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Embryo Transfer in Horses
- Method, Possibilities and Limitations

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Introduction

The horse breeding industry is dominated by stallions. They can produce hundreds of offspring in one season, and still be used for competition. A high-level performance mare on the other hand, would have to take a long time out of training and competition, to be able to produce a foal. Therefore, mares that are no longer used for competition because of injury or low performance will often be used as broodmares instead. This is handy and economical for the owner, but this mare might pass on undesirable traits to her foal. Embryo transfer (ET) makes it possible to choose a top stallion and a top mare, producing a valuable foal with minimal disturbance to the sports career of the biological parents. More often than not, a horse in intensive training will get some kind of injury during its lifetime. Could embryo transfer be a solution to breed sports horses with improved physical soundness? When I started my veterinary studies and learned more about embryo transfer, I decided to write my thesis about this topic. I would like to write a detailed manual for embryo transfer and discuss possibilities and limitations. I would also like to find out the situation in Norway and why it is not more common here. During my 10th and 11th semester at Szent Istvan Veterinary University I visited Dr. Juliane Kuhl the University of Veterinary Medicine of Vienna, Austria and Dr. Joanne Smith at the Weatherford equine clinic in Emarald, Queensland, Australia to learn more. This thesis is based on scientific articles and books on the subject as well as personal communications.

History

Horse breeders have always been looking for ways to improve their breeding. Although there are legends about artificial insemination (AI) of horses in 1300, the first known equine AI was done by Sir Walter Heape in 1898.[6] Embryo transfer, however, has had a slower development. The first successful embryo transfer in animals happened more than a 100 years ago in rabbits, again by Sir Walter Heape. In 1972, the first reports on equine embryo transfer were published. It was done with surgical method and was between horses and donkeys. In 1974 the first horse ET offspring were successfully produced. This was quickly followed by the first non-surgical horse ET offspring. However, ET was not available commercially until 1983.[1]

In Argentina, embryo transfer has been very popular in polo ponies since the 1990s. During the last decade, it has become more and more popular in Europe and America. Especially in

Quarter horses after the change in rules of registration from the American Quarter horse association, allowing registration of more than one foal per mare per year.[6] The last 30 years, a lot has happened in the area of equine reproduction, but the development has been a lot slower than in other species.[1]

The advantages of embryo transfer

An obvious reason for using embryo transfer is the possibility of breeding high performance mares, with minimal disturbance to her training. Not only can a valuable mare be bred and still compete, she can also produce more than one offspring per season.[12] Up to eight foals can be obtained from a mare during one season with embryo transfer.[28]

Mares that for some reason cannot carry a foal to term may still be good candidates for embryo transfer. Her infertility can be caused by problems with the genital tract, like endometrial periglandular fibrosis, cystic glandular distension, endometrial cysts or cervical problems. It is, however, important to exclude problems from the ovum or embryo itself being the cause of inability to maintain pregnancy.[13] Other problem mares, like mares suffering from musculoskeletal disease or a systemic disease, making her inappropriate to carry a pregnancy, can also be good candidates.[12]

Mares at an age inappropriate for carrying a pregnancy may also be used as embryo donors.[12] Mares reach puberty around 1 year of age, but most will not be able to carry a foal to term at this stage. Usually they are not bred before the age of 3 years. To be an embryo donor, the mare must be able to keep an embryo till day 6 or 7 for embryo flushing. Research shows that two years old mares can produce a viable embryo, and this has also been tested in one-year-old fillies with promising results.[22] Also, older mares at risk can be good embryo donors, as long as they can provide a healthy 7 day old embryo.[12]

Another advantage is the preservation of genetic material in endangered domestic horse breeds or wild equids. Several members of the genus Equus are now endangered or even extinct, like the tarpan and the quagga. The process has been much faster than it would be if it were only caused by natural evolution. ET has been used in Prezewalski's horse, zebras and Poitou donkey for species preservation.[15]
Embryo transfer can also be used to assess fertility, both of mares and stallions. It is especially useful in testing semen fertility after processing, for example freezing.[13]

An overview of seasonality and physiological oestrus cycle of the mare

To understand the process of embryo transfer, it is important to have a good general understanding of equine obstetrics and reproduction. Horses generally reach puberty at the age of 12-18 months, depending on season. Mares keep cycling their whole life, but fertility decreases.[11] 3-10 years of age are considered to be top fertility age in mares.[12]

Seasonality

Horses are seasonal breeders, and go into oestrus when the days get longer. Mares have approximately 11 months gestation so going into oestrus in the spring will give spring foals.[11] This seasonal pattern has evolved through time, since foals born in the spring has the greatest chances of survival in the wild.[12] June has been said to be the best month for breeding in the northern hemisphere.[13] The seasonality is controlled by melatonin secretion by the pineal gland. Melatonin is released in response to darkness and suppresses the synthesis and release of gonadotropin releasing hormone (GnRH). Opioids are also thought to play a role in the suppression of GnRH, but more research is needed on this topic.[13]

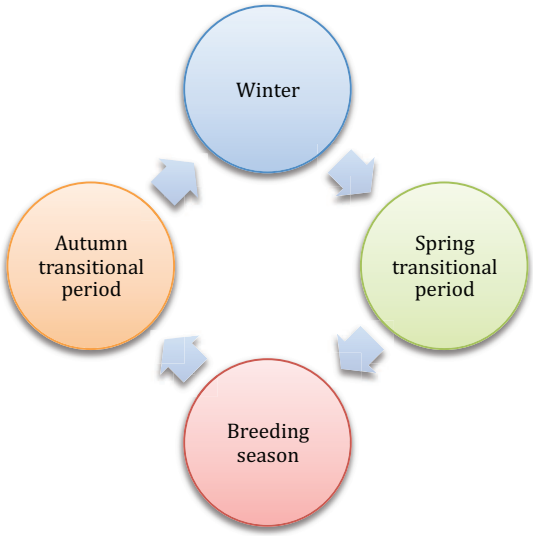

Figure 1: Annual reproduction rhythm

The annual reproduction rhythm can be divided into four phases: anoestrus, spring transitional period, physiological breeding season and autumn transitional period (Fig. 1).

During anoestrus most mares cannot reproduce. It occurs during the shortest days in the winter, when melatonin production is high, and gonadotropin releasing hormone (GnRH) concentration is low.[12] In the northern hemisphere, this is from November through January,

while in the Southern hemisphere from May till July.[24] If examined by transrectal ultrasound, anoestrus ovaries are not active and few follicles are bigger than 5mm. Some mares (20-25%) can cycle all year round, especially in areas closer to equator and more often seen in Arabian mares.[24] At Weatherford Equine, Australia, up to 60% of the mares cycle year round due to the warm climate. However foals must be born after the 1st of August to be registered as foals born that year. Therefore, breeding season is started at the 1st of September.

With increasing day length comes spring transitional period and the follicles starts to grow.[12] This will occur from February till mid-April in the northern hemisphere, and in the southern hemisphere from August till mid-September. This period is a source of frustration for many breeders because large follicle can often be seen, but they do not ovulate. Also, oestrus signs may be seen, which may fool an inexperienced owner. These mares may be bred with no following pregnancy. Breeding in this period should be avoided, since excessive breeding may lead to endometritis.[24] To avoid being tricked by these spring transitional follicles, their growth rate can be monitored. They have a much slower growth (1-2mm per day) compared to follicles that ovulate (10mm per day). The circulating oestrogen is also lower during spring transition period. An increase will be seen 5 to 6 days before the first ovulation of the season.

The breeding season starts with the first ovulation. It is usually in the beginning of April in horses and a bit later in ponies in the northern hemisphere. Once the breeding season has started, regular polyoestrus cycles will occur until the days get shorter again, and the autumn transition period begins.[12]

The oestrus cycle of the mare

One oestrus cycle has an average length of 21 days[13] with 5 to 7 days of oestrus. The length of the oestrus cycle may vary in different mares and is often longer in ponies than in horses, and also in lactating mares. During each oestrus cycle, one ovum is usually released. The double ovulation rate is low, ranging between 7% and 35%.[11]

The oestrus cycle is divided into follicular phase and luteal phase. The follicular phase is the oestrus. During this time, the mare accepts the stallion and the genital tract is ready to accept the sperm and transport it to the oviducts for fertilization.[13] The dominating hormone in the follicular phase is the oestrogen, produced by the growing follicle. Increasing oestrogen levels leads to relaxation and softening of the cervix, oedema of the uterus, increased fluid

production and secretions, growth of the primary follicle and usually oestrus behaviour. Oestrogen peaks around 24-26 hours before ovulation. Oxytocin leads to an increase uterine and oviduct contraction, helping the transport of the ovum and sperm so they will meet, and helps uterine clearance after breeding. Oxytocin release is stimulated by sexual arousal in the mare.[24] Ovulation happens 1 to 2 days before the end of oestrus. The ovulating follicle is at this stage between 30 and 70mm. The size is usually between 40 and 45mm, but often larger in the beginning of the breeding season. Ovulation takes 2 to 7 minutes and corpus haemorrhagicum is formed.[13] The corpus haemorrhagicum develops into corpus luteum due to blood clotting and organization. Intraluteal cavity may or may not be prestent, and has no effect on pregnancy rate. The ratio of luteal to non-luteal tissue increases gradually and is greatest at the middle of dioestrus.[15]

After ovulation and the formation of the corpus luteum, the mare goes in to luteal phase, also known as dioestrus. At this point, the mare is non receptive to the stallion and the genital tract is ready to recognize potential pregnancies. This phase is longer in the beginning of the breeding season.[13] The main hormone of the luteal phase is progesterone, produced by corpus luteum. Its concentration increases rapidly after ovulation, and peaks after 48 hours ovulation. Measuring its levels can help confirming ovulation. Progesterone leads to contraction of the cervix, induces proliferative changes of the uterine endothelium and endometrial glands and increases the viscosity of the vaginal secretions.[24] Luteal phase ends in regression of corpus luteum induced by prostaglandin 2 alpha (PGF2α), 13 to 16 days after ovulation. PGF2α is released by the endometrium and reaches the ovary by systemic route. This causes luteolysis and a decrease in progesterone. When the progesterone levels decrease, the block on lutenizing hormone (LH) is released. The follicles grow and mature and a new follicular phase begins.[13]

Oestrus detection in mares

In embryo transfer, the oestrus detection is very important to control the synchronization of the mares.[13] Early and accurate oestrus detection will give better pregnancy rates since the mare is bred at the optimal time.[24]

Oestrus can be detected by looking for oestrus behavioural signs. During oestrus, the mare will be receptive to the stallion. If in contact with a stallion, the mare will lift the tail, squat

and tip the pelvis, urinate and evert clitoris. If she is not in oestrus, she will switch tail, kick, try to bite the stallion and move away from him. It is important to remember that there will be differences from mare to mare.[13] Some mares might not show clear oestrus behaviour, especially maiden mares or mares with a foal by her side. Using a teaser stallion or testosterone implanted gelding can provoke oestrus behaviour.[24]

The cervix can be observed using a vaginoscope or be examined by vaginal palpation. Be aware that using a vaginoscope is semi-invasive and may lead to vaginal inflammation![24] During oestrus the cervix is pink, short, wide and relaxed with soft texture. When using speculum, you can see pink and drooping vaginal floor.[13] Thin mucoid secretion is typical, and the mucosal surfaces are shiny.[24] By palpation, the cervical lumen is open and 1 to 3 fingers can be pushed through.[13] During dioestrus, the cervix is long, pale and narrow with firm texture. Using the speculum, you can see the cervix pale and dry located cranially in the vagina. The lumen is closed.[13] During anoestrus, the vaginal mucosa will almost be white and the cervix may be open. If the mare is pregnant, the cervix is tightly closed, very pale and covered by a mucous plug. The vaginal mucosa is dry. As parturition approaches, the vaginal mucosa is covered by sticky mucous and the cervix is not as obvious as before.[24]

The uterus can be examined by rectal examination and transrectal ultrasound. During oestrus it is relaxed with soft texture and you can see oedematous endometrial folds with ultrasound.[13] The image on the ultrasound machine will resemble a cartwheel. This "cartwheel" will be visible up until 24 hours before ovulation, giving a good indication on when to inseminate.[24] During dioestrus, you can feel a firm tone by transrectal palpation and the echogenicity is uniform on ultrasound.[13] When examining the uterus by ultrasound, also notice any pathological changes, like fluid in the uterus or uterine cysts.[24]

The ovaries can be palpated transrectally or visualized by ultrasound. During oestrus, large follicles can be seen by ultrasound and they may feel soft on palpation. On the ultrasound you can see and measure the follicles and as ovulation approaches, the preovulatory follicle may become triangular with scalloped edges. During dioestrus, corpus luteum can bee seen and follicles varying in size. Day 0 of the oestrus cycle is determined by the first day a corpus haemorrhagicum can be seen on the ovary with transrectal ultrasound.[13]

A blood sample showing low levels of progesterone (<1ng/ml) also indicates oestrus.[24]

Embryo transfer method

Embryo transfer means collecting a fertilized embryo from a donor mare and transferring it to a recipient mare that will carry the foal to term (Fig. 2). The owner must be prepared for high economical costs and be aware that it is not always successful.[13] The most important points for success is the fertility of the donor and recipient mares, the fertility of the stallion and the experience and technical skill of the veterinarian. Sterile conditions should be maintained through the entire procedure. Contamination can come from the environment or the embryo.[26]

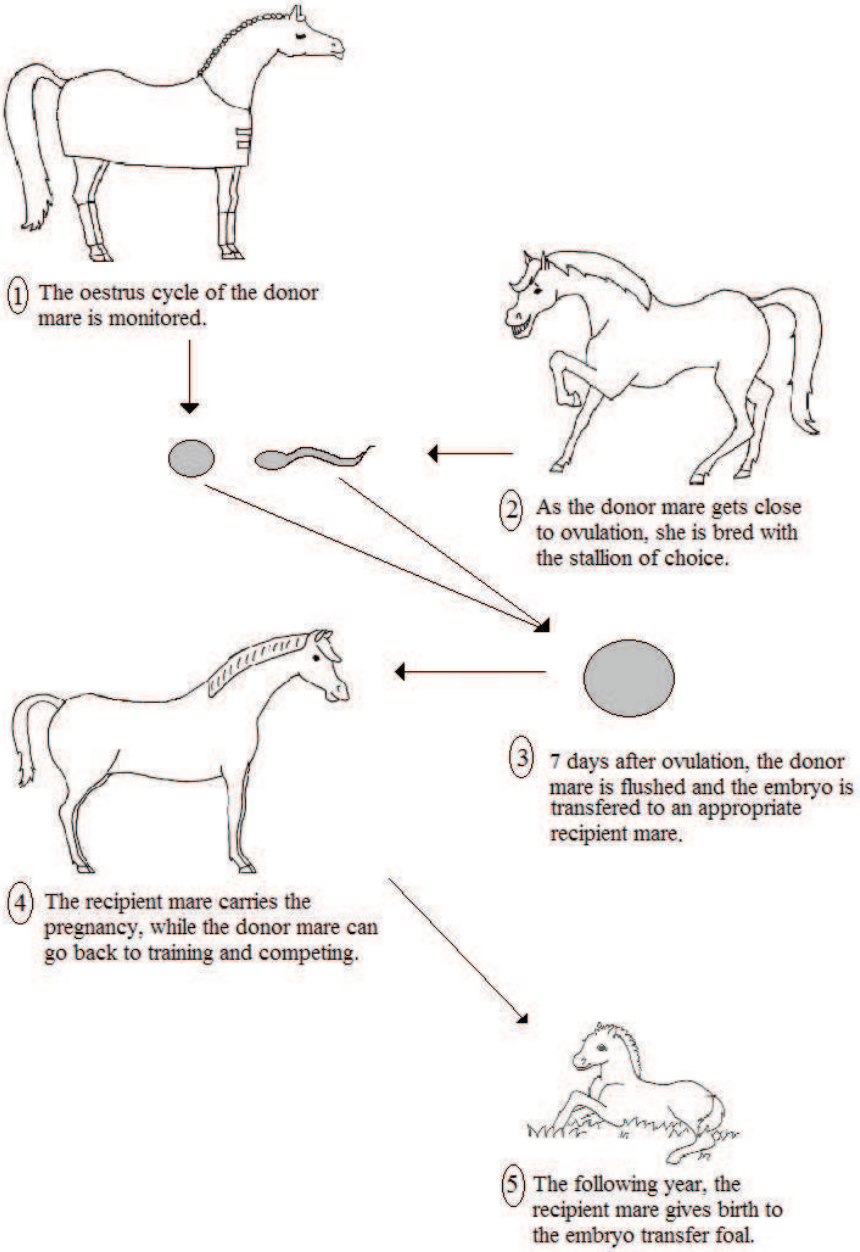

Figure 2: Illustration of the basics of embryo transfer

The recipient mare

Most veterinarians in embryo transfer programs considers the selection and management of the recipient mares to be the most important step for a successful program.[27, 28] To have a large number of recipient mares available will make the synchronization easier and a donor can be matched with a recipient who ovulated spontaneously. If only a limited number of recipient mares are available, the recipient and donor can be synchronized using hormonal treatment. This will be discussed later, under "oestrus synchronization by hormonal treatment" on page 28. At least two recipient mares should be available for each donor.[15, 28]

The selection and preparation of the recipient mare should start already in the autumn of the previous year. Due to risk of contamination, no additional mares can be added during the breeding season. The recipient mares have to fulfil several requirements. They should be easy to handle and have permanent identification. They should be reproductively sound and also healthy in other aspects. It is especially important that she has good udder health so the foal will get enough milk. Preferred age is 3-12 years, to avoid older mares with subclinical endometritits.[3] The mares should be vaccinated according to the rules of the country they are in.[28]

A "breeding soundness" examination should include checking that the mare has been properly vaccinated and de-wormed. Previous diseases and treatments should be known and you should decide whether this is going to affect her pregnancy or not. Her cycling activity should be monitored and note the length of the oestrus, and interovulatory interval. Also note if hormonal treatment has been used earlier. Previous pregnancies and their outcomes should be considered.[12]

Perineal and pelvic anatomy of the mare affects her fertility. High fertility mares often have long sloping hip, their sacro-iliac joint is positioned dorsal to the tail root and the vulva should be straight with more than 80% of its length below the tuber ischiadicus.[15] Good anatomy of perineum and vulva is important, since poor anatomy can predispose to bacterial contamination.[3]

The size of the recipient mare will affect the size of the foal. This was proven already in 1930, when a Shetland pony mare was bred with a Shire horse stallion and the other way around. The Shetland pony gave birth to a much smaller foal (less than half the weight) than the

Shire horse's foal. The size difference remained as they grew up.[6] This is also seen after embryo transfer. Research shows a positive correlation between surface area of the placenta and the foetomaternal contact area to the birth weight of the foal.[15] Recipient mare should ideally be the same size or slightly larger than the donor mare.[13] 450-500kg is the general recommended weight.[28]

It is very important to know exactly when the recipient mare is ovulating. When showing behavioural oestrus signs, the mares should be examined by rectal palpation and rectal ultrasonography every day.[3] Horses seems to be the least strict when it comes to synchronization of donor and recipient ovulation for embryo transfer. For cattle it is only +/- 1 day, and in pigs preferably the same day.[6] In horses +1 to -3 days between donor and recipient is recommended.[19] "The donor should be the one ahead, since washing and handling the embryo may lead to delay of maternal recognition of pregnancy" says Dr. Juliane Kuhl, Univeristy of Veterinary Medicine, Vienna. There is however reports of day 10 embryos successfully being transferred to -7 and +2 days asynchronous recipients. Transferring an older embryo later in the cycle will give less time for the embryo to transmit its anti-luteolytic maternal recognition of pregnancy signal, resulting in the mare failing to recognize the pregnancy. This report concluded that embryos transferred to recipient mares ovulating >2 days before the donor mare leads to reduced pregnancy rates and increase in embryonic loss, but after more research it may be possible to transfer older embryos to more asynchronous mares.[18]

To avoid the planning and work needed to synchronise mares, hormone treated ovariectomised mares have been used with good results. Progesterone is produced by the corpus luteum in the beginning of the pregnancy. Later the placenta takes over the production, increasing the progesterone levels throughout pregnancy. The mares are given progesterone or progestins for 4-7 days before transfer, till 100-140 days of pregnancy. Pregnancy rates for hormone treated ovariectomised mares were about 70%, which is similar to the control group of intact mares. The parturition and lactation was also normal. However, more research is needed, and giving progesterone to a mare for around 120 days is expensive and inconvenient.[15]

Figure 12: Standardbred recipient mare with embryo transfer Quarter Horse foal

At Weatherford equine in Emerald, Queensland, Australia, they use standardbred harness racing mares unsuitable for racing (Fig. 12). These mares are economical to purchase, have a suitable size and age, good fertility and they usually have great maternal instincts. The fact that they are used to being handled and have been contained in a harness with a sulky, makes them easier to put in a stock for examination. The mares are evaluated and graded based on reproductive potential to know which mares are the best ones. The recipient mares are owned by the clinic and will be leased to the clients from 30 days pregnancy until after weaning of the embryo foal. If it is a very valuable foal, or inexperienced clients, the pregnant recipient mare may be left at the breeding centre and foal down at the clinic. Even though it is recommended not to add any new recipient mares during the season, this is not so easy in reality. If more recipients are needed during the breeding season at Weatherford equine, Australia, they will first be examined and isolated at a separate location. Only healthy horses will be added to the recipient herd.

The donor mare

Donor mares are usually valuable mares that are worth spending the money on.
They should be healthy and even though a mare with endometritis can become pregnant and the embryo is washed, it is rare that these embryos survive after embryo transfer. Examination up to once a day as she gets close to oestrus is good to be sure that the reproductive tract is

healthy and to control follicular development and ovulation.[3] As with the recipient mare, the donor mare should go through a "breeding soundness" examination as described above.

Ideally, the donor mare should be between 3 and 10 years old. Often this is a problem, since her performance and the performance of her offspring may not be available this early. Older mares often have a decreased embryo recovery rate. This is due to oviductal and uterine pathology. Older mares also have a higher occurrence of early embryonic death.[20] The embryos flushed from older mares have more morphological abnormalities and their development are sometimes delayed. More embryos are produced and recovered in mares under the age of 10.[12] Breeding mares with frozen semen can also lead to smaller and/or less developed embryo than expected. If you do decide to use an older mare or use frozen semen, it is even more important make sure her nutrition is correct and minimise stress.[12,14] Also, take extra care when selecting a recipient mare. She must be at the right stage considering the embryo, and not picked according to when the donor mare ovulated.[14] Progesterone treatment may help in mares that is older or for other reasons ovulate small follicles.[3]

Around the time between ovulation and flushing, it is important to avoid stress in the donor mare. This may be a challenge in competing sports horses, but is important to increase the chances of embryo recovery.[3] Even though a valuable mare being an embryo donor can still compete at sport events, the owner must be prepared to give her some time off for the preparation and embryo flushing. Usually 1 to 2 weeks are needed.[13] The use of competing mares in embryo transfer programs will be discussed later, under "embryo recovery from exercised mares" on page 37.

The stallion and insemination

The stallion should also have gone through a "breeding soundness" examination.

Fresh semen by natural cover or collected at the farm, has the best results with 65% pregnancy rates.[21] The mare should ovulate within 48 hours of the last insemination.[24] Chilled semen, has a medium success rate, with an average of 55%.[21] Ovulation should occur within 24-36 hours after insemination.[24] Frozen semen has the lowest success rate, with an average of 45% pregnancies.[21] Ovulation should occur within 8-12 hours after insemination with frozen semen.[24]

Be aware, that post-ovulatory insemination may cause pregnancy loss.[12]

Dr. Joanne Smith at Weatherford Equine, Australia, prefers to inseminate as close to ovulation as possible for frozen semen AI. This is achieved using ovulation drugs (HCG/Des Combo) and ultrasonic scanning to predict the time of ovulation. It takes 4-6 hours for the semen to reach the oviduct. 8 hours post-insemination she recommends treating or flushing the uterus in mares bred with frozen semen, and other mares if she is concerned. Penicillin and gentamycin is given intrauterine at the same or following day of transfer, followed by oxytocin injection a few hours later and the next day to ensure emptying of the uterus. Sometimes only oxytocin is given to promote emptying of the uterus.

Embryo flushing

Embryo flushing is done by administering fluid into the uterus and retrieving it through a special filter.

Day 0 is the day of ovulation. The embryo usually enters the uterus on day 5. At this point it has developed to a morula or early blastocyst stage.[12] Embryo flushing is usually done on day 7 by transcervical uterine lavage.[3] At this point most embryos has reached the expanded blastocyst stage[12] and its diameter is approximately 400-500 m. This may however vary due to different factors like age and individual differences in the mare, stallion fertility and at what time the insemination is done compared to the ovulation.[3] If the embryo is intended to be cryopreserved or micromanitpulated, day 6 is the best day for embryo flushing. Day 6 however, has a lower embryo recovery rate, because the embryo may not have reached the uterus yet. It is more difficult to see the day 6 embryo in the recovery medium and its small size makes it easier to loose during the flushing.[15] In some mares, the embryo flushing should be done on day 8. This includes mares older than 18 years, mares bred with older or not so fertile stallions and mares inseminated with frozen semen.[3] Flushing embryos after day 8, when they are bigger, makes it easier to find with the microscope. It may even be seen with the naked eye. However, they are more fragile[13] and can be too big to fit comfortably into a 0,25ml pipette.[15]

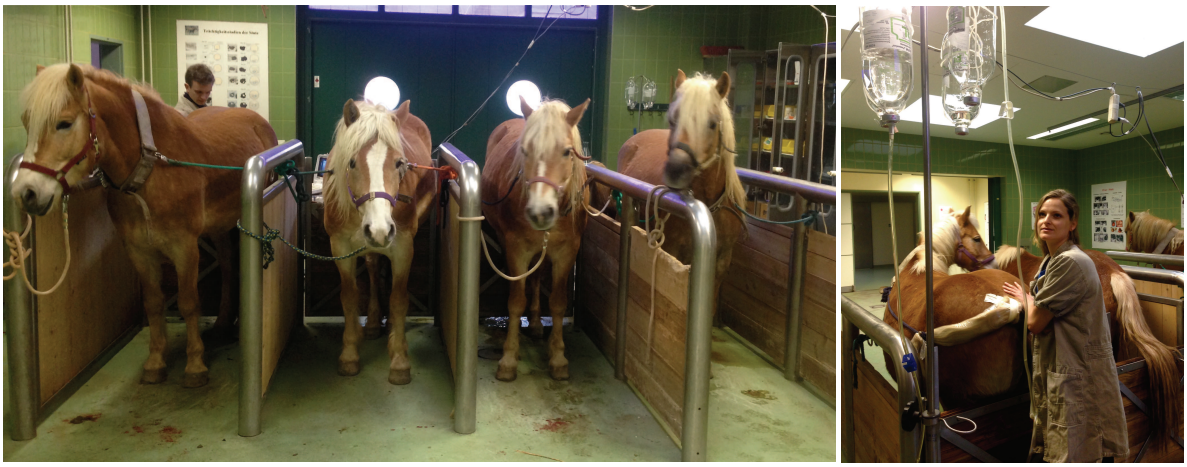

Figure 3: Embryo flushing at University of Veterinary Medicine, Vienna, Austria

Embryo flushing is quite easily done. The mare should be placed in a stock and the tail is wrapped and elevated.[13] Before starting the flush, ovaries should be examined by rectal palpation and transrectal ultrasound. The uterus should also be checked for fluid. The bladder should be empty to avoid displacement of the uterus when filled with fluid, which may block the way for the embryo. By examining the mare prior to the flushing, you will also notice if she will need to be sedated to safely retrieve the embryo.[3] The perineal area and hindquarters should be carefully cleaned to provide as aseptic conditions as possible.[13]

At Weatherford Equine, Australia, they use a silicone catheter with balloon tip, and a two-way Y-junction flushing tube where you can easily reverse the flow. Sterile plastic sleeves should be used to cover the operator's arm. Dr. Joanne Smith recommends cutting of the fingertips of the exploration gloves and using powder free, sterile surgical gloves on top of that to increase sensitivity. She use as little sedation as possible in the donor mare, since it causes a decrease in uterine tone.

Sterile, nontoxic water-soluble lubricant is used to prevent injury.[13] The catheter is introduced into the vagina and through the cervix, reaching about 5cm into the uterus.[28] The balloon tip of the catheter is filled with air or sterile saline to make sure no fluid (and maybe an embryo) will get lost at the junction of the uterus and the internal cervical opening.[13] After filling the cuff, draw the catheter back, so that it blocks the internal opening of the cervix properly.[28] Stabilise the external os of the cervix with your thumb and index finger while flushing.[3,12]

The flushing medium mostly mentioned in literature is Dulbecco's phosphate buffered saline.[6,12,15] Foetal calf serum or oestrus mare serum can be added to avoid embryo sticking to the walls of the tube when being collected.[3,6] However, calf serum may lead to excessive foaming, making it difficult to find the embryo. Polycinil alcohol is an alternative. You can also use flush medium with antibiotics, usually kanamycin.[3] At the University of Veterinary Medicine in Vienna, they use ringer lactate (Fig. 3), while at Weatherford Equine, Australia, they use a commercial flushing fluid (complete flush by viGRO, BIONICHE), containing surfactant and antibiotics. Since the commercial fluid is quite expensive, Dr. Joanne Smith uses a closed environment when she flushes the uterus. This way she can reuse the flushing media in the same mare. The fluid enters the uterus and is emptied out in a sterile empty saline bag and can be reused. The flushing medium can be prewarmed (30-37°C),[13] but room temperature also works. Most importantly the fluid should not be above body temperature.[3] Connect the flush medium container to the tube connected to the catheter and use gravity to administer the fluid into the uterus.[13] The operator can manipulate the uterus rectally to create movement.[12] This gives higher chances of recovering the embryo, in case it is stuck[6] and to be sure that both the horns are filled with fluid. In mares that have had a foal, often only the horn that carried the foetus will be filled. By controlling rectally, you can monitor the filling of the horns and know when to stop filling and start recovering the fluid. How much flushing medium needed for each flush depends on the mare. Young maiden mares may need only 750-1000ml, while some older or lactating mares can take 2 litres.[3] Oxytocin can be given if 2 litres does not fill the uterus. 20IU intravenously is suggested. Oxytocin makes the uterus contract.[6] Oxytocin should also be given if less than 90% of the fluid is retrieved.[13] You should do at least three flushes[3] even though the embryo is most often retrieved during the first flush.[12] Up to 8 flushes may sometimes be necessary.[25] The flushing medium is also collected by gravity.[12] The catheter should be moved around in the uterus, to retrieve all of the fluid. For the last flush you can leave the fluid in the uterus for about 3 minutes.[3] 93-98% of the fluid should be recovered.[28] After the last flush, check the uterus with ultrasound to make sure there is no fluid left. Not only to make sure you got the embryo, but also to avoid endometritis.[3]

When collecting the fluid it is passed through a special filter that will capture the embryo.[3] Make sure there is always some fluid left in the filter. It is important to keep the filter wet so the embryo doesn't get dehydrated or stuck in the filter.[13] Different filters are available. Dr. Juliane Kuhl at the University of Veterinary Medicine in Vienna, prefers using the Em-com

filter designed for cattle (Fig. 4). At Weatherford equine, Australia, Dr. Joanne Smith has better experience with the "EZ Way Embryo Collection Filter" (Fig. 5). The advantage with the second filter is the built in grid. It minimizes trauma to the embryo, since you don't have to pour the liquid into a petri dish to examine it in the microscope. Also less trauma will be caused to the embryo, since the filter and exit is located on the side of the filter, a bit elevated, so there will be less turbulence in the water.

Figure 4: Em-com bovine embryo collecting filter

Figure 5: EZ Way embryo collecting filter

Larger embryos can be seen with the naked eye, but for smaller sized embryos you will only see it in the microscope.[15] Embryo handling should be done under the microscope, making it easier to see what you are doing.[28] If you are not using a filter with a built-in grid, you have to pour the fluid from the filter into a gridded petri dish. Rinse the filter with flush medium to make sure you got the embryo.[3] The search dish should have been maintained at 37^{o}C to avoid injury to the embryo.[12] The flushed fluid should be clear but some debris is normal. It is a good idea to take a culture from the flushed media. [3]

Important factors influencing embryo recovery rate are number of ovulations, day of recovery (age of embryo), skill of technician, fertility of mare, fertility of stallion and treatment done to the semen (fresh, chilled or frozen) used and season.[28]

Finding, handling and evaluation of the embryo

Searching for the embryo can be a challenge for inexperienced people.[3] You should use an electronmicroscope[25] (Fig.6) of good quality and measurement of the embryo should be possible with eyepiece micrometer.[14] 15x magnification is recommended.[12] The embryo will sink to the bottom of the dish, so when searching for it with the microscope, make sure you are focusing on the bottom. This can be achieved by focusing on the grid or debris that is found on the bottom of the dish.[3] After finding the embryo, it should then be washed with flush medium or holding medium[14] to remove as much microorganisms as possible. You can use multi well dishes and move the embryo from well to well up to ten times.[3] It is also possible to make several drops in a petri dish and move the embryo from drop to drop (Fig. 7).[14]

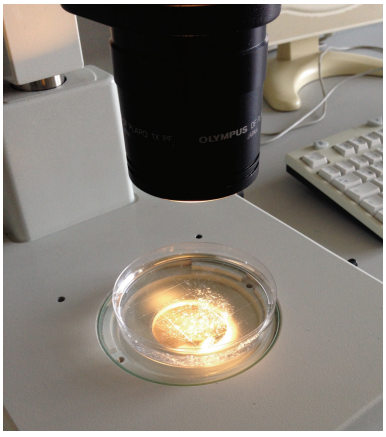

Figure 6: Searching for the embryo with the

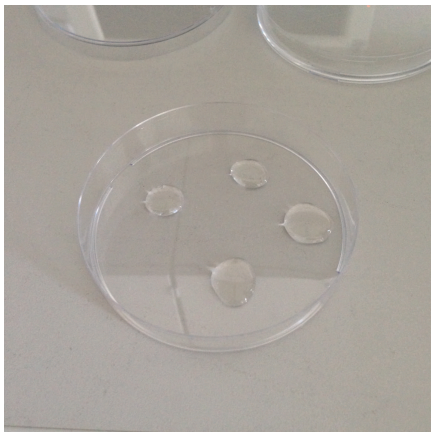

Figure 7: Drops in a petri dish for cleaning of the embryo.

Dr. Joanne Smith at Weatherford Equine, Australia, prefers using a "Drummond microdispenser" (Fig. 8) to transfer the embryo from well to well. If the embryo is too big for this pipette, she will use a 0.25ml insemination straw (Fig. 9), connected to a tuberculin syringe with a pipette tip (Fig. 10). She uses a 4-well cluster dish, with numbering. The wells are sloping for better embryo handling and it has an extra small well in the centre. The wells are filled with holding media flushed through a special syringe filter (minisart, Sartorius stedim, biotech, 25□m) to make sure it is as clean as possible. When picking up the embryo, first let some fluid into the pipette, then the embryo in fluid and at the end a little bit of air. This way you know that when the airdrop goes into the well, the embryo will follow. The pipette should be cleaned with a bit of flushing media between each time you move the embryo. At Weatherford Equine, Equine, the lab room holds a constant temperature of 24°C and the flushing and holding media is taken out of the 37°C incubator at the same time.

When you find an embryo you should examine it carefully at a higher magnification[12] to see developmental stage, quality, shape and size of the embryo, thickness of zona pellucida, uniformity and compactness of blastomeres, presence of perivitelline space, presence of damage of zona pellucida or capsule and presence of degenerated blastomeres. It is especially a challenge to differentiate a morula from an unfertilized oocyte (UFO). Other non-embryonic structures may also lead to confusion.[14] Morphologic assessment is very important to have good success rate in an embryo transfer program. Transferring an UFO, confused with an embryo, or transferring poor quality embryos is a waste of time and will give you lower success rate.[28]

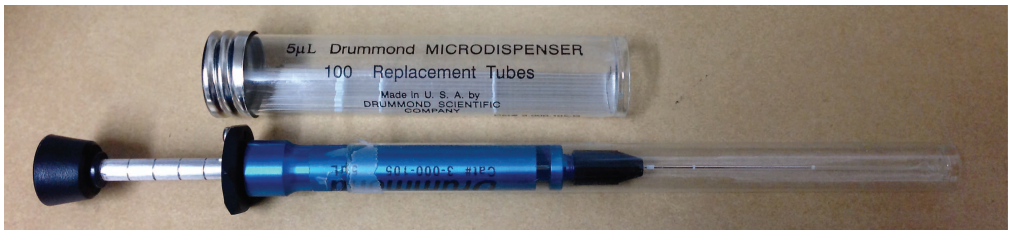

Figure 8: Drummond microdispenser, with 5µl tube

Figure 9: 0,25ml clear insemination straw

Figure 10: Tuberculin syringe with pipette tip

Evaluation of embryonic developmental stage:

- Morula is the earliest developmental stage of an embryo that you can obtain from the uterus.[28] It's size is 150-200µm with thick zona pellucida[14] surrounding a tight compact mass of at least 32 blastomers. As the blastomers divide they eliminate the perivitelline space.[28]
- The early blastocyst is 150-250µm, thick zona pellucida surrounding blastomeres with blastocele cavity beginning to form.[14]
- The blastocyst measures 150-300µm.[14] The outer layer is a thin zona pellucida, followed by an early stage of the capsule and then a thin layer of trophoblast cells (future placenta) surrounding the inner cell mass[28] with a large blastocele cavity.[14]

- Expanded blastocyst is 300- >1000µm, zona pellucida may be present. A thin layer of trophoblast cells with a cell mass protruding into the blastocele cavity.[14] After the loss of the zona pelucida, the blastocyst expands rapidly. The expanded blastocyst can be seen approximately 7,5 days after ovulation.[28]

Differentiate:

- Unfertilized oocyte (UFO): 125-150µm, oval and flat with thick zona pellucida with cytoplasm that may make the UFO look like a morula if degenerated or fragmented. In contrast to an embryo, the UFO does not roll when manipulated!
- Non-embryonic structures, for instance urine crystals or cellular debris can be differentiated by the lack of zona pellucida or its difference in shape and size.[28]

Grading:

Examine compactness of blastomers, damaged or extruded blastomers, size of perivitelline space, zona pellucida damage and embryo shape, colour and developmental stage.[28]

1. Excellent: Spherical, uniform, developmental stage according to age after ovulation.[14] Transfer of grade 1 embryos shows a high pregnancy rate at day 50 (70-80%).[12]
2. Good: Slight irregularities, for instance some extruded blastomers or trophoblastic separation.[28]
3. Poor: Moderate imperfection. For instance extruded blastomers, collapsed blastocel, degenerate cells but the embryonic mass still seems viable.
4. Degenerate/dead: severe problems or complete embryonic death.[14] Pregnancy rates with grade 4 embryos are much lower than the others (30-40% at day 50)[12]

In many ET projects, most embryos are graded as 1 or 2. This is probably because UFOs, poor quality or dead embryos are usually retained in the oviduct. UFOs are most often found when there is also an embryo recovered. If you find only an UFO it is a good idea to flush the mare again, because the embryo may still be in the uterus. It can also still be in the oviduct, so flushing again later may also give a viable embryo.[14] Dr. Joanne Smith, Weatherford Equine, Australia, explains that the reason for this is that the UFO would be from a previous ovulation and would not exit the oviduct by it self. It would have been pushed out from the oviduct by an embryo.

When having found and evaluated the embryo it can be put into a 0,25 or 0,50 ml semen freezing straw, depending on the size of the embryo. This is done by using a urethral catheter adapter attached to a tuberculin syringe[12] or by connecting a pipette tip to a tuberculin syringe and then to a clear French straw as they do at Weatherford Equine, Australia.

The common method, and also the way Dr. Joanne Smith fills the straw is the following: you start by drawing up holding media twice the length of the section with media and embryo. This needs to be larger since it will wet the cotton plug and push the embryo out of the straw. The first holding media is followed by a section of air, then holding media with the embryo, another section of air, holding media and air again. You pull up the columns until the media touches the cotton plug of the straw. Once the plug is wet, it seals the straw (Fig. 11).
The air is there to control the location of the embryo, to push the embryo out of the straw and minimize the amount of liquid administered into the uterus.

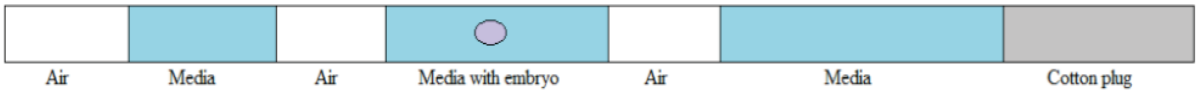

Figure 11: Illustration of the embryo transfer straw

Embryo transfer

The next step is the actual transfer of the embryo from the straw to the recipient mares uterus.

At Weatherford Equine, Australia, the recipient mares are sedated to avoid excessive handling and irritation of the cervix, which decreases the success rate. She recommends 1ml of romifidin with ½ ml of butorphanol administered intravenously 5 minutes prior to cleaning of the recipient mare. If the mare is stressed, increase the romifidine dose to 1 ½ ml.

The collected and washed embryo should be transferred to the recipient mare as soon as possible.[13] Preferably within 2 hours.[28] Today over 95% of equine embryos transferred are fresh.[8] It is most convenient to have the recipient and donor mare at the same location. If transported, the embryo should be placed in holding medium.[13] The results of cooling, freezing and transport of embryos are variable, and will be discussed later, under “cooling, freezing, transport and storage of equine embryos” on page 33.

The recipient mare should, as explained earlier, have gone through careful examination and at the time of embryo transfer, she is examined again with ultrasound and palpation per rectum. Corpus luteum and some follicular development should be detected. The uterus should have good tone with uniform echotexture and no signs of oedema and endometritis. The cervix should be tightly closed, indicating adequate levels of progesterone.[3]

Embryo transfer can be done surgically or non-surgically.
Surgically, it can be done either by ventral laporatomy under general anaesthesia or in a heavily sedated horse, by flank laporatomy. Either way, the embryo is injected into the uterine lumen by opening one uterine horn. Surgical method of embryo transfer has a high success rate, probably because it is very sterile and the embryo is put exactly where we want it in the uterine lumen.[6] In the beginning, the standing flank laporatomy was the most common method.[3,12] The disadvantages of surgical transfer are the economic costs, the discomfort of the mare and the work load.[6]

Non-surgical embryo transfer is the preferred method nowadays and shows equal or even better results than the surgical method.[3,12] Hygiene is very important. Perineum, vulva and vestibulum are cleaned properly and the tail bandaged and held away. At Weatherford Equine, Australia, the cleaning step of the transfer is considered as very important. First, the perineal area is cleaned with diluted chlorhexidine, then rinsed with water, then sterile saline, and at the end it is dried off with sterile swabs.
The embryo should carefully be deposited into the recipient mares uterus.[3] This is done transcervically with a sterile embryo transfer gun or an insemination catheter.[6] AI catheter is preferred in case of large embryos (day 8) since it has a larger inner diameter than an embryo transfer gun.[25] Different insemination or embryo transfer guns are available and depends on the preferences of the veterinarian.[12] Vaginoscope can be used to increase visualisation[6], but may lead to vaginal inflammation.[24] Use sterile lubricant and insert the device transvaginally with your hand to the cervix. The external os may be stabilized with thumb and finger. Advance the device through the cervix. At this point, you can remove your hand from the vagina and move it to the rectum to guide the device into the free lumen of a uterine horn close to the bifurcation. The embryo is expelled into the uterus and the device is removed.[3,12]

Dr. Joanne Smith at Weatherford Equine, Australia, uses the following method:
An ET transfer gun with a sleeve and a chemise on top of that is used for the embryo transfer. Use transrectal gloves with cut off fingertips and sterile powder free latex surgery gloves. Put on the plastic sleeve and the chemise. Fold the chemise over the tip of the transfer gun, to avoid piercing through it too early, which may lead to contamination. Push the ET gun with the plastic sleeve through the chemise at the opening of the cervix. When removing the ET gun out of the mare protect the tip with your hand. Sometimes the embryo may be stuck at the tip of the ET gun! Place the tip into the last well in the multiwell dish used for cleaning the embryo to make sure the embryo was deposited into the uterus. If the embryo has ”hung up” in the ET gun it must be cleaned again in a 4 step cleaning process prior to being transferred again.

The recipient mare after ET

After ET, there are some treatments that can be done in the recipient mares to optimize pregnancy rates. Remember that each mare is different, and individual needs are different.[13]

Embryo transfer will lead to a mild inflammation in the uterus, and this can lead to loss of pregnancy due to PGF2α response causing regression of the corpus luteum. By adding non-steroid anti-inflammatory drugs, this may be avoided. NSAIDs inhibit cyclooxygenase, an enzyme that is important in the PGF2α synthesis. In the embryo transfer programs of 2008 in Brandenburg State Stud, Neustadt, Germany and the veterinary teaching hospital in Vienna, Austria, higher pregnancy rates was seen after administering flunixin meglumin IV at the time of embryo transfer and Vedaprofen orally twice a day for the following 3 days. This result was also seen in a similar trial in cattle.[23]

It is a good idea to take a uterine culture after the embryo transfer since bacterial contamination may lead to death of the foetus. Antibiotics (for instance sulfmetoxazole and trimethoprim) can be given until the first pregnancy examination.[3] Antibiotics are sometimes used routinely for prevention in embryo transfer programs.[15]

Progesterone treatment after ET may help maintain pregnancy. Especially in marginally acceptable and acyclic recipient mares, and maybe even in all recipients.[19] Signs of decreasing progesterone levels are softening of the cervix and decreased uterine tone.

Serum progesterone can also be analysed.[13] If progesterone needs to be administered, the administration should continue till day 100-140 of pregnancy.[15]

At Weatherford equine, Australia, the routine treatment of recipient mares at flushing are the following: 10ml of Flunixin intravenously, 20ml Depocillin intramuscularly, 25ml Gentamycin intravenously and 10ml Regumate orally. Sedivet and Turbogesic are the drugs used for sedation, dosage depending on the mare. After ET, the recipient mare's corpus luteum, uterine tone and cervix is evaluated. If necessary, in mares with a poor quality CL (or minimal luteal tissue), poor uterine tone or soft cervix, she will receive 10ml progesterone intramuscularly at transfer. Her progesterone blood levels will be tested after 7 days and if under 10nmol/L she will get progesterone treatment up until day 120. By then, the placenta will have taken over the progesterone production.

Different treatments are used in different clinics. It may decrease pregnancy loss, but there are also reports where anti-inflammatory treatment and antibiotics showed no change in pregnancy rates.[19]

At Weatherford Equine, Australia, the mares are given antibiotics during the pregnancy if necessary. If the mare has a history of placentitis or uterine problems she will get antibiotics for a week every month. If she goes over her due date or there are any indications of problems, she will get antibiotics from the day of diagnosis till the parturition. In the recipient mares, antibiotic treatment is rarely needed, since these mares have been selected for reproductive health. It would be more relevant in older mares or mares with reproductive problems brought to the clinic by clients. The antibiotic used is a specially prepared BOVA drug of Sulphadimidine Trimetoprim oral paste. 4,5g/0,9g per 10ml. 25ml/500kg is given twice daily.

The majority of recipient mares at Weatherford will be caslicked at the time of transfer. This will be removed four weeks before foaling due date.
Caslick's operation is very effective to prevent pneumovagina and following infertility due to contamination by air, urine and faeces into the vaginal vault. The need for a Caslicks's operation depends on the vulvar conformation. Mares having reproductive problems often have a sunken in anus and an angled vulva.[15]

The first pregnancy examination can be done 4-5 days after the embryo transfer, by transrectal ultrasound. This is 11 days after ovulation.[13,12] If the embryonic vesicle cannot be seen at this point, the mare should be checked every 48 hours until it is. Research shows that pregnancies detected this early, have higher chances of lasting. Mares not having an embryonic vesicle until later examinations have higher incidences of pregnancy loss before day 50. This is also the result in case of undersized embryonic vesicles.[12] Examination is suggested on day 11, 13, 17, 23 and 29 after ovulation and more often if needed. Examine the uterus, looking for signs of endometritis, and check the corpus luteum. The mare should be checked again for pregnancy at day 60. The loss of pregnancy before day 60 does not seem to be higher after embryo transfer, than in mares inseminated with fresh semen.[15]

Mares that are not pregnant at the first or second attempt, should be excluded from the recipient herd.[28]

The donor mare after flushing

After flushing the donor mare, there are a few points we should think about.
Since there is a risk of the mare becoming pregnant if the flush was not successful or she ovulated more eggs, $PGF_{2\alpha}$ is often given routinely after flushing.[13] Also, this will make her return to oestrus quickly.[28] However, this is not always successful, because a second ovulation may have led to a corpus luteum refractory to $PGF_{2\alpha}$ at the time of flushing.

Another problem is endometritis following incomplete emptying of fluid from the uterus. The donor mare's uterus should be checked with ultrasound the day after flushing. Her oestrus cycle should also be monitored to make sure she goes into oestrus again.[13]

If the mare is healthy and the fluid obtained from her during the embryo flushing is clear, with minimal amount of debris, she can be inseminated again at the next ovulation.[28]
At Weatherford Equine, Australia, the donor mares are given 1ml of $PGF_{2\alpha}$ (Cloprostenol 250□g/ml) after flushing. All donor mares are examined with transrectal ultrasound 4 days after flushing.

Main challenges of embryo transfer in horses

Hastening the spring transition period

When registering a foal, 1st of January is set as their birthday in the northern hemisphere (1st of August in the Southern hemisphere)[24] regardless of their actual birth date. This means that breeders want foals born as early in the breeding season as possible. Therefore it is beneficial to speed up the process by inducing follicular growth and ovulation in transitional mares from the middle of the transitional period.[21]

Artificial light program

Since horses are long day breeders, they can be stimulated with artificial photoperiod. Artificial lighting will induce follicular growth and can be used alone or in combination with exogenous hormones. Artificial light is needed for about 60 days, 14-16 hours a day. A good tip is that the light should be strong enough for you to be able to easily read a newspaper in all areas of the stable. This can make the mare cycle as early as in the middle of February in the northern hemisphere. Some places, artificial lighting is also done outside in paddocks with floodlights.[24] However, using a lighting program is expensive, labour intensive and not very practical.[21] New research shows that directing blue light to one eye of the horse suppresses melatonin release. This means that using individual light masks for mares, can replace the older methods. Using individual masks for the horses is easier than to control the lighting of their surroundings.[30]

Hormonal treatment

To start hormonal treatment, follicles between 25 and 35m should be present on the ovaries.

By giving progesterone, the first ovulation of the year can be synchronized. The drugs commonly used are altrenogest (regumate) orally or progesterone in oil with injection for 10-15 days.[4] Intravaginal progesterone-releasing devices are also available, for instance PRID and Cu-mate. The problem with intra-vaginal devices is that they may cause vaginitis, typically by *Streprococcus equi* var *zooepidemicus*.[15] Giving progesterone to control the ovulation is very beneficial, because it can be given over a longer period of time without causing any side effects.[4] After the last progesterone treatment, oestrus behaviour can usually be seen after 1-5 days.[25] Ovulation usually happens after 4-8 days.

Prostaglandin should be given on the last day of progesterone treatment to induce luteolysis of any corpus lutea that might be present. Human chorionic gonadotopin can be given on the second day of oestrus to promote ovulation. For this to be successful, a follicle larger than 35mm should be present and the mare should have shown oestrus behaviour for at least 3 days.[24]

Gonadotropin releasing hormone agonist (desorelin or buserelin) can also be given to get ovulation earlier in the year. Buserelin is given as injections twice a day. This means that the mare must be handled twice a day and this causes more work and can be a problem for horse owners.[4] Desorelin can be given as a subcutaneous pellet every other day, giving 6-8 doses. Price is a limiting factor on this drug.[24]

An other alternative is equine follicle stimulating hormone (eFSH). This must be given twice a day for 5-7 days leading to ovulation in about 7 days. There is the same problem here as with Busorelin, that it must be administered twice a day and it is also very expensive.[4]

Dopamine antagonist (domperidone or sulpride) has also been used to induce ovulation earlier in the year. Dopamine has an inhibitory effect on prolactin and GnRH, and its levels are lower during the breeding season.[24] Research has shown that the first ovulation may occur 2 ½ months earlier in mares treated with dopamine antagonist, than in control animals. Sulpiride is another dopamine antagonist and led to ovulation about 1 month earlier than in control animals. In a study, they even managed to induce follicular growth and ovulation in mares that were in anoestrus by giving estradiol and sulpiride.[4]

Oestrus synchronization by hormonal treatment

Oestrus synchronization in mares is mostly used in embryo transfer programs, or when a stallion is only available for breeding for a limited time. It is a very important step for good results of embryo transfer. It is a much bigger challenge in horses than in for instance cattle. While synchronizing cattle with exogenous hormones will give a quite accurate time for ovulation, mares have a lot more individual differences. In mares, the oestrus lasts for 5-7 days and the ovulation occurs during the last 2 days in 69% of mares, while 14% will ovulate after the end of oestrus. In embryo transfer, the recipient mare should ovulate 1 day before to 3 days after ovulation of the donor mare.[15]

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

$PGF_{2\alpha}$ is used to induce oestrus, synchronize oestrus, treat mares with persistent corpus luteum, for removal of uterine fluid and for abortion.[21] It is normally produced by the endometrium.[15] Administration will lead to luteolysis of the corpus luteum and the mare will return to oestrus. The most commonly used drugs are dinoprost (naturally occurring, dosage: 9µg/kg) and cloprostenol (synthetic, dosage: 0,55µg/kg). For this to work, a functional corpus luteum must be present. Be aware that during the first 5 days after ovulation, the corpus luteum shows little sensitivity to $PGF_{2\alpha}$. This is the refractory period. After drug administration, most mares returns to oestrus in 5 to 7 days and will ovulate in 9 to 11 days. This depends on the size and stage of the follicles in the ovary at the time of administration. If a large follicle is present, ovulation may occur within 2-3 days (maybe even after 24 hours according to Dr. Joanne Smith, Weatherford Equine, Australia). Variations may be large and the ovulation can occur between 2-15 days after administration![15] It will take longer in the spring than in the summer.[15] If two doses are given, the mare ovulates averagely 7-10 days after the second injection. Due to this big variations between individual mares, this is not a very reliable drug for oestrus synchronization when used alone.[15]

Research on $PGF_{2\alpha}$ used in oestrus synchronisation shows different results when it comes to its effect on pregnancy rates. So far it does not seem like using $PGF_{2\alpha}$ has a great negative effect on later pregnancy rates, but risk may be increased when mares are repeatedly treated. Also, if mares ovulate shortly after $PGF_{2\alpha}$ administration, the uterus may not have gone trough the changes necessary for a pregnancy from the low progesterone and high oestrogen levels that would normally occur. This may affect early embryo development, so it is more important in recipient mares.[16]

Progesterone and $PGF_{2\alpha}$ possibly combined with estradiol-17β and hCG or desorelin

Progesterone has an inhibitory effect on LH, and exogenous progestins are used to lengthen the luteal phase.[15] This is beneficial to suppress oestrus, synchronize ovulation and help maintain pregnancy in high-risk mares.[21] The most common drugs are progesterone (natural) in oil and altrenogest (synthetic).[15] Progestins alone (150mg intramuscularly per day, oral administration is also possible[4]) or with estradiol-17β (10mg in oil per day, intramuscularly) will maintain the corpus luteum until the treatment is stopped.[15] A ten days treatment ending with a single injection of $PGF_{2\alpha}$ on the last day, is recommended. Combining progestins with

estradiol-17β is recommended in randomly cycling mares to give a better negative feedback on the GnRH release. Estradiol-17β will give more uniform follicular development, giving a more synchronized ovulation. To get even better synchronisation, hCG (5 IU/kg IV) or desorelin acetate can be given when a follicle measuring 35mm is present. This protocol leads to 70% of mares ovulating 10 to 12 days after the $PGF_{2\alpha}$ injection (8-17 days span).[15]

hCG and desorelin acetate

It is difficult to predict when ovulation will take place in a horse, but you can predict if the follicle will ovulate in 1-4 days. If you use an ovulation-advancing drug at this time, ovulation will most likely take place within the next 48 hours, usually 36-48 hours. In the spring, the interval is generally longer than in the summer. In case of multiple follicles, the smaller twin follicle may be too immature and ovulation may take up to 96 hours. Human chorionic gonadotropin (hCG) and deslorelin acetate are used to induce ovulation.[15]

hCG is produced by the human placenta during pregnancy and has LH effect in horses.[15] It is a very popular drug used for oestrus synchronization in horses, and has been available since 2003.[5] hCG can be given intramuscularly or intravenously. The disadvantage with hCG is that if it is used repetitively it may loose its effect. Also, older mares do not respond very well to it. hCG should not be used more than twice during one breeding season. Research has also been done on giving hCG in case of smaller follicles with fairly good results.[4]

Deslorelin acetate is a synthetic GnRH analogue. It is available as subcutaneous implant (Ovuplant) or injectable fluid. Using desorelin acetate instead of hCG, reduces the risks of antibody development after several injections in one season. The implant will however lead to a pituitary down regulation and prolonged interovulatory interval in some mares. But this side effect can be avoided by removing implant after ovulation. This is not a problem with the injectable form. If the injectable form is used, one shot of 1,5mg is given when a 35mm follicle is seen in a mare in oestrus. Ovulation will occur 36 +/- 4 hours after deslorelin acetate injection. These drugs are mostly used to induce ovulation post breeding.[15] They are also efficient in older mares.[4]

At Weatherford Equine, Australia, a specially ordered compounded drug is used: Deslorelin acetate and hCG combination injection (2mg2500IU/3ml). Give 3ml intravenously to induce ovulation in a mare in full oestrus with a dominant follicle >35mm. The results of

this combination are very accurate with >80% mares with follicle >35mm (in oestrus) will ovulate at 40 hours +/-2 hours.

After mentioning all these possibilities of hormonal treatment, it is important to think about the consequences it may lead to. Prostaglandin and hCG has shown a tendency to produce more twin foals, and the use of aldrenogest in pregnant mares may lead to mares that will be depending on this to maintain a pregnancy in the future. Hormones are definitely a very helpful tool, but should not be used without careful planning and concideration.[21]

Superovulation in mares

Most mares are monovulatory, releasing one ovum each ovarian cycle. The average occurrence of double ovulation in mares is 16%. It is more often seen in thoroughbreds, warmbloods and draft horses. It is most rare in quarter horses, appaloosas and ponies. Superovulation in mares used in embryo transfer programs would be very beneficial economically, since one mare can give more ovums for fertilization and embryo transfer.[13] Also, it would give higher pregnancy rates per embryo flushing, making it possible to freeze more embryos.[15] One of the biggest expenses of an embryo transfer program is the keeping of a large recipient herd and an unwanted cost is the keeping of those who are not with foal. With superovulation, more embryos would be available for transferring and fewer recipients would be empty.[20]

In several species, including sheep and cattle, superovulation has been introduced by using equine chorionic gonadotropin or follicular stimulating hormone with very good results.[15] In horses, superovulation has not been so successful. This has had a great importance in limiting the embryo transfer in horses for commercial use.[6] The reason for this is still under research, but it may be caused by the unique anatomy of the equine ovary. The ovary is inverted, so the structures found in the cortex in other species are in the middle, while the structures found in the medulla are on the periphery. Horses have an ovulatory fossa, which is the only area lined by germinal epithelium and where the follicle can rupture,[5] the rest is covered by tough tunica albuginea. The follicles will compete to reach this area, and the ones that don't will not be able to ovulate and will luteinize without rupture.[6] A good drug for inducing superovulation in horses is not yet found on the market. Several hormonal products have been tested but are both expensive and not consistent in their efficacy.[15]

Crude equine pituitary extract (EPE)

EPE has been used in several studies both in seasonally anovulatory mares and in cycling mares. The results have been promising, but in seasonally anovulatory mares, there are some problems. Usually the mares do not continue cycling after the induced ovulation, and may have problems maintaining a pregnancy if they get pregnant. This is due to the failure of maintaining a corpus luteum. The dose in the seasonally anoestrus mares also has to be higher, leading to additional costs. In cycling mares, giving EPE for a week during dioestrus, has shown god results with 2 to 4 ovulations per mare. This is not a very good result compared to cattle, but in horses it is considered as promising.[15]

Porcine FSH (FSH-P)

FSH-P has been tested, but the results have not been very promising. This might be caused by the lower affinity to porcine FSH compared to equine FSH.[5] Repeated administration of high doses may give results, but it is very expensive.[13]

Equine follicle stimulating hormone (eFSH)

eFSH has been used for many years and several research projects has been done. Administration of eFSH has shown to induce multiple ovulations. It is even more efficient in combination with human chorionic gonadotropin (hCG). eFSH treatment should start 5-7 days after ovulation or when the follicles are 23-25mm. hCG should be given twice a day 1,5 – 2 days after the end of eFSH treatment.[5] Administer twice a day for 6-8 days.[6] Later research has shown that using recombinant LH instead of hCG to induce ovulation after eFSH treatment is even more effective. The challenge in using eFSH for superovulation is that the results are very varying. The results depend on the mare, and research has shown that the best candidates for using eFSH for superovulation are young mares with normal oestrus cycles. Finding the correct dose for the individual mare is also a challenge. Overstimulation with too high doses can lead to luteinisation without ovulation or development of anovulatory follicle.[5]

Equine chorionic gonadotropin (eCG)

eCG is used in other species, but has no effect in the horse. This is probably because eCG only binds to LH-receptors in the mare and not to both LH and FSH as in other species.[13]

Gonadotropin releasing hormone (GnRH)

GnRH in higher doses has led to ovulation of multiple follicles, in one study the result was 3,5 ovulations. However, it has only been successful in seasonally anoestrus mares and has no effect in cycling mares.[5] The response to exogenous gonadotropin treatment was good in some of the seasonally anoestrus mares, but the others showed no difference. Mares with multiple follicles seen by ultrasound examination were given hCG when the most of the follicles were around 35mm. But when flushing for embryos a week later, there were fewer embryos than expected.[6] In conclusion, GnRH is not very effective in inducing superovulation in horses.[15]

Inhibin vaccination

Inhibin from the granulosa cells of the dominant follicle suppresses FSH production in the mare. Inhibin immunisation leads to increase of FSH and therefore increasing ovulation rates in sheep and cattle. This has also been tested in horses with promising results using active immunization. The mares in one study all double or triple ovulated after active inhibin immunization.[15] Passive immunization against inhibin has also been tested, and has given an average of over 2 ovulations per mare, per cycle. Unfortunately there were some side effects on the injection site, and the super ovulation continued over years.[13]

As of today, superovulation is expensive and not very efficient. Therefore it is not very commonly used. At Weatherford they do not do it, but Dr. Joanne Smith says the best alternative so far is the EPE. Also, there is suspicion that multiple ovulations can be associated with a reduced number of embryos and reduced embryo viability. More research is needed on this topic.[15]

Cooling, freezing, transport and storage of equine embryos

Short term cooling of embryos

To be able to cool down an embryo for transport would be a huge advantage for ET. If the embryo can be transported, the recipient and donor mare can be at separate locations. Transporting the embryo is much cheaper than transporting the horse.[7] Also, it would reduce the need for a large recipient herd, because the time of transfer will be more flexible.[15]

Today, short term cooling of embryos is possible. The first technique for transporting equine embryos was to put them into ligated rabbit oviducts surgically. 40-49 hours later, the embryos were transferred to the recipient mares. The results were good, and 3 out of 4 mares became pregnant. To cool down the embryo for transport would obviously be a better option.[7]

The embryo can be cooled to 5°C with Ham's F-10 medium for 24 hours.[7] Ham's-10 is the commercial holding medium mostly used today. The disadvantage with Ham's 10 is that it needs to be gassed to be in a steady state. Buffered Ham's F-10 with 10% foetal calf serum and antimicrobial agent is the transport medium most commonly used in the United States. New holding media that do not need to be buffered are under development.[12] Dulbacco's PBS has also been tested and showed good results at 4°C for 48 hours.[7]

The holding media is warmed to 37°C and 4,5ml is put into a 5ml sterile plastic tube with screw or snap cap before the embryo is put into it. The medium is added up to 5ml, closed with the cap and sealed with parafilm sealing film. A 50ml centrifuge tube is filled with saved flush commercial flush medium. The 5ml plastic tube is put into the centrifuging tube and the centrifuge tube is closed and sealed with parafilm. It is then placed into a passive cooling device for transport (Equitainer). The cooled embryo is sent to its destination with documentation including the results of the examination of the embryo.[7]

When receiving a cooled embryo, the embryo is emptied into a petri dish. Make sure it is not left in the plastic tube, check the lid too. Have holding medium available for rinsing. Rinse the embryo a few times before transfer.[7]

Cooling equine embryos may give adverse effects, but the reports are conflicting. Some researches found that cooled embryos had delayed development, leading to delayed appearance of embryonic vesicle and a smaller size vesicle when it appeared. It seems like large equine embryos handle cooling better than small ones, and had better pregnancy rates. However larger embryos are more fragile to physical handling, and may be damaged during transfer.[7]

Embryo transfer of cooled embryos is done some places commercially and the pregnancy rates seems to be similar to those done with fresh embryos.[7,15] However, research shows that

higher losses will be seen if the embryo is transported and cooled over 8 hours. More research is needed on this field.[13]

Cryopreservation of embryos

Successful cryopreservation would be a great advantage and important step in the equine embryo transport industry.[8] It means that both the time and location of embryo collection and transfer can be dissociated, with greater distance than by just cooling. It would make the synchronization of donor and recipients very flexible.[10] "For instance; an embryo could be collected from a valuable mare competing in sports outside of the sport season, preventing it from interfering with the training. Then it can be cryopreserved and later transferred to a mare in early breeding season "says Dr. Juliane Kuhl at the University of Veterinary medicine in Vienna. Transporting frozen embryos would also be a good way to transport genetic material across borders, with lowers risks of contamination.[26]

Cryopreservation of oocytes is also currently under research, because of the possibilities of saving gametes in mares dying suddenly. Cryopreservation of gametes in general gives the possibility to save genetic material from endangered species to later enlarge the gene pool.[9] Recently, another advantage of cryopreserving embryos has been discovered. The embryo can be tested for inheritable diseases and be frozen until the results are ready.[8] Once frozen, the embryo can be stored indefinitely.[15]

The first pregnancy in horses after cryopreservation of an embryo, was in 1981, but the mare lost the foal one month before term. The first live born foal was born in 1982. This was ten years after the first mammalian frozen embryo was transferred in mice and the year after in cattle.[10]

Two methods for gamete and embryo preservation are available today: slow freezing and vitrification. Slow freezing means lower concentration of cryoprotectants, decreasing the problems with its toxicity to the embryo and decreases osmotic shock. The disadvantage is that more ice-crystals are formed. The vitrification method is quicker and high concentrations of cryoprotectants are used to decrease the formation of ice-crystals. However, the toxicity of the cryopreservants suggests that the embryo should only be exposed to them for a short period of time.[10] Even though the process is time consuming and the start up costs are high

(because a programmed freezing machine is needed), slow freezing method is the one that has been used most so far.[8]

By slow freezing, the suggested method is to use a cryoprotectant, most often glycerol, which the embryo is incubated in. This is done gradually through five steps with increasing concentration of glycerol reaching a final concentration of 10%.[10] The embryo is then taken up into a 0,25ml freezing straw and is then cooled down to -6 to -7°C at 3°C per minute. This leads to formation of ice crystals. Then the straw is slowly cooled (0,3-0,4°C per minute) to –30 to –35°C and stored in liquid nitrogen.[8]

Vitrification is done with high concentration of cryoprotectants and direct immersion into liquid nitrogen. This can lead to a freezing rate of 2500°C per minute and even up to 20 000°C per minute if the freezing straw is stretched to decrease its diameter (open-pulled straw technique). This procedure will only take up to 15 minutes and is also cheaper and easier than the slow freezing method.[8]

For thawing, the straw containing the embryo is reheated by being placed in water holding 37°C for up to one minute. The cryopreservant must be diluted. This is done by moving the embryo through 4-6 baths for 5-10 minutes with decreasing concentration of cryopreservant.[10]

The problem with cryopreservation of embryos is the changes it leads to in the embryo. Ice-crystal formation and osmotic shock seems to be the most important causes of morphological and functional embryo damage.[9] Ice crystals are formed and dehydration follows due to the increase in extracellular osmolarity of the extracellular fluid and the fluid flowing out of the cells.[8] However, there are species differences. Bovine and ovine embryos seems to handle the freezing better than porcine and equine embryos. Porcine embryos has high lipid content and the equine embryo has a mucin-like embryonic coat on the inner surface of the zona pellucida of the early blastocyst, possibly explaining their low resistance.[9] Equine embryos usually don't reach the uterus before day 7 or 8 after ovulation. Best results have been reported with embryos with a diameter between 200 and 300µm.[10] With this size the expected pregnancy rate is 70%, while only 10-20% in larger embryos.[15] This means the embryo recovery should be done at day 6, when it is a morula or early blastocyst, which is a challenge.[6] This gives only a few hours possible for embryo recovery: when the embryo has reached the uterus, but has not grown too big. There are individual differences in the mares, concerning when the

embryo arrives in the uterus. This makes it difficult to decide when to do the embryo flushing. For better results, a new method is needed, making it possible to freeze expanded blastocysts. Several methods are under research. One method is to aspirate fluid from the blastocele, but large numbers of equine embryos are needed to get reliable results.[8]

Because of the poor results, cryopreservation of equine embryos is not common. Not only because of the difficulties with freezing larger embryos, but also that many breeding associations does not accepted foals made from frozen embryos for registration. Also, since superovulation is rare, it is not that common to have spare embryos for freezing.[20] Dr. Juliane Kuhl from the University of Veterinary medicine in Vienna, says that cryopreservation is not used commercially yet at their clinic, since the success rate is still low. The situation is the same at the Weatherford Equine in Emerald, Australia. According to Dr. Joanne Smith, only two facilities offer cryopreservation in Australia at the moment. Research is currently being done on the topic and hopefully better methods will soon be available.

Embryo recovery from exercised mares

Embryo transfer is especially an advantage if you want to breed a mare used in competition. By the time their career is coming to an end, they may be too old to breed, or at least have reduced fertility. However, this may not be as easy as it sounds. The heat and stress caused by training and competition may have a negative effect on fertility. Also, repeated flushing and hormonal treatments may decrease fertility. When a mare owner requests embryo transfer for their competing mare, it is important to educate them about the possibility of failure and that they must be prepared to take the mare out of training for a period of time.[16]

There may be several reasons for decreased fertility in a competing donor mare. One reason is change in body temperature during exercise. A horse in exercise will have an increase in body temperature, especially in warm and humid areas.[16] When a horse is exercising, the stored energy is transferred to mechanical energy. About 80% of the energy is turned into heat. In addition, they will not be as good at thermoregulation as they normally would, due to the stress caused by moving a large body mass. A research program found an almost 50% decrease in embryo recovery and a lower number of grade 1 embryos in mares exercised in >30^{o}C and >50% humidity. This effect of heat on fertility is also seen in cattle, showing a decrease in embryo recovery rates in the warmer seasons of the year.[17]

Research has also shown that blood flow in the ovarian arteries is greater in exercised mares and the vascular perfusion of preovulatory follicle wall was less the day before ovulation, compared to mares not exercised. Decreased vascular perfusion of the follicle was correlated with a decrease in embryo recovery. This is the same results that was seen in trials with cattle and humans.[16]

The stress hormone cortisol is released through the activation of the hypothalamic-pituitary-adrenal axis. The corticotropin releasing hormone has an inhibitory effect on GnRH secretion, and increased cortisol levels leads to a decrease in LH. The level of cortisol is increased in mares in training and/or under stress. Both an increase in follicle blood flow and a rapid increase in LH are important to complete the maturation of the oocyte. A mare in exercise may therefore have problems with the dominant follicle and have an increase in interovulatory intervals. The connection between stress, increase in circulating cortisol and the decrease in ovarian function has been proven in women, and is very likely the same in the horse. Not only exercise will cause stress to the mare. Transport and other stress factors may also affect fertility.[16]

It seems that even moderate training in not so warm temperatures will lead to decreased embryo recovery, maybe due to its effect on the follicular development and maturation. However, research projects showing no difference in fertility of exercised and non-exercised mares has also been seen. So there are probably individual differences between mares. Also, the horses in the project that showed no difference were very fit and maybe therefore had a smaller increase in rectal temperature after exercise. Research has been done in women, showing that exercise have beneficial effect on fertility and reproductive health, but ovarian dysfunction may occur in case of excessive exercise. This shows that a horse lacking fitness which is exposed to exercise is likely to have problems with reproduction due to heat stress. It would be a good idea breed outside sport season, but unfortunately, breeding- and sport season are at the same time in most equine sports. A suggestion would be to give the mare some time off during the periovulatory period and the time between ovulation and flushing. Also, a decrease in hormonal treatment, controlling post-flushing endometritis and careful management may increase the success rate.[16]

The problem most commonly seen in both old maiden mares and high performance mares by Dr. Joanne Smith at Weatherford Equine, Australia, is the lack of cervical dilation during

oestrus. These mares may need manual cervical dilation both pre- and post breeding to assist with cervical relaxation/softening to allow the uterine contents to be expelled post breeding. If you inseminate a mare with a firm cervix, without doing anything about it, you may end up with fluid retention and severe endometritis, since the uterus will not be able to get the semen out and cleanse the uterus.

Several equine clinics have no problems getting their sports mares pregnant. These mares should definitely not be excluded from embryo transfer programs, since their owners would be the ones to benefit the most from it. Great success rates will be seen if the procedure is carefully planned. In the majority of ET programmes world wide a large percentage of their donor mares are high performance sports mares. If the mare owners are flexible on the timing of breeding and cooperate well with the veterinarian, good results will be seen.[16,23]

Registration of embryo transfer foals

One of the reasons for the slow start in embryo transfer was the resistance from the breeding associations in registering foals from this method. In Thoroughbreds, no modern breeding technologies are allowed and to this date they are still strict on this.[6] One of the first embryo transfer foals to be registered was an American Quarter horse, which also had a natural born full sister. When they tried to register both, the American Quarter horse association said that this horse was already registered and it could not be done. After this, they decided to allow one ET foal per mare per year if the mare was over 15 years old or if it was her first foal in three years. In 1986 the rules were changed again and one ET foal could be registered per mare in general per year.[1] A big step was when the American Quarter Horse Association allowed registration of multiple foals from one mare in 2002.[13] After this, many other breeding associations followed.[28] Today, most equine breeding associations in the USA allow registration of embryo foals. But the Jockey club (Thoroughbreds), the United States Trotting Association (Standardbreds) and the American Miniature Horse Association will still not register embryo transfer foals.[13] The leader of The Norwegian Warmblood Breeding Association, Mette Hansson, says that registering a riding horse from ET is not a problem, but Thoroughbreds, harness racers and ponies will not be accepted by their respective organisations. Race horses (Thoroughbreds and harness racers) are horse industries involving a lot of money. If they started allowing embryo transfer, they would probably bring money into the industry and help the development.

Economical aspect

Even though getting a healthy foal with embryo transfer might be worth the money, it is a risk that it will not work, especially in small-scale practices. The mare care, the veterinary bills and stallion fee may reach over 8000 Euros. The mare owners should be aware of the risks and be willing to pay the bills regardless of the result.[12]

When it comes to starting up an embryo program in Norway, the costs of keeping a recipient herd would be the biggest challenge. The climate is rough and large amounts of rain require more from the keeping facilities here, than for instance in Australia. Also, since there is no one offering embryo transfer commercially at this point, it would be very expensive to get a veterinarian to help a breeder with few mares.

Situation in the world today

Embryo transfer has been a major step in equine reproduction. Even though superovulation, cryopreservation and cooling of embryos are still not very effective, equine embryo transfer is being done at large scale across the world.

The International Embryo Transfer society (IETS) has tried to register embryo transfer in animals world wide for many years. Unfortunately, not everyone registers their embryo transfers, but it gives us an idea of the development. In 2012, ten countries reported their equine embryo transfers. Brazil and Argentina reported over 15 000 in vivo embryos collected, while the other eight countries reported under a 100. Since embryo transfer is offered commercially in several other countries, the numbers are most likely much higher. However, looking at the numbers and comparing them to bovine, shows that equine embryo collection and transfer is still a challenge. Using the numbers from Argentina and Brazil, the result is 0,7 embryos per flush. The global average in bovine is 6,68 embryos per flush.[29]

According to the scientific articles, the success rate of embryo transfer programs is ranging from 65-90%.[19,21,25] Dr. Joerg Aurich from the University of Veterinary Medicine, Vienna, Austria, thinks that programs getting bad results are not doing it properly, since the theory behind it is easy enough. Dr. Joanne Smith from Weatherford Equine, Australia, has a very good success rate (around 90% foals born after ET). She thinks the reason behind the good results is that she follows a regime and does the same every time. Consistency is crucial! If changes are made, it is one at the time, and never without careful consideration. This way she

will immediately know the cause of change in success rate. Also being thorough with hygiene from start to end will give better results. However, never use disinfectants in areas that may get close to the embryo! Contact with disinfectants will kill the embryo. Be especially careful with flushing equipment.

In Norway, I was only able to find one example of embryo transfer being done. It is likely that others have tried, but today, no one offers ET commercially in Norway.

Conclusion

There are a lot of challenges with embryo transfer in horses. The difficulties with synchronising mares, the lack of superovulation and the fact that freezing equine embryos is not working very well, are all limiting factors. Regardless of this, there are several big scale embryo transfer programs in the world with high success rate. Breeding two talented and healthy parents will increase the chances of a talented and healthy foal. Embryo transfer makes this possible without interfering too much with the biological parents' sports careers. Breeding healthy horses is something most people want, but trying embryo transfer is expensive, especially in small-scale programs. Also, lack of knowledge makes people reluctant to try. I think the development will be slow, but that embryo transfer in horses will become more common. Especially after more research is done, making it more efficient and economical.

To start up an embryo transfer program in Norway is definitely possible, and I think there is a market for it. The biggest limitation would be the keeping of a recipient herd, since keeping a horse in Norway is very expensive. Also, lack of experience from Norwegian veterinarians on this field could cause poor results in the beginning. With enough money invested, I think Norway can have a good embryo transfer program up and running in a few years.

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