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DEPARTMENT AND CLINIC OF REPRODUCTION

THE CURRENT STATUS OF EQUINE EMBRYO TRANSFER

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Innhold

List of Abbreviations:

AI = Artificial Insemination

AQHA = American Quarter Horse Association

CPA = Cryoprotective agent

DPBS = Dulbecco`s Phosphate Buffered Saline

EPE = Equine Pituitary extract

ET = Embryo Transfer

FSH = Follicle Stimulating Hormone

GnRH = Gonadotropin-releasing hormone

hCG = Human Chorion Gonadotropin

ICSI = Intracytoplasmic sperm injection

IVF = In Vitro Fertilization

LH = Luteinizing hormone

PCR = Polymerase Chain Reaction

PGD = Preimplantation genetic diagnosis

PGF2α = Prostaglandin F2 alpha

1. Introduction:

Embryo transfer has been under development for over a 100 year and has developed greatly since its first successful attempt in 1890 in rabbits. Embryo transfer itself involves the removal of an embryo from the uterus of one mare, and the transfer of the embryo into the uterus of another mare. The first foal produced by embryo transfer was born in 1974. Since then, there has been a tremendous amount of research performed (Patrick M. McCue, 2009). The development of equine embryo transfer has met several challenging obstacles on its way to where it is today, and some of them are still under development to make embryo transfer (ET) available and profitable to be used in commercial breeding performed by clinical equine practitioners.

The number of ETs performed annually world-wide has grown enormously during the last 20 years. It has become more and more normal that practitioners are able to perform ET, which was previously considered a highly specialized technique. The rapid expansion of ET due to technical changes including the development of a non-surgical embryo transfer and the possibility to cool embryos to 5℃ and transport them for 24h without affecting pregnancy rates. Not to mention the downregulation of the strict rules for studbook breeding, which now allows more than one foal per mare each year. Together these changes have created an environment in which horse breeders appreciate the potential of ET for hasting genetic progress. With an increased interest for invitro fertilization via intracytoplasmic sperm injection, and even reproductive cloning by nuclear transfer. (Stout, 2012)

Now embryo recovery and transfer has become a very common technique used in equine industry that can readily be performed by the veterinarian. In the beginning of its use ET was mostly a tool to obtain pregnancies from subfertile mares. (Squires, 2016), mares with poor reproductive history that have been unable to conceive with natural mating or artificial insemination (AI) (Squires, McCue and Vanderwall, 1999). Now breeders have started to take more advantage of the possibilities that ET has given them to obtain multiple foals from a mare in a given breeding season, or mares foaling late in the breeding season are often used as embryo donors instead as broodmares carrying their own foal. This practice allows the foaling mare to remain open so that she can be bred early the following breeding season. (Squires, 2016). Other important benefits of ET are that valuable mares may have more than one foal per year and mares in athletic competition

can donate embryos and remain in training (Patrick M. McCue, 2009). Embryo transfer makes it possible to breed high performing competition horses without taking them out of competition for over a year.

Even with this great progress within embryo transfer there is still room for improvement when it comes to reliable methods/ products for stimulating multiple ovulation and the failure to develop techniques by which the majority of day 7 embryos can be successfully cryopreserved.

1.2. History:

The first successful transfer of mammalian embryos is given to Walter Heape. In 1890 Heape transferred two 4-cell Angora rabbit embryos into an inseminated Belgian doe which subsequently gave birth to four Belgian and two Angora young. A later paper described Heape`s technique for handling rabbit embryos, which involved spearing them on the tip of a needle and transferring them to a recipient without an intermediary step to placing them in holding medium (Squires, McCue and Vanderwall, 1999)

In the late 1970`s, only American Quarter Horse Association (AQHA) accepted equine embryo transfer, and only for use in older, barren mares (Squires, McCue and Vanderwall, 1999). The first successful ET in horse was performed in Japan in 1974 (Patrick M. McCue, 2009).

The ET in the AQHA increased due to change in the regulations of the AQHA. The new regulations permit the registration of an unlimited number of ET foals per mare each year, whereas only one per year was previously allowed. The use of frozen semen for insemination in ET procedures was allowed at the same time. In 2006, the AQHA voted to allow the cryopreservation of embryos starting 2007 (Hasler, 2008). Opening up the possibility of long distance transport of embryos, genetic testing and genetic banking.

Transfer of equine embryos in other countries was generally not used until the mid-1980`s. Today the collection and transfer of fresh embryos is permitted in most breeds, and shipment of cooled and frozen embryos is gained without acceptance. The use of embryo transfer has increased the last decade, but there are fluctuations in the frequency of its use, due to the high expenses connected to embryo transfer. With the high expenses connected to ET only genetically superior animals are used as donors. In addition to the high cost, the unique biological features and technical problems that needs to be overcome to perform a

successful ET in horses, limits its use. Even with the increase in ET only 1500 of 300 000 foals born every year in the united states originate from ET. (Squires, McCue and Vanderwall, 1999)

The number of embryo collections and transfer has increased dramatically in the United States, Brazil, and Argentina. Nearly all breeds in the United States use ET, with the American quarter horse being the leader in ET, because this breed produces 60% of all registered foals each year. The primary driver for ET in Argentina would be the polo horse and in Brazil the sport horses and the native breed, mangalara (Squires, 2016).

2. Brief summery of the mare`s oestrus cycle

Mares are seasonal polyoestrous, during spring the increasing hours of daylight triggers gonadotropin-releasing hormone (GnRH) production in the hypothalamus which stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) production and release in the pituitary gland. This will activate the ovaries in the spring transition period, the mare`s follicles will grow and produce oestrogen without ovulation, until an LH surge will trigger ovulation of the dominant follicle and mark the end of the transition period (Sandra Dögg Gardarsdòttir, 2017). The mare will then cycle regularly until the end of October. One oestrus cycle lasts for approximately 21 days, but may vary between 18 to 24 days, with ovulation being designated as day 0 (Làszlo Solti, 2018).

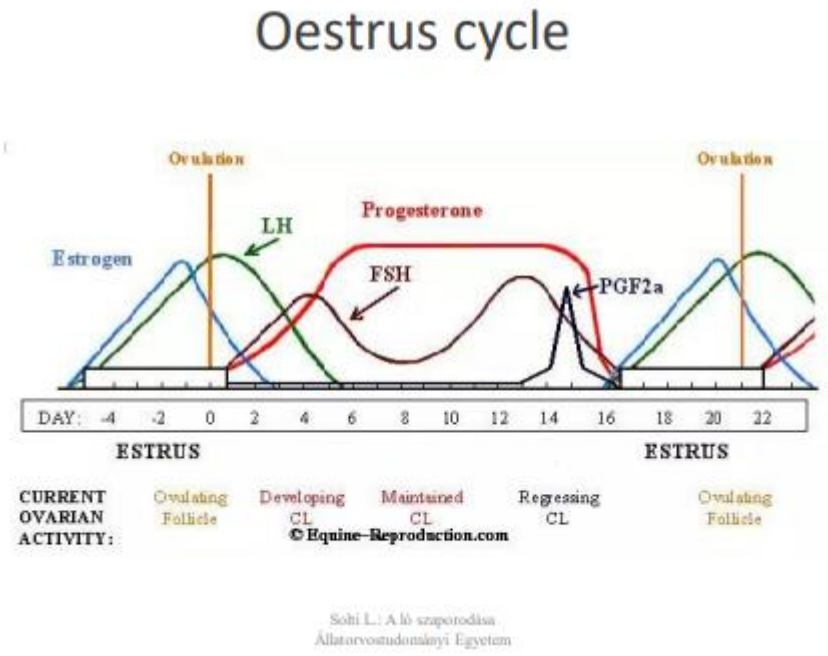

Figure 1:*Showing the change of hormones during the mares estrus cycle (Làszlò Solti, 2018).*

The oestrus cycle can be divided into 4 stages based on behaviour or gonadal events:

- **Proestrus:** is the building up phase. Follicles are developing and produce estrogen.
- **Estrus:** is the phase of sexual receptivity. Increased production of oestrogen by the mare's follicles. Ovulation occurs 24 to 48 hours before the end of oestrus.
- **Metestrus:** in this phase the mares sexual reproductivity ends. After ovulation, the granulosa cells from the developing corpus luteum (CL) starts to produce progesterone.
- **Diestrus:** a mature CL characterize this phase with high concentration of progesterone and low levels of estrogen. In this phase follicles may start to grow but are not able to ovulate. (Sandra Dögg Gardarsdòttir, 2017).

Another way the mare's oestrus cycle may be divided, is into 2 phases based on secretory patterns of the ovary:

- **Follicular phase:** includes Proestrus and estrus. The duration of this phase is 5 to 7 days. In this phase follicles are growing and producing estrogen to provide optimal environment for oocyte maturation, and eventually one dominant follicle is left to ovulate. The increase in estrogen and decrease in progesterone during this phase is responsible for the behavioural changes of the mare and 24 to 48 hours before the end of estrus the mare ovulates.
- **Luteal phase:** includes metestrus and diestrus. When the follicle ovulates, it undergoes structural and functional changes. The granulosa cells transform into luteal cells and start to produce progesterone (P4) instead of estrogen (E2). It takes the CL about 5 days postovulation to reach high levels of progesterone production which will remain until the end of diestrus. The average length of the luteal phase is 14 to 15 days, during which the mare does not show signs of sexual receptivity but follicular waves are already present to prepare here for the next estrus cycle. (Sandra Dögg Gardarsdòttir, 2017).

3. Embryonic development:

The equine pregnancy lasts for 337 days, starting at ovulation and the penetration of the zona pellucida by the sperm cell. In the tubular phase of development, the pronuclei formation and fusion takes place, followed by the mitotic cleavage (morulation) of the zygote. The early blastocyst enters the uterus at day 5/ day 5.5 where it further develops, and embryonic collection will occur by the help of uterine flushing.

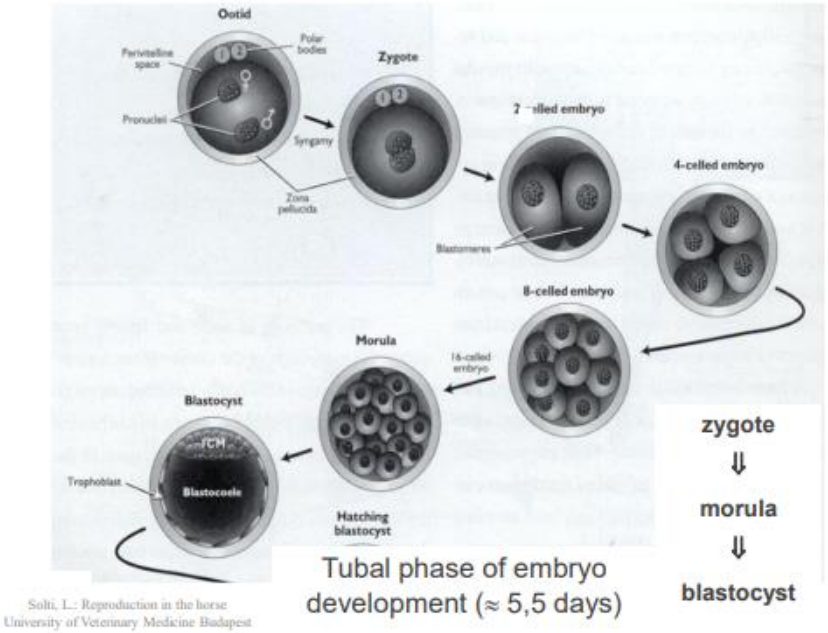

Figure 2: Showing tubal phase of embryonic development until day 5.5 (Làslò Solti, 2018).

- Day 6: Capsule formation.
- Day 8: Hatching from Zona pellucida.
- Day 9: Start of yolk sac formation.
- Day 12: Yolk sac.
- Day 12 to 15: maternal recognition of the pregnancy occurs due to the migration of the embryo between the two horns almost 13 times per day. The production of INF-like protein and estrogen leads to maternal recognition which in turn prevents luteolysis by inhibiting the production of PGF2α.
- Day 15 to 16: Fixation, plus the start of chorionic development.
- Day 17: At this stage the pregnancy may be detected with the help of ultrasonography.

- Day 19: Start of organogenesis, chorion formation and vitelline circulation. Increased cervical tone can be palpated trough the rectum.
- Day 22: Decapsulation.
- Day 24: Heart beat may be detected by the help of ultrasonography.
- Day 25: Development of the chorionic girdle.
- Day 25 to 30: pregnancy may be detected trough rectal palpation as a flexible spherical bulge (tennis ball), close to the bifurcation and on the cranioventral side.
- Day 30 to 40: Development of endometrial cups.
- Day 35 to 40: Placenta starts to develop.
- Day 60 until term: fetoplacental unit. The pregnancy has expanded into the uterine body and the uterus is less tonic (Làslò Solti, 2018).

4. Oestrus synchronisation of the donor and recipient mare:

The viability of a commercial embryo transfer program relies heavily on maintaining and managing surrogate mares, as caring for those animals represents the costliest component of the program (Oliveria Neto et al, 2017). One of the most critical steps for a successful embryo transfer is the synchronization of the oestrus cycle of the two mares, the donor and the recipient. It is very important that the recipient mare`s uterus is ready to receive and nourish the embryo (Christa Leste-Lasserre, 2015). Synchronization of the donor and recipient mare is important but does not need to be as precise as in cattle. In horses the pregnancy rates were similar for recipients that ovulated 1 day before and up to 3 days after the donor (Squires, McCue and Vanderwall, 1999), as indicated in **Figure 3**.

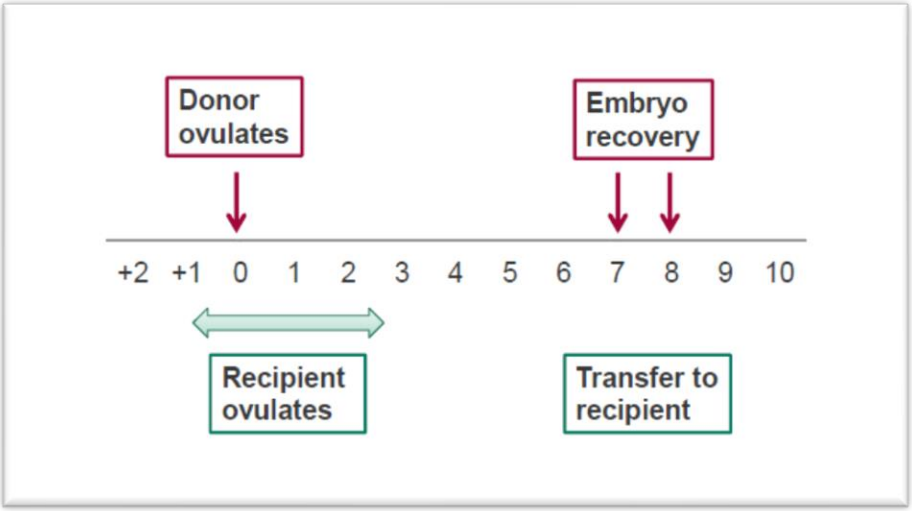

Figure 3: *Synchronisation of ovulation of donor and recipient mare and the timing of the embryo recovery (Juliane Kuhl, 2015).*

The best is to have several recipient mares to choose from that is naturally in oestrus at approximately the same time as the donor mare. Many embryo transfer centres have mare herds to choose from, and breeders can lease the appropriate mare for the season and return her to the facility after weaning. The checking of recipient mares is a costly and time-consuming activity that requires well-trained personnel to carry out large number of scans and proper determination of ovulation synchrony with embryo donor mares. Therefore, hormone treatments allowing synchronization of embryo recipients with donor mares could provide useful to maximize the use of recipient mares and minimize the number of ultrasonography examinations (Oliveira Neto et al, 2017). If the breeder desires a specific mare as recipient, it is possible to synchronize the two mares or cryopreserve the embryo until the recipient mare is at the optimal time in here cycle. Cryopreservation includes several risks for the embryo and the pregnancy, which is further explained later under cryopreservation of embryos.

For the possibility to do a direct transfer of the embryo from donor to recipient mare a precise scheduling of ovulation is crucial for the successful embryo transfer and optimal pregnancy rates. (Norman, Larsen and Morten, 2006). Reported synchronisation protocols may involve the use of prostaglandin F2 alpha (PGF2α) (i.e., dinoprost tromethamine) or its analogs (e.g. sodium cloprostenol), steroid hormones (progestins and oestrogens), and ovulation induction agents such as human chorion gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH) agonists (e.g. deslorelin acetate, histrelin). (Oliveira Neto et al, 2017). The mares cycle is monitored by ultrasound and ovulation is induced with hCG intravenous (IV) when a follicle ≥ 35 mm is detected (Panzani et al, 2014). PGF2α alone is commonly used in cyclic mares to bring them back into oestrus when necessary. Whereas, a combination of oestrogen and progestin is typically used in acyclic and transitional recipient mares or when there is a shortage of recipient mares, demanding a tight-synchrony between one embryo donor and one or two recipients (Oliveira Neto et al, 2017)”

A well-known technique that has produced acceptable and repeatable results; is a daily administration of progesterone and oestradiol by intramuscular injection over a 10-day period. This technique has the disadvantage of requiring daily injections (Norman, Larsen and Morten, 2006). Therefor in 2003 Norman, Larson and Morten looked at the follicular development, onset of behavioural oestrus, ovulation in mares and weather pregnancy could be achieved following the insertion of an intravaginal progesterone releasing device,

combined with a single intramuscular injection of 10mg oestradiol benzoate at the time of insertion and a single intramuscular injection of PGF2α at device removal. The ovarian follicular status was assessed by palpation and ultrasonography at the time of removal, then twice daily until ovulation became imminent. Than follicular size and tone was examined by palpation and ultrasonography trice daily. Once a follicle of 35mm or greater was detected, ovulation was induced by an intravenous injection of human chorion gonadotropin (hCG). Eight mares from the study was inseminated with fresh-chilled semen, while five mares were selected as embryo transfer recipient mares and were not bred to the induced ovulation (Norman, Larsen and Morten, 2006).

Their study showed that:

- The follicle diameter at device removal had an average of 29mm, ranging from18mm to 48mm.
- The time from device removal to the first day of behavioural oestrus was between 1 and 4 days, correlating to the size of the follicle at time of the intravaginal sponge removal.
- The time from device removal to the presence of a 35mm follicle was 2.9 days. All the mares in which ovulation was induced by a hCG injection ovulated within 36 to 48 hours after the follicle reached 35mm.
- Pregnancy results after the treatment was approximately 75% at both day 16 and day 45 post insemination.

This study demonstrate that the treatment regimen has potential to synchronise the commencement of oestrus in a group of randomly cycling mares and have them ovulate approximately 2 days from device removal. The treatment itself is inexpensive and well tolerated by the mares (Norman, Larsen and Morten, 2006). Collectively, hormone treatment of mares in all stages of the oestrus cycle or acyclic mares can be useful to synchronize embryo recipient with donor mares (Oliveria Neto et al, 2017).

5. The donor mare

Factors that affect embryo recovery include the day of recovery, number of ovulations, age of the donor and the donor mare`s reproductive history and the quality of sire`s semen. (Squires, McCue and Vanderwall, 1999)

Day of recovery: embryo recovered at day 6 after ovulation show slightly lower recovery rate than on day 7 or 9 after ovulation. The advantage of recovering the embryo at day 6 is that it is smaller and more viable after freezing and thawing than embryos recovered at day 7 or 9.

Number of ovulations: in single-ovulating mares there is a 50% chance of recovering an embryo. The low number of ovulation increase the time spent and the cost of equine embryo transfer. To increase the chance of recovering an embryo the use of superovulation can be advantageous.

Age of the donor mares: old mares with poor reproductive histories produce fewer embryos. The reason for the reduced embryo recovery can be uterine and oviduct pathology and increased early embryonic death. Oocytes collected from mares >20 years old and transferred into oviduct of young mares resulted in embryonic vesicles in 8/ 26 mares, versus 11/12 pregnancies from transfer of oocytes from young mares. The rate of early embryonic death following transfer from older donor mares is higher than that obtained from younger mares. Fewer embryos from subfertile mares resulted in pregnancies on day 14. The combined low embryo recovery rate from the old, infertile mares and the high rate of loss of transferred embryos from mares greatly limits the number of foals that can be produced from this category of mares. “On the other hand, it was demonstrated that fillies as young as 1 year old are able to produce embryos, although at a lower rate, and that embryos can be recovered from 2-years-old mares in a rate similar to the one of mature mares (Panzani et al, 2014). How age affects rate of ovulation is presented in **Table 2**.

Quality of the semen: generally, mares inseminated with fresh semen are more likely to provide an embryo than those inseminated with either cooled or frozen-thawed semen (Panzani et al, 2014). Table 2 clearly shows the decreasing embryo recovery rate from using fresh semen to frozen.

Type of semen	Embryo recovery rates
Fresh semen	70-80%
Cooled stored semen	50-60%
Frozen semen	30-65%

Table 1: Methods of semen preservation and embryo recovery rates. Adapted from Juliane Kuhl, 2015.

In a retrospective study performed by Panzani et al in 2014, 198 donor mares of different breed, age, reproductive category and use were monitored during 10 breeding seasons at the former Dipartimento di Clinica Veterinaria of the Pisa University, shown in **Table 2**. Out of the 661 cycles observed during this period, 937 ovulations were recorded (mean ovulations/cycle: 1.42 ± 0.58).

The mares were divided into 4 categories:

1. Healthy donor performing sport activity (n =39; SHD): all the donors of this category were non-resident at the university.
2. Healthy donors not performing sport activity (n = 87; NSHD).
3. Donors with reproductive pathologies (mares with an history of endometritis and/ or embryonic loss or abortion, n = 45; or persistent mating induced endometritis, n = 18; RPD).
4. Mares with nonreproductive pathologies (respiratory, n = 3. Neurologic or neuroendocrine, n = 5, and gastrointestinal, n = 1, diseases; NRPD).

Factors	Multiple ovulations/cycle (%)	Ovulation rate (average ± SD)
Age class		
2–10	25/130 (19.23%)[a]	158/130 (1.22 ± 0.47)[a]
11–15	55/163 (33.74%)[b]	223/163 (1.37 ± 0.54)[a]
16–20	115/219 (52.51%)[c]	351/219 (1.60 ± 0.64)[b]
21–24	52/149 (34.90%)[b]	205/149 (1.38 ± 0.54)[a]
Breed		
Show Jumping	206/453 (45.47%)[a]	685/453 (1.51 ± 0.61)[a]
Standardbred	21/85 (24.71%)[b]	107/85 (1.26 ± 0.47)[b]
Quarter Horse	16/77 (20.78%)[b]	95/77 (1.23 ± 0.48)[b]
Haflinger	3/35 (8.57%)[b]	38/35 (1.09 ± 0.28)[a,b]
Arabian	1/11 (9.09%)[b]	12/11 (1.09 ± 0.30)[a,b]
Reproductive category		
SHD	17/76 (22.37%)[a]	94/76 (1.24 ± 0.46)[a]
NSHD	120/348 (34.48%)[b]	477/348 (1.37 ± 0.53)[a,b]
RPD	85/202 (42.08%)[b,c]	298/202 (1.47 ± 0.60)[b]
NRPD	25/35 (71.43%)[c]	68/35 (1.94 ± 0.80)[c]
Total	247/661 (37.37%)	937/661 (1.42 ± 0.58)

[a,b,c]Data designated by different superscripts differ significantly ($P < 0.05$). Pearson Chi-square test.

Table 2: *Factors affecting the occurrence of multiple ovulations per cycle and ovulation rete in 661cycles of 198 donor mares (Panzani et al, 2014).*

The study shown in **Table 2** indicate that mares in the age between 11 and 24 had higher incident of multiple ovulation than mares ≤10 years old, whereas the ovulation rate was significantly higher for the mares between 16 and 20 years of age compared with the others. If we combine the information in **Table 2 and 3** we can clearly see that the ideal age for mares to be bred as embryo donors is between 11 and 15 years of age. This is probably due to the simultaneous presence of a good quality of uterine environment and higher multiple ovulation rate than in younger mares (Panzani et al, 2014).

Show jumping mares had the highest frequency of cycles with multiple ovulations (45.47%) and higher ovulation rate (1.51 ±0.61). Nevertheless, healthy donor mares not performing sport activity (NSHD) gives the most reliable results here since the number of mares in this group was significantly higher than in the others, and most likely the best donor candidate even though the number shows differently in this study (Panzani et al, 2014).

5.1. Superovulation:

Superovulation is defined by Saunders comprehensive veterinary dictionary as production of more than one ovum at ovulation. Reliable and successful induction of superovulation is currently not available for horses (Squires, 2016), even though the first report of successful superovulation was reported in 1974 in Wisconsin (Patrick M. McCue, 2009). The fact that there is a lack of reliable protocols for induction of superovulation of mares in clinical practice, is one of the major factors limiting the outcome of ET programs (Panzani et al, 2014). Consequently, embryo recovery is based on only one ovulation and generally ranges from 50% to 70% embryo recovery per cycle (Squires, 2016). But it has been well demonstrated that the occurrence of multiple ovulations enhances embryo recovery rate. (Panzani et al, 2014). For this reason, spontaneous multiple ovulations are highly desirable in embryo donor mares. Breed, age, reproductive status, season, and the use of drugs to induce ovulation have been reported as affecting the number of double ovulations (Panzani el al, 2014).

Embryo recovery rate in spontaneous multiple ovulating mares may vary depending on the ovulation being ipsilateral or bilateral." Ipsilateral double ovulations resulted in a lower embryo recovery rate and in more unsuccessful recovery attempts than bilateral double ovulations. This is probably due to an alteration of the mechanism of oocyte transport to the oviduct, caused by interference between two or more simultaneous ovulations in the limited space of the ovulation fossa (Panzani et al, 2014)."

Mares used as embryo donors, superovulation, or the induction of the maturation and ovulation of more ova than normal, is usually induced with the injection of gonadotrophin. In the horse follicle stimulating hormone (FSH) purified from porcine or ovine pituitary glands is not reliably to induce superovulation in mares. Equine FSH pituitary extract (eFSH) also called equine pituitary extract (EPE), has proven to successfully induce superovulation in the mare. (Hasler, 2008)

Responses to eFSH in mares are not as large as those seen in cattle and other species, and when large responses do occur, embryo collection rates are usually disappointing. This is because follicles in the mare can only ovulate and release their oocyte trough the ovulation fossa because of the fibrous tunica albuginea that covers the rest of the external surface of the mare ovary. Nevertheless, ovulation rates and embryo rates have proven to be about four times greater with the use of superovulation (Hasler, 2008).

In laboratories the induction of multiple ovulation starts with eFSH treatment 5 to 6 days after ovulation, then PGF2α is administer the day after the initial eFSH treatment, an eFSH treatment is continued until at least 2 follicles >35 mm is obtained. The mare is then treated with hCG to synchronize ovulations. This has induced multiple ovulations in approximately 75% of the mares, with a mean ovulation rate of approximately 2 embryos per mare, which is about 3 times more than for untreated mares. (Squires, McCue and Vanderwall, 1999)

5.2. Insemination:

During the donor mare's oestrus, follicular development on the ovary and changes in the uterus are monitored daily with ultrasonography. Mares are generally inseminated either with fresh, cooled or frozen-thawed semen (Squires, McCue and Vanderwall 1999). Artificial insemination is performed 48 hours or 24 hours prior to ovulation with fresh or cooled semen respectively, or within 6 hours of ovulation if frozen semen is used (Panzani et al, 2014). The response of the uterus to breeding is monitored by ultrasonography, and any mare showing post breeding-induced endometritis after insemination is treated with oxytocin, prostaglandin and/ or uterine lavage (Squires, McCue and Vanderwall 1999, Panzani et al, 2014).

A common belief is that fresh semen is more fertile than frozen one, which will negatively influence embryo recovery rate, was confirmed in the study by Panzani et al in 2014. AI with fresh, cooled and the association of cooled plus frozen semen resulted in significantly higher embryo recovery per cycle than frozen semen. While AI with fresh semen resulted in significantly higher embryo recovery per cycle than frozen semen AI and in a significantly higher embryo recovery per ovulation than cooled or frozen semen artificially inseminated (Panzani et al, 2014), as indicated in **Table 1** (adapted from Juliane Kuhl, 2015).

6. Embryo collection:

The factors affecting embryo recovery are age, breed, reproductive category, type of semen, number of ovulations, and location of artificial insemination (Panzani et al., 2014). According to the retrospective study done by Panzani et al in 2014 the flushing protocol and day of flushing had no effect on embryo recovery.

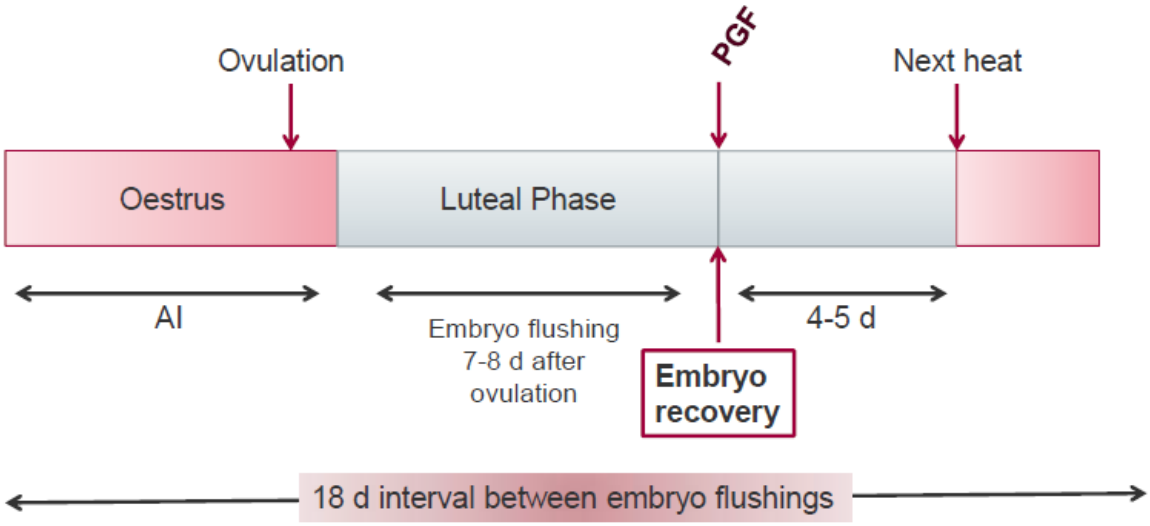

Figure 4: *Timing of embryo recovery in the donor mare (Juliane Kuhl, 2015).*

Embryo recovery is generally scheduled 7 to 8/ 10 days after ovulation, with day 0 being designated the day of ovulation, as indicated in **Figure 4**. Donor mares are placed in a stock, the rectum is evacuated of faeces, the tail wrapped and tied up, and the peritoneum washed three times with a povidone iodine soap, rinsed and dried accurately (Penzani et al, 2014). The embryo recovery procedure includes using a sterile technique, by introducing a silicone catheter (28–34 in. long, French size 28–37, depending on the size of the reproductive tract of the mare) through the cervix and into the uterus, and then inflate the balloon (typically 60–80 mL) with air or collection medium; the catheter is then gently pulled back so the cuff seals the internal cervical Os (Carlos R. F. Pinto, 2018).

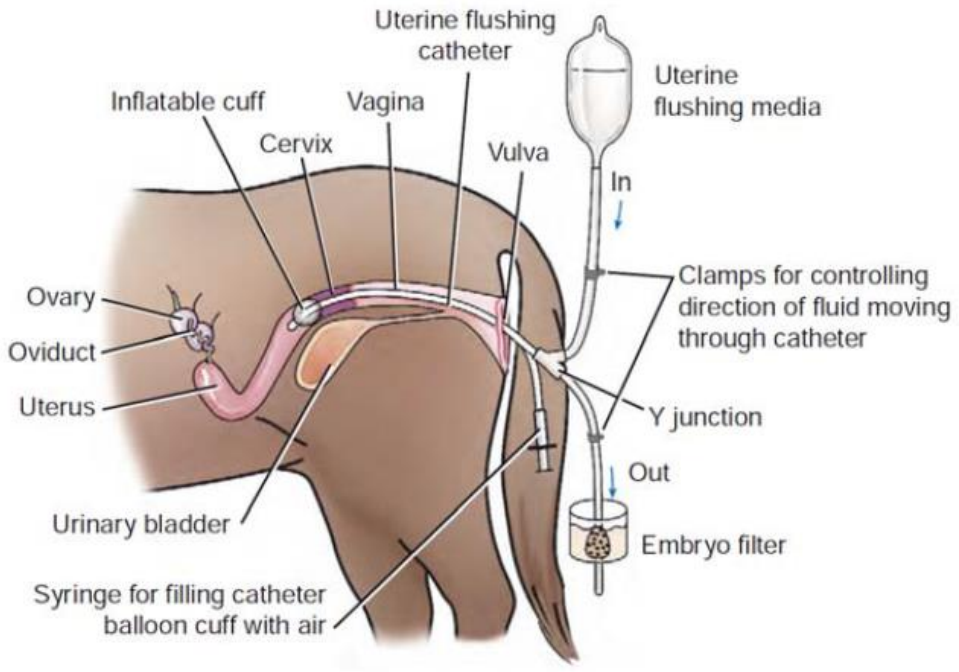

Figure 5: *Showing uterine flushing for embryo recovery. This picture nicely describes where the cuff is inflated, how the flushing media enters the uterus, the collection of the flushing media trough the catheter and embryo filtration (Juliane Kuhl, 2015).*

Type of lavage used during flushing can differ, using either **1:** 3 to 4 L of Dulbecco`s phosphate-buffered saline (DPBS) containing 1% foetal calf serum (FCS (Squires, McCue and Vanderwall, 1999), **2:** 3 L DPBS containing 0.4% BSA (Panzani et al, 2014) or **3:** 1 to 2 L Ringer Lactate (Juliane Kuhl, 2015). Flushing should be repeated 4 times (Juliane Kuhl, 2015) the fluid is recovered through tubing system by gentle manipulation of the uterus trough the rectum to aid the flowback of the flushing medium (see **Figure 5**), with a filter cup connected to the tubing system. The contents are poured into a search dish and examined under a stereomicroscope for the presence of an embryo. (Squires, McCue and Vanderwall, 1999 and Panzani et al, 2014). After the flushing, 3mg of alfaprostol is injected IM to the donor mare to induce luteolysis. The recovered embryos are washed and submitted to morphological evaluation or directly transferred to the recipient mare (Panzani et al, 2014).

In the retrospective study performed by Panzani et al in 2014, positive flushing per cycle and embryo recovery per ovulation were not different between day 7, 8, 9, and 10. In this study, the occurrence of multiple ovulations enhanced the embryo recovery rate, however the embryo recovery per ovulation was significantly lower after multiple ovulations compared with a single one. These data from this study is shown in **Table 3** and are similar to previous studies where multiple ovulations affected positively the embryo recovery in non-treated and superovulated mares (Panzani et al, 2014).

Factors	Positive flushes/cycle (%)	Embryos/cycle (%)	Embryos/ovulations (%)
Flushing protocol			
FP1	39/104 (37.5%)[a]	43/104 (41.3%)[a]	43/158 (27.2%)[a]
FP2	259/557 (46.5%)[a]	295/557 (53.0%)[a]	295/779 (37.9%)[a]
Age class			
2–10	61/130 (46.9%)[a,b]	66/158 (41.8%)[a]	66/158 (41.8%)[a]
11–15	85/163 (52.1%)[a]	100/163 (61.3%)[b]	100/223 (44.8%)[a]
16–20	89/219 (40.6%)[b]	105/219 (47.9%)[a]	105/351 (29.9%)[b]
21–24	63/149 (42.3%)[a,b]	67/149 (45.0%)[a]	67/205 (32.7%)[a]
Breed			
Show Jumping	183/453 (40.4%)[a]	215/453 (47.5%)[a]	215/685 (31.4%)[a]
Standardbred	49/85 (57.6%)[b]	52/85 (61.2%)[b,c]	52/107 (48.6%)[b,c]
Quarter Horse	49/77 (63.6%)[b]	54/77 (70.1%)[b]	54/95 (56.8%)[b,c]
Haflinger	13/35 (37.1%)[a]	13/35 (37.1%)[a,c]	13/38 (34.2%)[a,c]
Arab	4/11 (36.4%)[a,b]	4/11 (36.4%)[a,b,c]	4/12 (33.3%)[a,c]
Reproductive category			
SHD	41/76 (53.9%)[a]	42/76 (55.3%)[a]	42/94 (44.7%)[a]
NSHD	178/348 (52.6%)[a]	208/348 (59.8%)[a]	208/477 (43.6%)[a]
RPD	65/202 (32.2%)[b]	71/202 (35.1%)[b]	71/298 (23.8%)[b]
NRPD	14/35 (40.0%)[a,b]	17/35 (48.6%)[a,b]	17/68 (25.0%)[b]
Type of semen			
Fresh	81/148 (54.7%)[a]	90/148 (60.8%)[a]	90/197 (45.7%)[a]
Cooled	118/266 (44.4%)[a]	136/266 (51.1%)[a,c]	136/392 (34.7%)[b,c]
Frozen	87/226 (38.5%)[b]	99/226 (43.8%)[b,c]	99/318 (31.1%)[b,c]
Cooled + frozen	12/21 (57.1%)[a]	13/21 (61.9%)[a,c]	13/30 (43.3%)[a,c]
Number of ovulations			
1	178/414 (43.0%)[a]	178/414 (43.0%)[a]	178/414 (43.0%)[a]
2	108/220 (49.1%)[a]	140/220 (63.6%)[b]	140/440 (31.8%)[b]
3	12/25 (48.0%)[a]	20/25 (80.0%)[b]	20/75 (26.7%)[b]
4	0/2 (0.0%)[a]	0/2 (0%)[a,b]	0/8 (0.0%)[a,b]
Day of flush			
7	16/35 (45.7%)[a]	16/35 (45.7%)[a]	16/47 (34.0%)[a]
8	253/546 (46.3%)[a]	287/546 (52.6%)[a]	287/770 (37.3%)[a]
9	21/63 (33.3%)[a]	27/63 (42.9%)[a]	27/99 (27.3%)[a]
10	8/17 (47.1%)[a]	8/17 (47.1%)[a]	8/17 (47.1%16)[a]
Location of AI			
AI@department	155/384 (40.4%)[a]	179/384 (46.6%)[a]	179/569 (31.5%)[a]
AI@home	143/277 (51.6%)[b]	159/277 (57.4%)[b]	159/368 (43.2%)[b]
Total	298/661 (45.1%)	338/661 (51.1%)	338/937 (36.1%)

[a,b,c]Data designated by different superscripts differ significantly ($P < 0.05$). Pearson Chi-square test.

Table 3: *Factors analysed for embryo recovery in 661 cycles of 198 mares in the retrospective study done by Panzani et al in 2014 (Panzani et al, 2014).*

A number of studies agree that old age and history of subfertility are related to a lower embryo recovery rate. In this study, healthy mares, performing or not performing sport activity, showed significantly higher positive flushing than mares with reproductive pathology. Healthy mares, performing or not performing sport activity, had a positive influence on embryos per cycle and embryos per ovulations rate, whereas mares affected by reproductive pathologies had a negative influence on flushing rates (Panzani et al, 2014).

After embryo recovery the embryo is washed and attached cells are removed. During the morphological evaluation of the embryo a scoring system is used, the score indicates the embryo quality and size.

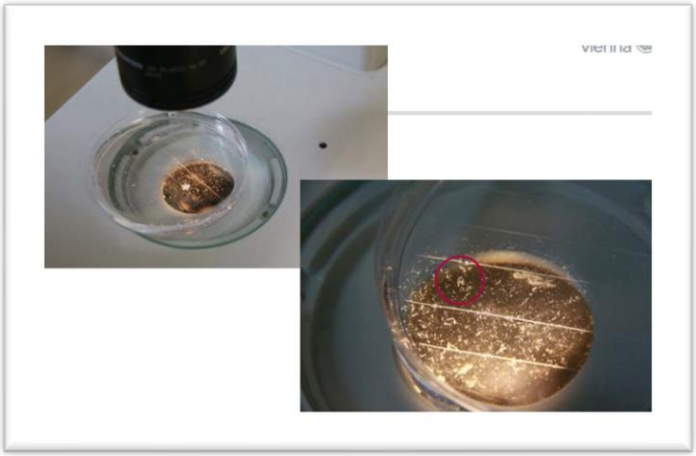

Figure 7: *Embryo detection and morphological evaluation of the embryo under stereomicroscope (Juliane Kuhl, 2015)*

The embryo is then either aspirated into 0.5ml straw as indicated in **Figure 8** and transferred directly into the recipient mare, or the embryo is cooled and shipped to another facility, sent for genetic testing or prepared for cryopreservation.

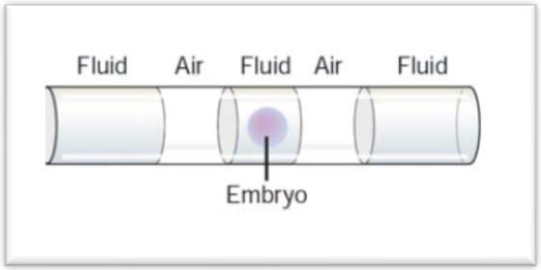

Figure 8*: Shows how fresh sperm is to be aspirated into a 0.5ml straw before embryo transfer into recipient mare (Juliane Kuhl, 2015).*

7. Recipient mare before embryo transfer:

Proper selection and management of recipient mares is an important factor affecting the success of an equine embryo transfer program. The selection criteria include optimal weight (400 to 450 kg), age (3 to 10 years), a gentle disposition, and a good mammary development (Squires, McCue and Vanderwall, 1999). Mares that have a history of producing foals or maiden mares may be preferred as recipients (McKinnon and Squires, 1988). Recipients need to have normal oestrus cycle and to be free of ovarian and uterine abnormalities such as uterine fluid, uterine cyst, air or debris in the uterus, ovarian tumours or other abnormalities of the ovaries.

On day 4 to 5 post ovulation, the recipient mare is examined with ultrasonography for the presence of corpus luteum (CL) and absence of any uterine fluid or endometrial folds. The mare is also examined trough the rectum to check the uterine and cervical tone (Squires, McCue and Vanderwall, 1999). In a study performed by Carnevale et al in 2000 a relationship between the uterine tone and progesterone serum concentration in the mare was detected. Showing that a low concentration of progesterone correlates with a low uterine tone and that a high concentration of progesterone correlates with a high uterine tone (Carnevale et al, 2000). If the mare "pass" the test, she can be used as a recipient for the next 5days (Squires, McCue and Vanderwall, 1999).

Ovariectomized, progesterone treated mares can also be used as recipients, and have similar pregnancy rate as intact progesterone treated mares. Progesterone treatment of ovariectomized mares must be continued for approximately 120 days. (Squires, McCue and Vanderwall, 1999) While non-cycling mares that has entered the transition period early in the year can be used as recipient mare if treated with progesterone for 5 to 7 days or until the mare ovulate and develop a functional CL. (Squires, McCue and Vanderwall, 1999)

Another important factor to take into consideration is the size of the donor and recipient mare must be approximately the same. A research done by the Pauline Peugnet and her team at the French Agricultural Research Institute showed that embryos put into recipient mares smaller or bigger than the donor mare affected the size of the foal and the duration of the pregnancy. Embryos donated from saddle horse mare and put into a recipient pony mare weight 37% less than foals born from saddle mares, but they spent on average two weeks longer in the uterus. While embryos donated from a pony mare and transferred into a recipient draft horse mare were 57% heavier at birth and spent one week less in the uterus (Christa Lesté-Lasserre, 2015).

8. Embryo transfer:

Embryo transfer (ET) is the process of moving one or more embryos from the reproductive tract of one female to another. Transfer may be conducted by either surgical or nonsurgical procedures and may involve transferring the embryo from either the oviduct or uterus of a donor, into the oviduct or uterus of a recipient. Embryo transfer also may involve the transfer of a laboratory-produced embryo, such as those made by in-vitro or cloning procedures, into the reproductive tract of a recipient (Hasler, 2005).

8.1. Surgical embryo transfer:

Surgical ET is done by a flank incision. The mare is restrained in a stock, tranquilized, and site of incision anesthetized. The tip of the uterine horn is exteriorized, and the embryo deposited through a small puncture site (Squires, McCue and Vanderwall, 1999). This procedure gives a pregnancy rate ranging from 65% to 75%. Although surgical transfer of equine embryos was once thought to yield higher pregnancy rates than nonsurgical transfer techniques, the latter are now the preferred method to transfer horse embryos (Carlos R. F. Pinto, 2018).

8.2. Non-surgical embryo transfer:

A majority of equine embryos are transferred non-surgically into the uterus of recipient mares (Patrick M. McCue, 2009). Non-surgical embryo transfer is done transvaginal. Recipients are generally sedated, and the perineal area scrubbed prior to transfer (Hasler, 2008). The embryo is loaded into a 0.25 or 0.5 ml straw and inserted into the body of the uterus using an embryo transfer gun (Squires, McCue and Vanderwall, 1999), or a standard insemination pipette/ rod which is covered with a protective sheath may be used to transfer the embryo trough the cervix and into the uterine body or one of the uterine horns of the

recipient mare where the embryo is deposited (Patrick M. McCue, 2009 and Hasler, 2008) . Transfer of the embryo must occur within 30 – 60 minutes after flushing when stored at room temperature, but if the embryo is cooled to 5℃ the embryo transfer can occur within 24 hours (Juliane Kuhl, 2015). The success of non-surgical ET depends on the skill and experience of the practitioner, the pregnancy rate can range between 50-75% (Squires, McCue and Vanderwall, 1999).

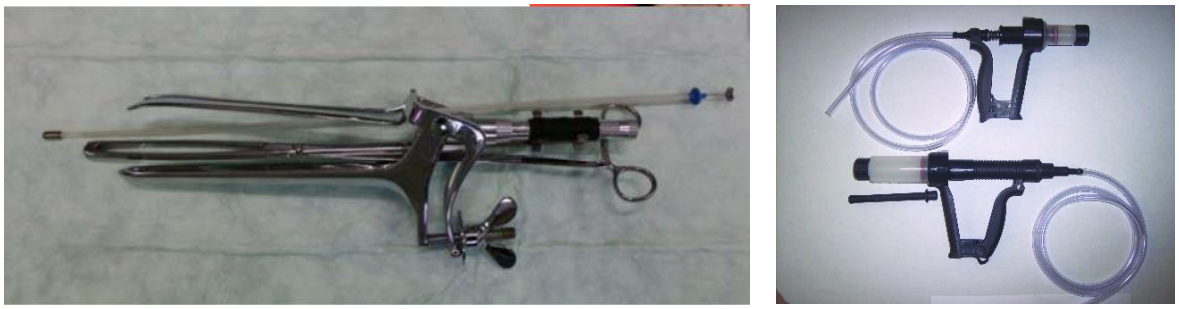

Figure 9: the left picture is showing a standard insemination rod (Juliane Kuhl, 2015). The right picture shows an embryo transfer gun (Har-vet.com, 2018).

9. Recipient mare after embryo transfer:

Factors that may affect pregnancy rates after embryo transfer include method of transfer, synchrony of the donor and recipient, embryo quality, and management of the recipient (Squires, McCue and Vanderwall, 1999). As with all species, the health and management of the recipients is probably the single most important factor in ensuring a high pregnancy rate following embryo transfer (Hasler, 2008).

After the embryo transfer the owner do not have to wait long to find out if the recipient mare ‘accepted’ or ‘took’ the embryo since the embryo is already 7 or 8 days old at the time of transfer. If the transfer is successful, the embryo can usually be observed on ultrasound examination 5 to 7 days after transfer. Follow up pregnancy examinations are encouraged as some recipient mares do not stay pregnant to term. A majority of embryo losses occur prior to day 50 of pregnancy and the loss rate is generally highest for embryos recovered from older mares (Patrick M. McCue, 2009). During the pregnancy proper nutrition and care is important for a healthy and optimal embryonic development, for the mare’s health, milk production after delivery, the delivery itself and her recovery after foaling.

10. Storing embryos:

10.1. Cooling of embryos:

It is possible to store embryos at 5°C and transport them to recipient mares. In that case the embryos are collected by practitioners on the farm, cooled to 5°C in a passive cooling unit and shipped to an embryo transfer station without a major decrease in fertility (Squires, McCue and Vanderwall, 1999).

Figure 10: Showing a equitainer used for storing cooled embryos (Systems, 2018).

After embryo recovery the embryos are washed with several drops of DPBS with 10% FCS; they are than placed into a snap-top plastic Falcon tube containing HAM`s F10 that has been previously gassed with a mixture of 5% CO2, 5% O2 and 90% N2. Next, the tube is immersed in 45 ml of the same medium within a 50ml centrifuge tube, which is also sealed with a cap and parafilm. The tubes are then placed into the specimen cup of a passive cooling unit. The equitainer is taken to the nearest airport and shipped counter-to-counter or by an overnight courier service" (Squires, McCue and Vanderwall, 1999).

10.2. Cryopreservation of embryos

Cryopreservation of equine embryos has been a work in slow progress, from the first ET of a frozen-thawed equine embryo was reported in 1981, the pregnancy failed at approximately day 60. The first frozen embryo foal was born the following year in 1982, it was the only survivor from 3 pregnancies resulting from 11 transferred embryos. The development has been slow due to high cost of obtaining sufficient embryos for research, the lack of reliable protocols for superovulation and that IVF have also been slow in its development (Stout, 2012).

Cryopreservation of embryos opens up opportunities for the breeder to choose a specific recipient mare which oestrus cycle is not appropriately synchronized with the donor mare for every flush (Stout, 2012). This will minimize the number of recipients and, thus, decrease the cost of embryo transfer (Squires, 2016). The embryos can be frozen for years

until the breeder wants to use them, sell them or ship them to other countries (stout, 2012), opening a marked for export or import of embryos (Squires, 2016). The possibility to transport frozen embryos pose less health risk than transporting live animals across the borders (Squires, 2016).

Embryo cryopreservation would also allow temporary genetic banking which would be advantageous to the owners of young mares from interesting bloodlines that has still to prove themselves in competition; embryos could be frozen when the mare was young and fertile and transferred at a later date only if the mare proved its worth. Unfortunately, equine embryos do not tolerate being frozen as well as other species (Stout, 2012)

A more recent development for which cryopreservation is desirable is pre-implantation genetic diagnosis, during which a small number of cells are recovered from ET-stage embryos to test for specific heritable diseases. After the biopsy procedure, the embryos are ideally stored until the test results are available so that only embryos free of the genetic defect are transferred (Stout, 2012). Cryopreservation of embryos has also opened up the possibility of in-vitro produced embryos to be taken out of culture as a morulae or early blastocyst and frozen at the appropriate size and stage of development (Squires, 2016).

10.2.1. Freezing Techniques:

Two major techniques have been described for the cryopreservation of equine embryos, slow freezing and vitrification (i.e. ultra-fast freezing).

Slow-freezing involves the gradual exposure of the embryo to a cryoprotective agent (CPA), prior to carefully controlled cooling in stages (Stout, 2012). Classical CPA used for slow-freezing equine embryos is glycerol (10%: 1,36M) with which the embryo is equilibrated by incubation in 2-4 solutions of increasing concentration (stout, 2012).

Other CPA are; DMSO, 1,2-propanediol, ethylene glycol (1,5M) and Methanol (2,5M) has also been tested because earlier studies suggested that exposure to glycerol was responsible for most of the damage to the embryos at the level of cellular ultra-structure and metabolic activity during cryopreservation, not the freezing itself. These alternative CPA had lower molecular weight, that was expected to facilitate their entry into embryonic cells (Stout, 2012).

As summarised in the article “the current status of equine embryo transfer” by Squires, McCue and Vanderwall, in 1999. In 1989 Seidel et al. evaluated the viability of equine embryos cryoprotected with 1.2-propanediol. All of the small embryos (130 to 175µ) showed good morphology at the end of the culture period, whereas none of the embryos larger than 1000µ survived cryopreservation. In 1993 Meira et al compared pregnancy rates resulting from embryos cryopreserved in either glycerol or 1.2-propanediol at different stages of embryonic development. Embryos recovered at day 6 and day 7 after ovulation showed this pregnancy rate:

- In glycerol:
    - 1/12 for blastocysts and expanded blastocysts resulted in pregnancy.
    - 6/15 for tight morula/ early blastocysts resulted in pregnancy.
- In 1.2-propanediol:
    - 0/15 for tight morula/ early blastocysts resulted in pregnancy.
- Fresh embryos
    - 12/15 resulted in pregnancy

They concluded that glycerol was superior to 1.2-propanediol as a cryoprotectant, and that blastocysts and expanded blastocysts were unsuitable for cryopreservation.

“During slow-freezing, the embryo is exposed to twin threats, ice crystal formation and dehydration. As cooling proceeds, extracellular ice forms gradually leaving solutes behind, such as the osmolarity of the remaining extracellular fluid rises. To balance the intracellular and extracellular solute concentrations, water flows out of the cells (the intracellular fluid is not yet frozen) causing them to dehydrate. Establishing the optimal cooling rate is there for critical, because, if cooling proceeds too rapidly lethal intracellular ice crystals will form, on the other hand, if cooling is too slow, the cell will be damaged by severe dehydration and solute toxicity. This explains why slow freezing of embryos requires a programmable freezing machine, thereby significantly increasing start-up costs, and why the straws need to be ‘seeded’ at -6 to -7°C. Seeding initiate coordinated ice crystal formation throughout the extracellular fluid with the aim of avoiding ‘supercooling’, that is, cooling below the normal freezing point without ice formation. Slow-freezing is also a time-consuming process with pre-freezing equilibration taking an average of approximately 40 min, while the freezing process itself lasts 1.5-2 hours (Stout, 2012).”

The advantage of the slow cool approach is the acceptable pregnancy rate (60% to 80%) that can be obtained when freezing small, early blastocyst embryos. The main disadvantage with slow freezing is that it is time consuming and the need for an expensive programmable freezer. Although many bovine practitioners doing ET would have the knowledge and equipment, it is unusual for bovine ET practitioners to also perform equine ET (Squires, 2016).

Vitrification: The other option for cryopreservation of equine embryos is vitrification. This technique is an ultra-rapid cooling method that prevents ice crystal formation. This method has the advantage of being fast and does not require elaborate equipment (Squires, 2016). In addition, with the use of vitrification instead of slow-freezing the embryo can be transferred directly into the mare without removal from the straw, because cryoprotectants can be diluted in the straw (Hudson et al, 2006).

The first reported pregnancy from vitrified horse embryos was in 1994 (Stout, 2012), and since then there has been a growing interest in the field, this is because it gives us an alternative approach to cryopreservation that enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice crystal formation. Vitrification simplifies and frequently improves cryopreservation because it eliminates mechanical injury from ice, eliminates the need to find optimal cooling and warming rates, eliminates the importance of differing optimal cooling and warming rates for cells, eliminates the need to find a frequently imperfect compromise between solution effect injury and intracellular ice formation, and enables cooling to be rapid enough to "outrun" chilling injury (Fahy and Wowk, 2014). It provides a simpler, faster and cheaper method for cryopreservation than conventional slow-freezing. It does not require an expensive programmable freezing machine, and equilibration and freezing combined lasts less than 15 minutes in total (Stout, 2012).

The challenges presented using vitrification as a method for cryopreservation is the complicated osmotic effects of adding and removing cryoprotective agents, and the increased risk of cryoprotectant toxicity during the addition and removal of cryoprotectant (Fahy and Wowk, 2014). Due to the high risk of embryo toxification during vitrification it is critical to adhere precisely to the recommended durations of immersion in the various solutions, in particular the final solution with the highest CPA concentration. This is more difficult than it sounds, because the solutions with such high CPA concentration are very dense and the embryo sinks surprisingly slowly; experience in finding and moving embryos in dense solutions is therefore a prerequisite for success with vitrification (Stout, 2012)

Vitrification uses ultra-fast freezing to induce and to instantaneous transition both the intra- and extra-cellular fluids from the liquid to the solid, gas-like phase ('solidification') without ice formation. Solidification is, however, only possible when very high concentration of CPA is used (approximately 4-5 times higher than for slow freezing), and when the temperature is reduced very rapidly, that is, by direct immersion in liquid nitrogen (Stout, 2012).

Vitrification of equine embryos is done by collapsing a 7- or 8-day old embryo to empty most of its fluid before storing it in liquid nitrogen. The collapsed embryo regains its normal shape and fluid volume after thawing and passing through a series of fluid. This vitrification process currently yields a 70% pregnancy/ live foal rate (Christa Lesté-Lasserre, 2015).

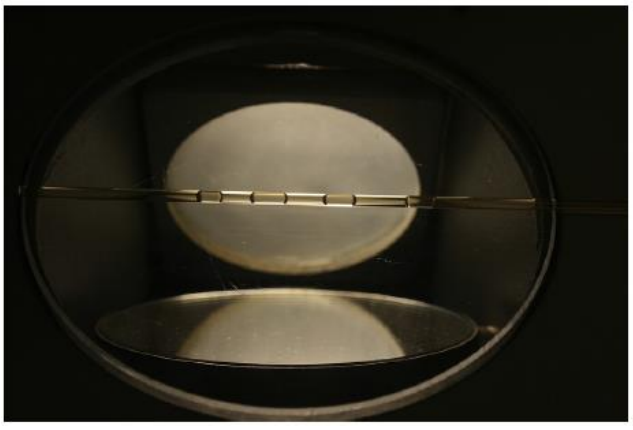

Figure 10: *Embryo in a straw ready for vitrification (Squires. 2016).*

Figure 11: *Embryo in the straw is placed in a goblet suspended in liquid nitrogen (Squires, 2016).*

CPA toxicity is a big problem in vitrification due to the high concentration of CPA needed; fortunately, a large number of remedies for the latter problem have been discovered over the past 30 + years, and the former problem can in most cases be eliminated or adequately controlled by careful attention to technique (Fahy and Wowk, 2014).

The volume of medium surrounding the embryo in the straws during cooling can decrease the temperature from 2500°C/min to 20 000°C/min by stretching the straw to diminish its internal diameter, this is called the open-pull straw technique, or by using cryotops or cryoloops. This accelerated cooling rate ensures rapid passage through the critical cryoinjury zones and reduces the concentration of CPAs required (Stout, 2012).

A study presented in 2006 by Hudson et al compared pregnancy rates after transfer of equine embryos vitrified within 1h of collection of cooled for 12 to 16 hours before vitrification. In the study 40 mares were superovulated using eFSH. Embryos were recovered 6.5 days after ovulation or 8 days after hCG treatment. The 40 morulae or early blastocysts with a grade of 1 to 2 and <3µm in diameter were randomly assigned to 1of 2 treatments:

- Group 1 (n = 20): washed 4 times in a commercial holding medium and then vitrified.
- Group 2 (n = 20): washed 3 times and then stored in the same holding medium at 5°C to 8°C in a passive cooling device for 12 to 19 hours before vitrification.

Thawing of the embryos were done by holding the straw in the air at room temperature for 10 seconds and then submerged in a water bath holding the temperature of 20-22°C for an additional 10 seconds. The content of the straw was transferred by the use of a special cassougun, directly into a recipient mare that had ovulated 4 to 6 days previously. Each embryo was transferred into the recipient transcervically within 8 minutes after removal of the straw from the liquid nitrogen tank.

Pregnancy rates were not significantly different between embryos vitrified after collection and embryos cooled for 12 to 19 hours before vitrification, giving an overall pregnancy rate of 70% as shown in **Table 4** (Hudson et al, 2006).

Effect of embryo developmental stage on pregnancy rates (%) after nonsurgical transfer			
Developmental stage	Cooled/ Vitrified	Vitrified immediately	Combined
Late morulae	5/7 (70%)	4/4 (100%)	9/11 (82%)
Early blastocysts	8/13 (62%)	11/16 (69%)	19/29 (66%)
Combined	13/20 (65%)	15/20 (75%)	28/40 (70%)

Table 4: *showing pregnancy rate on embryos cooled and then vitrified and embryos vitrified immediately after collection (Hudson et al, 2006).*

This finding means that embryos can be collected on the farm and then cooled and shipped to an embryo transfer facility for subsequent transfer and vitrification without a decrease in fertility (Squires, 2006).

10.2.2. Significance of the embryo size for successful cryopreservation:

The reason for the failure of large equine embryos to survive cryopreservation has not been clear, but it has been believed that it is related to the low permeability of the equine blastocyst to cryoprotectants and its size (Hasler, 2008). Recent experiments have proved that the problem lies with the structure of the embryo. When the embryo enters the uterus from the oviduct it is 150 to 220µm in size and has the morphology of a morula or early blastocyst. Within 0.5 to 1.0 days the embryo will increase in diameter to greater than 300µm and become a blastocyst. As an embryo expands beyond 300µm (day 6 and 7), the blastocoel fluid increases dramatically and the zona pellucida surrounding the embryo becomes thinner and is replaced by an acellular capsule. It is thought that the large volume of the embryo and capsule make it difficult for cryoprotectants to penetrate the inner cell mass in sufficient quantity to prevent chilling injury during cryopreservation (Squires, 2016).

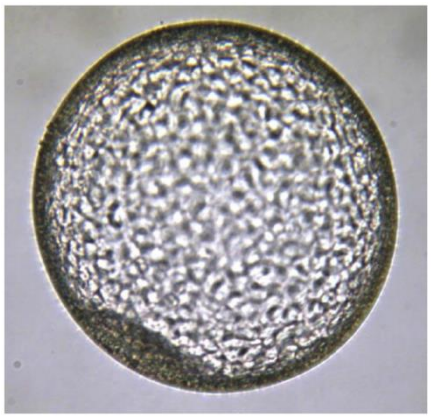

Figure 12: *Expanded blastocyst with an acellular capsule and no zona pellucida. (Squires, 2016)*

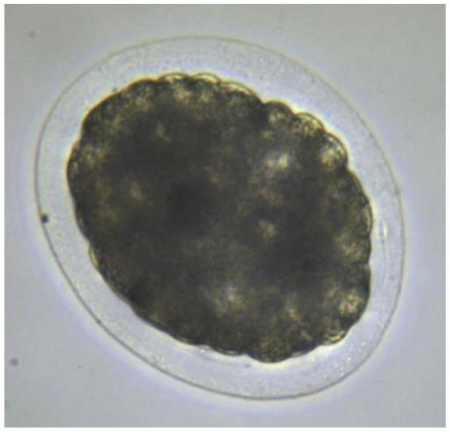

Figure 13: *Embryo with a zona pellucida (Squires, 2016).*

A major obstacle to the implementation of embryo cryopreservation in the field is that acceptable pregnancy rates (>55%) are, at present, achievable only with embryos recovered at an early stage of embryonic development; at day 6 to 6.5 at morula to early blastocyst stage, at the size <300µm in diameter, which is very shortly after their arrival in the uterus. Vitrified embryos > 300µm show a reduced ability to re-expand during post-warming incubation and very rarely results in pregnancy. Whereas larger embryos cryopreserved by slow-freezing do yield normal pregnancies, even though at a lower pregnancy rate (<20%) than for small embryos (Stout, 2012).

A solution to the problem would just be to harvest the embryos while still under 300µm. The embryo will only remain this small a few hours after the arrival to the uterus. In addition to this the exact time of uterine entry and rate of embryo development appear to vary, depending for example on the time of year, type of semen used (fresh vs. frozen) and age of the donor mare. Flushing to early risks a fall in embryo recovery because a proportion has yet to exit the oviduct, while flushing to late will result in recovery of an embryo too large to freeze successfully. (Stout, 2012).

The attempts to recover embryos of a size appropriate for freezing are labour intensive and by no means certain to succeed (Stout, 2012). Knowing the exact time of ovulation allows the veterinarian to schedule the flush at the appropriate time to recover an embryo less than 300µm. This generally requires performing ultrasound on the mare several times per day while in oestrus to pinpoint the time of ovulation (Squires, 2006).

Two methods have been developed to make early embryo recovery “easier” and more successful. The first method is done by flushing the mare with human chorionic gonadotropin (hCG) exactly 8 days after the induction of ovulation and assumingly the mare will ovulate 36 hours after hCG administration. Commercially this method is difficult to use because of the variation in both the rate of embryo development and the exact timing of ovulation after hCG injection. The second method is the application of PGE2 gel to the oviduct ipsilateral to ovulation, 4 days after ovulation this pharmacological induction of premature embryo transit trough the ampullary-isthmus junction allowed recovery of early stage embryos from the uterus one day later. This method has not been adopted for clinical use, primarily because the PGE2 has to be applied via laparoscope, and this does not really appeal to owners of valuable mares (Stout, 2012).

10.2.3. Freezing expanded blastocysts:

While it's not entirely clear why larger blastocysts are more susceptible to cryodamage, contributing factors probably include the increase in embryo size and blastocoel volume, the rapid increase in cell number during this period, the associated intense mitotic activity, the presence of the blastocyst capsule, and the failure to tailor freezing techniques to the specific requirements of expanded blastocysts. (Stout, 2012).

In the case of slow-freezing it is likely that the cooling rate currently applied are simply not suitable for expanded blastocysts, which may require more time than for morulae or early blastocysts to reduce their intracellular water content during the dehydration process that accompanies ice formation. The increase in surface area and volume will significantly affect their ability to take up CPA and to lose water during equilibration. Another explanation to the problem is the formation of the acellular glycoprotein capsule soon after the embryo arrives in the uterus, and in association with blastocyst formation. There has been attempts to improve CPA penetration by partially digesting the capsule with trypsin prior to freezing, this have produced conflicting results. Trypsin will make the capsule thinner decrease the cytoskeleton disruption during the freezing, but it will also make the capsule sticky and more prone to loss during subsequent embryo handling, where loss of the capsule is known to compromise embryo survival *in vivo* (Stout, 2012).

A more recent discovery during an embryo biopsy study was that embryo puncture and collapse did not compromise viability. In a follow-up study, they used a piezo drill to similarly penetrate the capsule and trophoblast, and then deliberately aspirated blastocoel fluid prior to vitrification. With one set of CAP they achieved promising pregnancy rates of 70% (5/7) for embryos up to 650µm.It is not known how blastocoel collapse improve cryosurvival of expanded horse embryos, it is suspected that the most important component was the reduction of blastocoel size per se, rather than improved access of CPA, because there was an association between post-warming survival and the extent of blastocoel collapse (Stout, 2012).

Diaz and colleagues published an article in 2016 on a study that evaluated several blastocyst micromanipulations and vitrification procedures for day 8 equine embryos. They compared single versus double puncture of the capsule and direct or indirect introduction to cryoprotectants. For the single-puncture technique, the pipette was inserted through one side of the capsule and for the 2-puncture approach the pipette was inserted through the

embryo. In both treatment 95% to 99% of the fluid from the blastocyst was removed. Cryoprotectant was either injected into the embryo or entered passively by exposure. There did not seem to be any benefit to double injection or direct exposure of cryoprotectants (Squires, 2016).

In a second trial, 6 embryos were punctured once, and fluid removed and exposed to cryoprotectants indirectly. Five of six vitrified embryos resulted in pregnancies at 25 days. Based on these trials high pregnancy rates can be obtained from large day 7 and day 8 equine embryos if most of the blastocoel fluid is removed before vitrification. Unfortunately, these studies were all done using a micromanipulator which are very expensive and require skilled technicians to operate. There are commercial companies that have both possess the micromanipulator and the skill to use it and it is quite likely that the facilities will provide a service whereby embryos can be shipped to them, deflated, and vitrified. The vitrified embryo could then be shipped back to the owner in a liquid nitrogen container or thawed and transferred to a recipient (Squires, 2016). This type of service will most probably be quite expensive, making the use of vitrification of larger embryos nonsensical. Since the point of developing vitrification for embryos >300µm is to make ET more cost and time efficient for practicing veterinarians and breeders.

In an effort to find a less expensive way to vitrify equine embryos >300µm Ferris and Colleagues reported in 2016 on a method to manually collapse large equine embryos (687 - 663µm) before vitrification. They compared a manual method against the standard micromanipulator method using a holding pipette and injection pipette. For the manual method, a 25-gauge needle was used to puncture the capsule and remove fluid. All embryos were vitrified in 1,5M ethylene glycol for 5 minutes, then 7M ethylene glycol and 0,6M galactose. Then they were loaded onto a cryolock device and plunged into liquid nitrogen for 40 seconds. There was a trend for pregnancy rates at 14 days to be greater for those embryos deflated by the micromanipulator (11of 15 vs. 7 of 15). Pregnancy rate of the embryos collapsed by the micromanipulator were similar to those of fresh transferred embryos (25 of 28) (Ferris et al., 2016). This study confirms the previous studies demonstrating excellent pregnancy rates with collapsed blastocysts after vitrification and transfer and provide encouragement to further develop a manual means of collapsing large day 7 and 8 embryos (Squires, 2016).

11. Intracytoplasmic sperm injection:

"Intracytoplasmic sperm injection (ICSI) is a method for in vitro fertilization in which one sperm is injected into the cytoplasm of a mature oocyte to achieve fertilization. This is currently the only repeatable and effective method for in vitro fertilization in the horse, because efficient methods for standard in vitro fertilization, that is, placing sperm and mature oocyte together in media to allow sperm penetration, have not yet been identified (Rader et al, 2016)".

Immature oocytes are recovered via transvaginal ultrasound-guided follicle aspiration of subordinate follicles existing on the mare's ovaries at any given time or mature oocytes are recovered from the dominant preovulatory follicle after gonadotropin stimulation. The immature oocytes are held overnight at room temperature before being placed into maturation culture the following morning. While oocytes from dominant stimulated follicles are cultured immediately upon recovery, until about 40 hours after the donor mare received gonadotropin stimulation. The fertilized oocytes are cultured to the blastocyst stage in vitro, then resulting blastocysts are transferred transcervically to recipient mares (Rader et al, 2016).

Indications of the use of ICSI in mares to obtain foals from mares that do not provide embryos for embryo transfer. The reason for this might be chronic endometritis, cervical defects, damage to the reproductive tract from previous foals, repeated incidence of haemorrhagic anovulatory follicles, old mares unable to produce embryos anymore, or possibly oviduct problems that problems that prevent fertilization or descent of the embryo into the uterus. Indications for ICSI in stallions include old age, in which stallions may no longer produce quantity or quality of semen needed for standard insemination, death of stallion where only frozen limited amount of semen is available. With ICSI it is possible to produce 6 to 10 embryos from one straw, or the frozen straw may be thawed, diluted and refrozen resulting in hundreds of ICSI doses (Rader et al, 2016).

Contraindications of ICSI is variable success, complication during oocyte recovery, higher rate of pregnancy loss than for transfer of naturally-conceived embryos. Because of these drawbacks, ICSI is not a recommended means of obtaining more foals in a given season from a normally-fertile mare with standard quality semen. ICSI should only be used when indications listed previously are present, or if all other assisted reproduction methods failed. It is also to be noted that this is a costlier method than standard embryo transfer (Rader et al, 2016).

Intracytoplasmic sperm injection is performed under micromanipulation, using one large diameter holding pipette and one fine injection pipette equipped with Piezo drill. One sperm is aspirated tail-first into the injection micropipette and when the mid-piece enters the pipette, is immobilized using pulses from Piezo drill. Following immobilization of the sperm, the entire sperm cell is aspirated into the injection pipette, which is then moved to the holding droplet containing the awaiting oocyte. The oocyte is manipulated with the injection pipette so that the polar body is at 12 or 6 o`clock, and the aspect of the oocyte nearest the holding pipette presents the clearest cytoplasm, see **Figure 14A**. A core of the zona pellucida is bored out with the injection pipette, using Piezo drill pulses, and is discarded. The sperm pipette is inserted through the hole in the zona and is advanced into the oocyte to the far side of the oocyte near the holding pipette, see **Figure 14B**. The oolemma is broken using a pulse from the Piezo, and the sperm is ejected into the oocyte cytoplasm. This is done without aspirating any of the cytoplasm into the injection pipette (Rader et al, 2016).

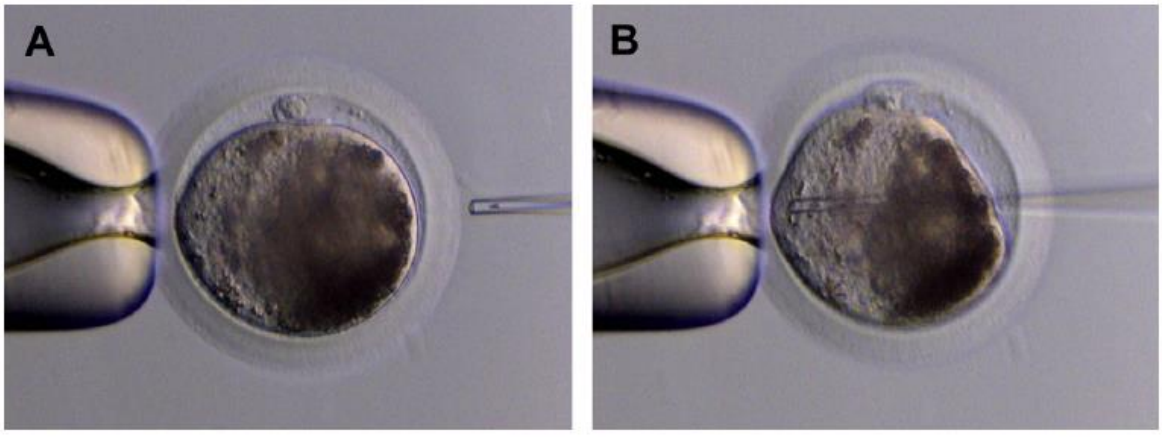

Figure 14 A & B: *Sperm injection (Rader et al, 2016).*

The embryo is then cultured in a holding medium. Embryos examined at day 5 for cleavage, non-cleaved embryos are discarded, while cleaved embryos are placed in a new droplet of culture medium and are checked daily for development to the blastocyst stage. Blastocysts are recognized by formation of an apparent trophoblast layer immediately inside the zona pellucida, with a suggestion of decreased density of the inner aspect of the embryo (Rader et al, 2016).

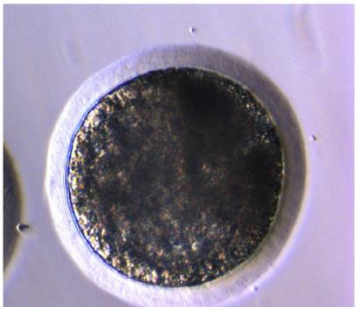

Figure15: *Equine blastocyst with trophoblast layer produced by ICSI and in vitro culture (Rader et al, 2016).*

12. Equine embryo biopsy:

One of the great advantages of embryo transfer is the ability to biopsy the embryos, to diagnose different genetic traits or determine the gender of the embryo before transferring it into the recipient mare (Matthews, 2017).

Biopsy is ideally done on embryos 7 to 8 days old. The embryo can be biopsied with the use of an inverted microscope and micromanipulators. The embryo is held in place with a suction pipette while the outer membrane (capsule and trophoblast) are pierced by the drill to retrieve cells from the embryo. These cells are the submitted to the laboratory for PCR testing (Matthews, 2017). The process of the biopsy itself takes less than an hour. This means that the embryo can be transferred to the recipient mare, the cells harvested from the biopsy are packaged and sent to the laboratory for testing. The test result takes several days to receive, and the owner can then decide to continue with the pregnancy or terminate the pregnancy if the results are unfavourable. Another alternative is to freeze or vitrify the embryo if < 300µm and take the biopsy from the frozen embryo. If the embryo is >300µm a biopsy of the embryo can be taken, and the blastocoel fluid aspirated to deflate the embryo for cryopreservation. Once the results of the biopsy are obtained the embryo can be transferred or remain in storage for future use (Matthews, 2017).

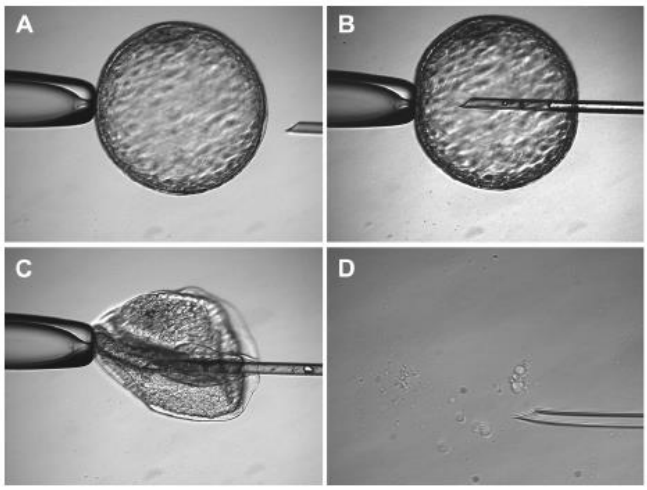

Figure 16: *Biopsy procedure of in vivo produced equine embryos.* ***A****: intact expanded equine blastocyst before biopsy.* ***B****: A 25µm micropipette is used to puncture the capsule.* ***C****: Using the micropipette, almost half of the blastocoel fluid is aspirated, and 10 to 30 cells are removed from the inside of the blastocoel cavity by gentle suction.* ***D****: Embryonic cells obtained during the biopsy procedure. Herrera et al, 2013.*

The biopsy sample is incubated before polymerase chain reaction (PCR) amplification. The sample is centrifuged for 5 minutes at 400x g to ensure that the samples were at the bottom of the tube. Than incubated for 10 minutes at 95℃ on a dry bath and spinned down to bring the condensation formed on the inner walls of the tube during sample heating, to the bottom of the tube. The PCR reagents were added directly to the 0.2ml tube containing the biopsy sample. For sex determination a duplex PCR is used to amplify equine sex-determining region Y (eSRY) and amelogenin (AMEL). (Herrera et al, 2013).

There are no studies that show performing a biopsy is detrimental to the embryo and at this time the embryo transfer success rate (pregnancy rate) with biopsied embryos equals non-biopsied embryos (Matthews, 2017).

13. Sexing embryos:

"Preimplantation genetic diagnosis (PGD) was first used in humans more than 20 years ago. Since then, this technology has been improved and currently being successfully used by human IVF clinics to determine various genetic traits of early stage embryos (Herrera et al, 2013)". The complete genome sequence of the domestic horse was published in 2009 and genes have been associated with 40 equine diseases and more than 20 phenotypes (Herrera et al, 2013)

In the Polo Argentino breed, females are preferred to males because of their ease of training and agility. Preimplantation genetic diagnosis (PGD) can be used to determine the gender of the embryo before transfer and thus allow the production of only female or male pregnancies. In a study performed by Herrera et al in 2013, equine embryos were classified by size, biopsied and transferred 1 to 2 or 7 to 10 hours after flushing. Pregnancy rates were recorded at 25 days of gestation. Foetal gender was determined using ultrasonography and compared with PGD results. Foetal gender determination by the use of transrectal ultrasonography was done by locating the genital tubercle in pregnant mares between day 58 to 70 of gestation.

There were similar results when transferring the embryos 1 to 2 hours as for embryos transferred 7 to 10 hours after flushing. These results did not differ from pregnancy rates of non-biopsied embryos undergoing the same holding time. By incubating the biopsy samples before PCR a 100% matched the results obtained using ultrasonography, while 87.8% in the non-incubated biopsy samples (Herrera et al, 2013).

A duplex PCR was performed using primers to amplify equine sex-determining region Y (eSRY) and amelogenin (AMEL). Amelogenin is a gene involved in the construction of teeth enamel and is located on the X and Y sex chromosomes. The coding region of this gene on the Y chromosome is shorter than that in the X chromosome. The amplified products were electrophoresed on 2% agarose gel stained SYBR Safe and visualized under blue light. The process from embryo biopsy to gender determination by PCR takes 6 hours if performed in the same facility.

This study concluded that embryos can be kept in the holding medium until gender diagnosis by PGD is completed without affecting the pregnancy rates, even if embryos of different size are used. This simplifies the management of an embryo transfer program willing to incorporate PGD for gender selection, by transferring only embryos of the desired sex. PGD can have a high economic impact on commercial programs, significantly reducing the number of recipient mares needed (Herrera et al, 2013).

14. Conclusion:

The development of equine embryo transfers the last 10 years has shown great improvements and development. A lot of time and resources has been put down to further investigate and develop the techniques for optimal equine embryo transfer. Equine embryo transfer entails so much more than just the act of embryo transfer itself.

It's important to carefully select both the donor and recipient mare to achieve optimal pregnancy rates when using embryo transfer. Synchronization of the donor and recipient mare is a crucial first step to a successful embryo transfer. Synchronization can be done by the use of drugs, or that embryonic centres keep a large flock of mares to choose from, so that they can find a mare ovulating naturally at the same time as the donor mare.

Superovulation is another obstacle for ET. There is still a lack of a reliable protocol for the induction of superovulation in mares, leaving embryo recovery rates at 50 % to 70% per cycle. Making this one of the most limiting factors of embryo transfer programs today.

Flushing is done 7 to 8 days post ovulation. The embryo is located and examined before embryo transfer. Embryo transfer itself has not developed much, but the use of surgical embryo transfer is hardly used anymore. This is because non-surgical embryo transfer is safer, quicker and gives the same pregnancy rates as surgical ET does.

Cooling of embryos at 5°C has opened up the possibility to transport embryos to the location of the recipient mare and transferred within 24 hours. Another option for storing embryos is cryopreservation of equine embryos, which has shown great development over the last 10 years and has opened up the possibility for embryos to be shipped across countries. The embryos can be frozen for years until the breeders wants to use them, sell them or ship them to another country. It has given the breeders the opportunity for genetic banking and pre-implantation testing of the embryo.

Equine embryo transfer has developed a lot over the last 10 years and its use has increased with it. More and more practitioners have become skilled in the technique, and breeders have become more and more aware of the possibility of ET as a tool in breeding. There is still a lot of challenges when it comes to keeping the cost down, making it available for everyone who desires to use this technique to optimise their equine breeding.

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