledge about sperm capacitation in this species. The duration of equine sperm capacitation in vivo remains unknown; therefore, more research needs to be done [55]. ICSI was developed to perform fertilisation of oocytes in vivo [56].

Success rates

So far, only two foals have been born from IVF in horses [16, 25, 32].
When high-quality semen is used, live foals can be successfully achieved; using frozen semen, the results of IVF lead only to the cleavage stage zygotes to 16 cells [25].
A fertilisation rate of 50% was described in 2009 after chemically induced hyperactivity of the sperm [57].
Recently, "the first successful production of IVF-derived blastocyst in horses using frozen semen following prolonged sperm capacitation" was reported [25].

Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic sperm injection (ICSI) is a method for in vitro fertilisation in which one sperm is injected into the cytoplasm of a mature oocyte to achieve fertilisation [53]. Nowadays, it is one of the most commonly used techniques for in vitro-produced (IVP) embryos in the field of equine reproduction [25].
The development of transvaginal OPU techniques for mares, along with improvements in IVM efficiency and culture media, has increased the adoption of ICSI among breeders recently. Current statistics from equine breeding programs show that around 60% of OPU-ICSI sessions result in at least one blastocyst, with an average yield of 1.8 blastocysts per successful OPU-ICSI procedure [58]. Nevertheless, there remains considerable variation in blastocyst formation rates across various ICSI laboratories, affected by factors like stallion fertility, mare age, and oocyte quality. Still, the blastocysts formed typically demonstrate acceptable pregnancy rates when transferred to recipient mares, with success rates reported between 50% and 80% [59].
Nowadays, ICSI is a common procedure for both infertile mares and stallions. In the beginning, the procedure was used for mares, which were not able to provide embryos via ET anymore. Nowadays, it is used to overcome stallion infertility problems as well, such as old stallions that have a lower sperm count, have lower quality semen or have died, and frozen semen is still

available. The thawed semen can be diluted and refrozen, which can extend the availability of semen [32, 51].

A significant advantage is that only a single sperm cell is needed for each oocyte [14].

Especially in Europe, the procedure is used as an alternative to ET. The number of ICSI stations is limited but exists in at least 13 countries throughout the world [16, 32]. Increasing numbers in such stations are not expected due to the high equipment costs and a shortage of trained personnel in this procedure [16]. Therefore, the oocyte and semen often have to be shipped to such facilities [32].

For some mares, especially if they have not had a foal and their ovarian ligaments are very tight, the OPU procedure can be uncomfortable. Some countries are beginning to question the ethics of undertaking the procedure. For example, starting from March 1, 2025, the Swedish Warmblood Association (SWB) will prohibit the registration of foals conceived through the OPU-ICSI-ET method [60, 61].

Procedure

The ICSI procedure requires specialized equipment, including an inverted microscope and paired micromanipulators, enabling precise movements. Two arms are essential: one equipped with a holding pipette and the other with an injection pipette. Sometimes, the injection pipette is connected to a piezo system (piezoelectric movements) [31, 59].

Spermatozoa used for injection into the oocyte can be either fresh or thawed after cryopreserved [46]. The sperm cells needto be immobilised so they can be aspirated into the injection pipette for injection into the oocyte. The semen is washed and often techniques are used (e.g. swim-up) to select the most motile sperm prior to a small quantity being placed in a polyvinylpyrrolidone (PVP) medium to reduce its motility so it can be aspirated with the micromanipulator [31, 46]. Prior to aspiration into the injection pipette the sperm tail is disrupted with the edge of the pipette to render it immobile and ensure it will be capable of fertilising the oocyte [31]. The oocyte is held with the holding pipette and positioned so the With the oocyte has the polar body at either 6 or 12 o’clock. From the opposite side, the aspirated and immobilised sperm can be injected into the oocyte [31, 59].

After injection the oocyte is placed in culture medium within an incubator for fertilisation [46].

Success rates

In 2018 [31] , reported success rates of a pregnancy post-transfer to a recipient mare of at least 65%, and a foaling rate per pregnancy of 80% or more.

Of the 515 OPU-ICSI procedures performed in [49], 78% yielded one or more blastocysts, resulting in a mean of 2.12 embryos per session. The blastocyst rate stands at 23% per injected oocyte derived from immature follicles, and at 38% for those from dominant stimulated follicles [53]. In contrast, embryo flushing following artificial insemination usually yields an embryo recovery rate of 50-65%, and it is difficult for the number of recovered embryos to surpass 2 [49]. The pregnancy rate is above 70%; approximately 15% of pregnancies are lost between one week after embryo transfer and foaling. Nearly 99% of the foals were born healthy and survived [49].

Data from [36] taking only transfers done in Avantea into account show an average success rate of "70.9% ±1.9 at 17 days, 63.2% ±1.8 at 30 days, and 58.6% ±1.3 at 50 days of gestation."

Various factors, including the donor mare, recipient mare, stallion, and embryo, affect the chances of generating an in vitro blastocyst or successful pregnancy [36, 49].

It can be said that pregnancy rates after transfer are better if the oocyte is from a young mare instead of an older one. Significantly more oocytes from young (6-10 years) than old (20-26 years) mares develop into an embryonic vesicle when transferred into a young (3-7 years) recipient. [62]

Oocyte Transfer

McKinnon was the first to describe a living foal from this procedure. Nevertheless, it was not a sufficient procedure as only one foal was born from two established pregnancies out of 15 transferred oocytes into inseminated recipients [63].
It was not commercially used until the 1990s, and since then, it has proven to be useful in obtaining offspring from mares with reproductive problems, such as ovulatory failure, uterine infections and other problems preventing them from a successful pregnancy [41].
But with the development of a less invasive procedure such as ICSI, there was no longer a need for oocyte transfer, and therefore, the use declined immensely [16].

Procedure

In this procedure, a mature oocyte is transferred into the oviduct of an inseminated recipient via a flank incision. Both the recipient and the donor mare should be synchronised, and the follicles should be receptive to gonadotropins, which are administered to them at approximately the same time. 24 to 35 hours after administration, the follicle of the donor mare is aspirated to ascertain that an oocyte is available for transfer; if so, the recipient mare follicle is also aspirated to ensure this will not be fertilised [56]. The oocyte need to be in metaphase II [31]. The transfer is done via a standing flank incision; the ovary is brought into the incision, and the oocyte is placed in the ampulla of the oviduct. The recipient is typically inseminated 12 hours before or 2 hours after the transfer, but a full dose of good-quality semen is required. [31, 41].

Success rates

Success rates for clinical oocyte transfer indicate a 77% recovery from donors and a 38% pregnancy rate [56]. In research studies, the pregnancy rates are higher, over 70%, than in clinical cases [31]. Oocytes from older mares produce lower pregnancy rates then oocytes from younger mares [56].

Gamete Intrafallopian Transfer (GIFT)

This procedure was introduced for the same reason as the oocyte transfer, as an alternative for ET in mares which show problems with ovulation, produce an embryo, maintain pregnancy or other reasons. GIFT can also be used as a solution for stallions which show a fertility problem or have limited availability of semen. As the oocyte and the semen are placed together in the oviduct of a recipient directly where the fertilisation takes place, a lower amount of sperm can be used, unlike in oocyte transfer. Since the first successful gamete intrafallopian transfer was performed in 1998, the procedure has been attempted with fresh, chilled and frozen semen [32, 41].

Procedure

In contrast to oocyte transfer, in gamete intrafallopian transfer (GIFT), both the sperm and the oocyte are transferred into the oviduct of the recipient mare [32, 41]. The procedure is prepared the same way as OT [56]. Once the recovered oocyte reaches metaphase II [17], it can be transferred to the donor similarly to OT, through the flank incision [56]. The oviduct is exposed surgically, he oviductal opening is located, and the oocyte is inserted into the ampulla [64]. However, other than in OT, the semen is placed into the oviduct for better fertilisation [41].

Success rates

In research studies, the procedure shows a pregnancy rate of 82% with fresh, non-extended semen [56]. But depending on the semen and the oocyte used, rates range between 27% and 82% [41].

Embryo Cryopreservation

The cryopreservation procedure is crucial for optimising ART protocols and their efficiency. For successful embryo cryopreservation, ice crystal formation and a prolonged display of toxic cryoprotectants must be prevented, while the normal cellular functions and viability of the embryo or oocyte must be maintained. In horses, cryopreservation of embryos is commonly used for in vitro-derived embryos, but it is still inefficient for in vivo embryos.
In the mid-1980s, the first living foal out of a cryopreserved embryo was reported.
Various protocols have been proposed using different types and concentrations of cryoprotectants, cooling rates, equilibration times, and cryopreservation devices [16, 32].

Procedure

Two major processes are described: slow cooling and vitrification.
Slow cooling in horses was uncommon due to its high costs. A programmable freezer was needed, and only one embryo was recovered at a time. There were also low success rates.
However, with the increased success of in vitro-produced embryos, the freezer can be justified, as several embryos can be frozen at a time.
The slow freezing procedure tends to be more forgiving of freezing mistakes than the vitrification. Nevertheless, vitrification is still used more commonly than slow freezing.
Vitrification is an ultrarapid cooling technique that allows cells to solidify into a glass-like state without forming ice crystals. The solution for this procedure contains a high amount of cryoprotectants. However, the embryo can be exposed to the toxic cryoprotectant only for a short time, and it should not be diluted excessively. Over the last ten years, it has been increasingly used in horse reproduction.
The size of the equine embryos has a major effect on the procedure's success. This is due to the large blastocoel and glycoprotein capsule, which reduces the penetration of cryoprotectants. In vitro-produced embryos tend to be smaller than those derived in vivo [16, 32].

Success rates

Embryos smaller than 300 μm freeze effectively and achieve a pregnancy rate of 50-60%. In contrast, larger embryos over 300 μm have poorer pregnancy rates of 20-30%. The cooling method used, whether slow cooling or vitrification, does not influence these outcomes. As a result, the majority of embryos are still transferred directly [16, 32, 65].

Using micromanipulators to penetrate through the embryo's wall aids in fluid aspiration, promoting its collapse. The aim is to remove nearly 90% of the blastocoels fluid. This technique shows improvement in post-thawing pregnancy rates from embryos which were initially bigger than 300 μm. The success is similar to embryos smaller than 300 μm. Nevertheless, this micromanipulator is prohibitively costly and complex for common use in the ET industry. As a result, embryos must be sent to facilities equipped with a micromanipulator and subsequently returned to the station with the recipient mare [16, 32].

Recently, a procedure where the blastocoel puncture is done manually without a micromanipulator was described [16].

Cloning and Somatic Cell Nuclear Transfer (SCNT)

Cloning is one of the most controversial techniques developed in recent years in the equine ART [66]. The first cloned equids were described in 2003. Many laboratories can use this technique [1, 3, 4, 17, 31, 39, 47, 66], and companies also provide clients with commercial cloning [66]. Cloning gained commercial interest after the Fédération Equestre Internationale (FEI) permitted clones to compete in sports in 2012, lifting their ban from 2007. This was when various stud books worldwide began permitting registrations of clones [67].

Cloning in horses is used for several purposes, primarily focused on improving breeding programmes, preserving genetics, and advancing scientific research.

A primary reason for cloning is to replicate exceptional horses with desired traits like speed, agility, and temperament, which are invaluable in competitive sports such as racing and show jumping. As geldings take part in competitions and win the major events as well, it would be logical to select such for breeding the next generation of champions if they were still entire. Therefore, reviving these geldings with functional testicles and leaving them as stallions solely for breeding purposes is a viable option for mating with such champions after all [17].

If a tissue sample is available, animals that have already performed outstandingly but are unfortunately no longer alive can be given another chance for breeding [4, 31].

It is certain that a cloned horse will not be an exact replica of the donor [4, 66].

This technology helps conserve endangered or rare horse breeds by preserving genetic diversity when natural breeding alone is inadequate [4].

Additionally, cloning contributes to the progress of genetic research by offering valuable insights into the impact of specific genes on performance health [3, 4, 17, 31, 39].

The technique used for cloning nowadays is called Somatic Cell Nuclear Transfer (SCNT) or, briefly, Nuclear Transfer (NT). In this technique, an enucleated oocyte from a mare that has reached metaphase II is combined with the somatic cell from the donor. The reconstructed embryo is then activated, and the embryo is transferred into a recipient [1, 3, 4, 17, 31, 39, 47, 66].

Procedure

To carry out nuclear transfer (NT), oocytes (the genetics of the oocyte donor are not relevant) are either matured in vitro or collected post-maturation in vivo [4, 68]. For in vitro matured oocytes, abattoir-derived or OPU-derived can be used [69]. Through micromanipulation, the cytoplasm area that holds the oocyte's chromatin is excised, resulting in an enucleated oocyte, also known as a cytoplast. Somatic cells from the genetic donor are cultivated in vitro from a tissue sample, such as a skin biopsy. A single somatic cell from the donor is chosen and fused with the cytoplasm. This can be done either by applying an electric pulse to merge the two cells or by piercing the donor cell membrane and injecting the donor cell directly into the cytoplasm of the oocyte. The recombined oocyte, now equipped with the donor's nucleus, is then artificially activated. As a result, the transferred nucleus decondenses, prompting the oocyte to begin embryonic development [4, 31, 68].

Success rates

The success rates vary drastically between laboratories [70] and the type of oocyte source [69]. The cloning procedure became a commercially viable option, and hundreds of equine clones worldwide were born [70]. There are differences between oocytes, which are abattoir-derived and OPU-derived. Pregnancy rates at day 14 were higher in the abattoir group; on day 42, they were nearly the same, but on day 90, a drop in p could be seen [69]. Pregnancy losses tend to occur in early gestation before day 50, but if the foal survives this stage [3]. The number of foals born live is higher in the OPU-derived group this group tend to have healthier foals post-partum as well [69]. Complications can be enlarged umbilical cord, limb deformities or other malformations [31, 69].

Conclusion

Assisted reproductive technologies (ART) have revolutionised equine reproduction by addressing fertility challenges, preserving genetic diversity, and enabling broader access to valuable bloodlines. Techniques such as artificial insemination (AI), embryo transfer (ET), and intracytoplasmic sperm injection (ICSI) have not only improved reproductive efficiency but also expanded the boundaries of equine breeding by overcoming species-specific challenges.

While established methods like AI and ET remain central to equine breeding programmes, advancements in oocyte recovery, in vitro maturation, and ICSI highlight the potential for infertility issues. Techniques such as cloning offer unique solutions for genetic preservation, though ethical considerations and high costs accompany them.

Despite their transformative impact, ART in horses faces challenges such as variable success rates, high economic costs, and regulatory and ethical constraints. Future developments in these technologies, combined with ongoing research, hold promise for optimising breeding strategies and improving the accessibility and efficiency of these methods.

In conclusion, ART continues to be a foundation of modern equine reproduction, enhancing the potential for genetic advancement and the sustainability of equine breeding. With further refinement and broader acceptance, these techniques are set to play an even more integral role in the future of equine industry practices.

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