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Practical aspects of equine embryo transfer

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Abstract. This article focuses on practical aspects and most specific points of equine embryo transfer (EET). Induction of polyovulations in donor mares is not possible. Only the repetition of embryo collections can increase the number of foals from the same mares. At each oestrus, follicular growth and artificial inseminations are conducted in the same way as for any brood mare. It is essential to know the number and timing of ovulations at least a 12-hours accuracy. Induction of ovulation is frequently required, GnRH agonists are used to do that. Embryos are flushed from the donor mare's uterus, typically on day 7 or 8 after ovulation using a long (~1.5 m) and large (diameter 8 mm) Foley-type balloon-tipped catheter introduced by vaginal route just in front of the cervix. Some practical details are important to EET practitioners, firstly, the embryos are easily located under stereo-microscope after filtration of flushing medium, and secondly most equine embryos

are immediately transferred or after cooling at 4°C less than 24 hours. To have a recipient mare in the right stage of her oestrous cycle (D5 to D8 after ovulation) is the biggest and the most difficult challenge for EET practitioners because the synchronisation of oestrous and ovulations is very difficult to obtain. Therefore, embryos are transferred transcervically to the uterus of a recipient mare. Recently, a modified method using a speculum and a specific forceps seems to give a much better success rate than the conventional technique which requires a large training. The EET has regulatory constraints: regulations of different breeds allowing or not allowing the embryo transfer and sanitary European regulation concerning intra-community trade of equine embryos (Annex D to Council directive 92/65 EEC) and, in some countries, additional national regulation. The possible sanitary risks of ET in equine have been still little studied, and without International or European official registrations of EET activity, it is difficult to know the extent of the use of the technique around the world. The average success rate of embryo recovery is of the order of 40 to 50% depending on the fertility of the donor and the type of sperm used, the transfer success rate can reach 70 to 80% if the technique is well mastered by ET practitioners.

Keywords: Equine; embryo; donor; recipients; transfer; practitioners.

Introduction and general considerations

The objectives of embryo transfer in mares are i) to produce several foals from the same mare during a single breeding season, eventually bred by different stallions; ii) to allow donor mares to pursue their sport career while recipient mares carry their foals; and iii) to obtain foals from mares with genital lesions or diseases which preclude them from carrying a pregnancy to term. Ten years ago, during the 25th annual meeting of AETE in Poznan, the father of equine embryo transfer, Twink Allen [1] gave a long lecture to present historical and modern aspects of equine embryo transfer (EET), readers could go to AETE website to see a complete review about this Assisted Reproductive Technique (ART) including more than 170 references. EET is a well-established ART described in many papers [2, 3, 4], chapters of books [5] or even books [6]. Herein, the aims of this paper is i) to focus on some specific practical aspects concerning mainly i) donor mares management, ii) uterine flush for embryo recovery and embryos collection, iii) identification and manipulation of embryos, iv) recipient management and selection of recipient mares, v) non-surgical

embryo transfer vi) practical constraints induced by rules of equine embryo transfer and vii) expected results to explain the main differences of the EET process in the mare by comparing to the ET in cow.

Donor mares management

The first limitation of equine embryo transfer (EET) is that superovulation of mares is problematic because of different peculiarities specific to equidae [7, 8]. Many such studies have shown that i) only the equine FSH can stimulate follicular growth in the mare, ii) only low results can be obtained with a maximum three or four ovulations per oestrous, because in each follicular wave there are few recruited follicles able to be stimulated by FSH, iii) the third difficulty is that equine FSH must be extracted from equine pituitary. Few years ago this equine FSH was available in America sold by a Canadian company, but now it is no more available on the market. Ten years ago, first studies about a recombinant equine FSH produced by genetic engineering were presented both in communications of congresses and in first papers, this new opportunity was therefore expected, but no new paper were published after 2014. However, recently, a start-up company (www.fertilplus.us) made the announcement of the imminent marketing of this recombinant equine FSH. But at the moment the price of this product, as well as its availabilities in Europe are not yet known. Moreover, without opportunity to induce superovulation in mares, only the potential embryo resulting from ovulation of one dominant follicle (or sometimes 2, if the donor mare spontaneously double-ovulates) is available. Therefore, to try to produce several foals, embryo collections can be performed at each oestrous cycle along year (or breeding season), and to increase number of cycles, each luteal phase is shortened by a luteolytic injection of PGFα2 at the day of embryo collection.

When induced and even spontaneous multiple ovulations occurred, the proportion of embryos recovered per collection is lower than in single ovulation. This fact is explained by equine ovarian anatomy with its ovulation fossa as well reported by Twink Allen [1]. In case of multiple

ovulations at the same ovary, a physical competition occurs between large preovulatory follicles to simultaneously reach the ovulation fossa, some of them are unable to release their oocyte before luteinization. This phenomenon seems to occur even during natural double ovulations. In this way, two Argentinian Embryo Transfer Centres reporting their data showed that recovery rates per ovulation are lower after double ovulations occurred into the same ovary than after double ovulations occurred into each the two ovaries (307/731, 42% *vs* 381/698, 55%) [9] and (574/1056, 54% *vs* 588/872, 67%) [10]. There are more unsuccessful collections (none embryo recovered) after unilateral double ovulations than bilateral (147/528, 28% *vs* 66/436, 15%) [10] and less twin embryos recovered after unilateral double ovulations than bilateral (193/528, 37% *vs* 218/436, 50%) [10].

The standard protocol for EET starts by a daily monitoring the preovulatory follicle of the donor mare via ultrasonography per rectum, due both to the long and very variable duration of oestrus and to the variability of ovulation time after the beginning of oestrus. The mare is inseminated when the follicle appears to be ready to ovulate, or in coordination with induction of ovulation by administration of hCG or a GnRH agonist. Induction of ovulation at each oestrus in donor mares needs to use GnRH agonists because after 2 or 3 injections of hCG per year, mares are immunized against hCG and became refractory to the human gonadotropin [11]. The preovulatory follicle is monitored daily after insemination so that the day of ovulation (Day 0) must be accurately determined. Donor mares are often in the same time used as sport mares and implicated in competitions at high level. Different studies tried to evaluate eventual effects of sport and stress of training and competition on success rate of embryo transfer, most of this studies showed that it seems to have no any side effect. However, in practice, the daily management of donor mares added to the embryo collection one week after the ovulation, and the repetition of the process at each cycle is very complicated to combine with the training and competition program.

Uterine flush for embryo recovery and embryos collection

The embryo is flushed from the donor mare's uterus, typically on Day 7 or 8 after ovulation [12], it has been established [13] that the timing of embryo entry into uterus is somewhat variable ranging between 144 hours (6 days) and 156 hours (6.5 days) after the ovulation but probably sometimes more later in old mares (>18 years), and in mares bred with frozen semen or inseminated in the first 8 hours after ovulation. In addition to this great variability of the moment of entry of the embryo into the uterus after ovulation, another peculiarity of equine embryonic development is that once arrived in the uterus, embryonic growth in size and in number of cells are dramatically accelerating almost exponential [14, 15, 16].

Due to the anatomy of the equine uterus, and to the fact that embryo once into the uterus move from the top of one uterine horn to the other several times per day, the flushing catheter is introduced just front of the cervix (at the level of the internal os) and uterine lavages concern the entire uterine cavity.

A wide variety of embryo collection supplies are readily available from commercial distributors. A Foley-type balloon-tipped catheter of sufficient length (~ 1.35–1.5 m) and diameter (internal diameter 8 mm) is prefilled with flushing medium to prevent introduction of air into uterus and in the system. Then catheter is introduced through the cervix by vaginal route; balloon is inflated (30 to 60 mL) to ensure the fixity and the watertightness of the device. Flushing is performed by filling the uterus with ~1 L of flush solution by gravity, then draining the fluid by gravity too. Fluid is collected either directly in empty bottle, either through an embryo filter. This process is repeated multiple (3 to 6) times with a new medium litre at each time.

Some important points need to be highlighted.

- A majority of donor mares do not require sedation to safely and effectively perform the uterine flush procedure. Sedation may be warranted in young, excited or dangerous mares; in this case, xylazine

or detomidine should be used preferentially rather than acepromazine that relaxes the uterus.

- To avoid the contamination of the catheter and the introduction of pathogens into the uterus, a strict hygiene must be applied. After emptying the rectum, the perineal area and vulva area are thoroughly washed with antiseptic soap and rinsed three times, then dried. The tail is packed in a disposable tail guard. In the same way, only sterile gloves are used to perform embryo collection.
- Before the introduction of tube the operator has to check that the balloon remains inflated. It is also necessary to fill the catheter with medium before its introduction in genital truck to avoid to introduce air into the uterus and to prevent a bad return of the fluid.
- To siphon the medium from the uterus, the operator must be careful to lower the bottle very slowly, if it is too fast it causes a collapse of the uterine wall against the tube, and the flow stops. Normally the return of the fluid is very fast producting swirls in the bottle.
- Once the fluid recovered in the bottle, operator clamps the catheter and changes bottle to introduce a new sterile medium, to do that correctly, the team must be well-trained as to end the collection to be sure to recover the entire fluid.
- Finally, PGF2α or agonist is injected to the mare both to prevent pregnancy if embryo has not been recovered and to induce a new estrus by inducing luteolysis.

Identification and manipulation of embryos

After filtration, directly behind the mare or in laboratory, embryo(s) is (are) located in retained in the filter under stereo-microscope. For a practitioner trained to search ruminant embryos, equine embryo identification is easy because flushing media contains significantly less organic matter and embryos are often bigger mainly at blastocyst or expended blastocyst stage. But in contrast to bovine embryo collection, there is only one embryo, rarely 2, in case of double-ovulations, or nothing. Equine

embryos passed actively through the uterotubal junction into uterine cavity by secreting PGE2. Unfertilized oocytes and early-degenerated embryos stayed into oviducts… sometimes when an embryo entered into uterus, unfertilized oocytes from previous ovulations could pass with it. The most challenging decision would be to differentiate between an unfertilized oocyte and a morula (6-day embryo); however morula is spherical while unfertilized oocyte appearing round or somewhat oval and flat from the side.

Because of its active transit into the uterus, recovered embryo is normally non-degenerated and in most of cases it is of good quality. Morphological gradation could be done as for bovine embryos [6]… However in practice, even a low graded embryo is transferred because it is often the only one recovered, and sometimes a pregnancy and then a foal are obtained.

As indicated in European rules and as well as for ruminants embryos, equine embryos should be washed 10 times using a dilution factor of at least 1/100. Following the washing procedure, embryos may be transferred immediately if recipient mare is on site or they may be transported the same day or overnight to an alternate location for transfer to a recipient mare. For transportation, embryos can be placed into one of several types of commercially available holding medium. The customary method for embryo transport is to cool them in the same systems that are used for cooling transported semen [17]. However, a study presented during the last International Symposium on Equine Reproduction (2018) showed that embryos placed in a room temperature in a commercial holding medium can wait until 14h without effect on transfer success rate or on embryonic mortality [18]. In this study 1745 D7–10 embryos were transferred immediately or after a wait of 1 to 14h at room temperature 18–22°C, there was no significant difference in pregnancy rate at D16 and D45 between groups (respectively 336/509 (72%) and 333/509 (65.5%) for group 0–4h ; 638/890 (72%) and 587/890 (66%) for group 5–9h ; 252/346 (73%) and 232/346 (67%) for group 10–14h).

Deep freezing and vitrification are problematic and this is another limitation of EET [13].

Recipient management and selection of recipient mares

To have a recipient mare in the right stage of her oestrous cycle (D5 to D8 after ovulation, D5 and D6 seem to be the best) is the biggest and the most difficult challenge. Both after PGF2α injection made D5 to D8 of oestrous cycle and either after a PGF2α injection made the last day of 7 to 10 days of progestin treatment, the occurrence of the ovulation is highly variable; the synchronisation of ovulations between donor and recipient mares is problematic. Figure 1 shows results obtained in donor and recipient mares (n=555) treated on the 6 or 7th day after previous ovulation by an intramuscular injection of 250 αg of cloprostenol (PGF2α agonist) the next ovulation occurred in mean 9.4 days after injection but with a great variability between the 1st day to later than the 20th day after injection. Figure 2 shows the same thing after daily treatment with alternogest (40 mg) given orally during 7 days and a cloprostenol intramuscular injection the 7th day of treatment.

Taking into account this great variability, a probability calculation shows that with a 95% chance to have a recipient mare that ovulate be-

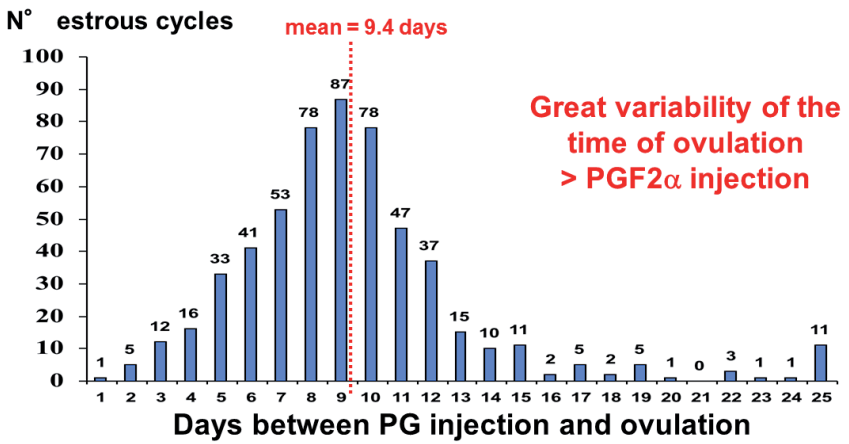

Figure 1. Distribution of days between PGF2 injection performed in donor and recipient mares Day 6 or Day 7 (n=555) and the following ovulation

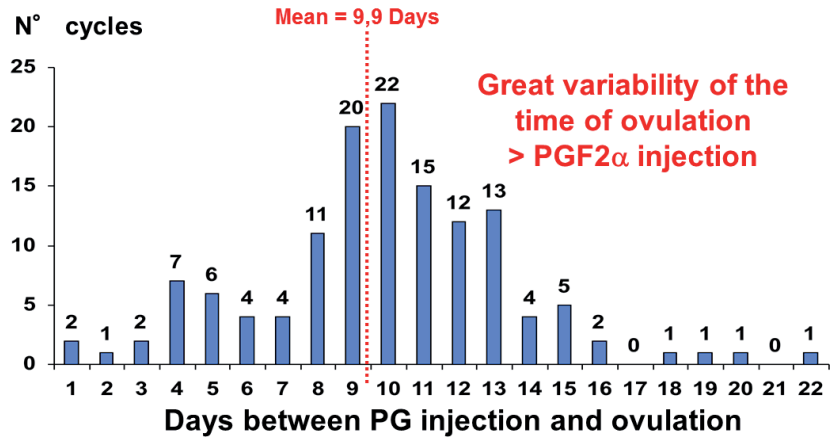

Figure 2. Distribution of days between PGF2 injection performed in donor and recipient mares the last and 7th day of oral treatment with altrenogest (40 mg) (n=134) and the following ovulation

tween 2 days before and 2 days after the donor we should treat 12 recipient mares in the same time, and with only a 80% chance to have a synchronous recipient we need to treat 3 recipient mares in the same time.

Several equine embryo transfer units have large herds of recipient mares and statistically they have quite always few mares in oestrus in the same time as each donor mares, and ovulations of recipient mares are induced once an ovulation of donor occurred. When they are few synchronous recipients mares for an embryo, the selection of the recipient used the day of transfer includes a genital palpation and ultrasound examination per rectum. The mare should have good uterine tone and tightly closed cervix, the echo structure of its uterus should be uniform with no oedema or fluid.

Non-surgical embryo transfer

In the early years of equine embryo transfer, most of embryos were transferred surgically through a standing flank laparotomy; from many years,

they are transferred transcervically to the uterus of a recipient mare that ovulated from a day before to 3 days after the donor mare. Catheters used to transfer equine embryos are similar as used in bovine, they are longer (+15 cm) and exist in 2 sizes. Physiologically once embryo is into uterus, its size increases dramatically; accordingly the time elapsed between its arrival in the donor's uterus and its recovery, and it is more or less large (diameter from 0.25 to 2 mm). Smallest embryos could be introduced into 0,25 mL straw, but 0,5 mL straw should be used for biggest ones. A sedation or strong contention of recipient is needed to prevent any movement of the mare during transfer. A perfect sterility of material has to be applied: sterile glove and transfer gun enclosed with a sterile plastic sleeve. To increase the sterility, it is recommend using double-gloved technique in which an extra sterile glove is used to cover the inner glove and transfer gun up to vestibular-vaginal ring. With this technique the inner glove and the gun are totally protect from contamination and arrive perfectly sterile front the cervix.

As faster and easier is the cervical catheterization, higher is the chance of success; practitioner's skill, training and experience are a key point of success. Once the transfer gun is near the cervix, the external os may be held between the practitioner's index and middle fingers and then pulled back gently, this stretch of cervix make its duct straight, the gun (free from plastic sleeve) is then advanced through the cervix. To do that, the easiest is to put his hand back against the floor of the vagina. The external os of cervix is then wedged between the index and middle fingers and pulled back to make the cervical canal rectilinear. The transfer gun can then slide gently against the palm of the hand and the cervical canal.

An alternative method of non-surgical transfer described by Twink Allen [1] could be used by a poorly trained practitioner: a Polansky's speculum is introduce to 1) see the external os of cervix, 2) grasp its ventral quadrant with the Wilsher equine embryo transfer forceps and 3) perform a gentle retraction straightening the cervical canal and allowing an easy catheterization of cervical duct by the transfer gun manipulated from outside the mare's vulva.

In a study conducted in Utrecht University and recently published, embryos were transferred by seven different operators with different levels of previous experience in embryo transfer [19]. When they were trained, they transfer with conventional method and when they have never performed any transfer they performed the method using Polansky's speculum and Wilsher's forceps. The pregnancy rate was significantly higher after transfer using Wilsher's technique (156/170, 92% *vs* 127/179, 71%) and early embryonic loss before Day 45 was not different between groups (8 *vs* 9%). Data of this study show also the strong influence of skills and/or experience on the success of conventional transfer; an operator designed as medium trained (only 50 embryo transfers performed before the study) obtained a lower success rate (27/53, 51%) than 2 other well-trained (more than 100 for one and more than 500 embryo transfers before the study) operators, with respective success rates of 79% (41/52) and 80% (59/74)). On the opposite, there was no difference in the success rate of transfer (91 or 93%) between the 4 novice operators transferring embryos with speculum and forceps method [19].

Practical constraints induced by rules of equine embryo transfer

Equine embryo transfer also has regulatory constraints. Firstly, breed regulations may or may not allow the transfer or set limits. For example, in France there are 58 different breeds with its own regulation for each of them. For 8 breeds including thoroughbred (certainly as everywhere in the world), Highland and Shetland ponies embryo transfer is prohibited. Two other breeds whose French Trotter limits to one colt born by transfer per year. Rules of other breeds in Europe have to be studied and there are probably few limitations. For example, for Polish Arabian breed, the owner of a mare must obtain authorization to produce a foal per year by embryo transfer.

On sanitary point of view, there is, as in the case of livestock species, an European regulation concerning intra-Community trade of equine se-

men, oocytes and embryos. Annex D to Council directive 92/65 EEC concerning equine embryos (embryo collection and production teams, and conditions for donor animal) is regularly updated, the last version was published in 2014.

Each European country, has normally transposed the European regulation into its national laws, this concerns the intracommunitary exchanges of equine embryos. In addition, in France, there is also a regulation for embryos collected and transferred at the national level**,** it is probably a French particularity, but it is necessary to know what it is the situation on this point of view before to begin to perform embryo transfer.

European regulations focus on the prevention of sanitary risks and among legal recommendations, during in vitro manipulation of embryos they should be washed as for bovine embryos by moving them in 10 successive bathes of fresh sterile media by using a new sterile micropipette at each step.

However, unlike in ruminants and pigs, there have been very few studies to actually assess the sanitary risks of embryo transfer in horses. It seems that there are only 3 experimental studies on this topic. One study showed that donor vaccinated or unvaccinated mares inseminated with equine arteritis virus infective semen collected from a carrier and shedder can produce infected embryos [20]. After 10 washes in flush medium and 2 washes in trypsin medium embryos remain infected. Few unvaccinated seronegative for EAV antibodies recipient mares had seroconverted to EAV 28 days after transfer of washed embryos [20]. Two studies performed by our team showed that equine embryos placed *in vitro* in a medium contaminated with the EHV1 virus remain carriers of the virus after washing in a trypsin bath followed by 10 washes in holding medium [21, 22]. Few years before those studies, a Brazilian team reported that they have collected an equine embryo naturally contaminated by EHV-1 from a clinically healthy donor mare [23]. Sanitary risks of infectious transmission by embryo transfer should be more investigated in equine and other pathogens should be tested on equine embryos. In addition of European regulations for the intra-European and international trade of equine embryos, the last editions of both the manual of the International

Embryo Society [24] and the Terrestrial Animal Health Code published by the World Organization for Animal Health recommend, to apply the same sanitary procedures for equine embryos as for ruminant and porcine embryos.

Expected results to explain the main differences of the EET process in the mare by comparing to the ET in cow

Expected embryo recovery rate published is often between 50 and 75%, and expected pregnancy rate after transfer about 75%. Success rates depend on the fertility of the donor and the stallion and the mode of preservation of his sperm (insemination with fresh, chilled or frozen sperm). *nota* in the European rules, natural covering is not allowed in donor mares to produce horse embryos for intra-European trade!

It is very difficult to have a global overview of equine embryo transfer in the world and even in each country. In a recent survey [25] we have analysed data from 11/62 French equine embryo transfer Centres during breeding season 2014 and 2015, the recovery rate was 41.5% (277/669) (in 9.5% (26/277) successful collections 2 embryos and once (1/277) 3 embryos were recovered) this rate was significantly lower in oldest mares (>14 years); pregnancy rate after embryo transfer was 75% (229/305). Data about commercial embryo transfer activity in Europe in 2017 presented in the last AETE (Association of Embryo Technology in Europe) scientific meeting (September 2018) reported for only 9 countries (Finland, France, Italy, Netherlands, Poland, Russian Federation, Spain, Sweden and Switzerland) in total 2173 embryo collections and 1211 viable embryos recovered (recovery rate: 55,7%). [*in Poland 7 embryo collections reported and 7 embryos recovered (100%.) and there is no data from Belgium and Germany where there is a big equine embryo industry.*] In France, for the different official studbooks, all mares bred and all foals born are officially registered by IFCE (*Institut Français du Cheval et de l'Equitation* – French

institute of Horse and Riding). Figure 3 shows data officially registered for equine embryo transfer activity at the date of 2 march 2019 (foals born in 2018 were not yet reported) : about 800 donor mares were collected in 2 past years and an average of about 1 foal/ donor mare born by embryo transfer born each.

Statistics of embryo production and transfer in domestic farm animals in the world are published every year, in December, by IETS in its Newsletter; for 2017 there is only data from 3 other countries in addition to those published by AETE [26]. If those of Brazil seem be close to reality (19560 embryos recovered for 31650 embryo collections (61.8%)), it misses those of the other countries of South America including Argentina (historic world leader) and those of USA and Canada are very largely underestimated with respectively reported only 60 embryo collections in USA and 19 in Canada! Those data from IETS reported in total 20821 embryos recovered for 33902 embryo collections (recovery rate: 61,4%). There are no world statistics about success rate of transfer. For USA, Dur-

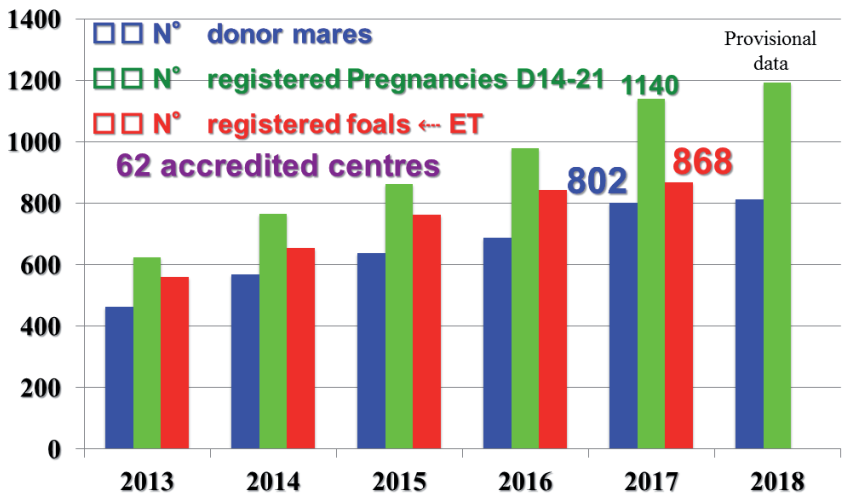

Figure 3. Evolution of official registered data of equine embryo transfer activity in France (provided by IFCE-SIRE 2nd March 2019)

ing a congress in Paris in 2015, Squires gave a lecture where he showed data from US equine embryo transfer from 2005 to 2013 with more three thousand donor mares per year, and more 4 thousand registered foals born by embryo transfer per year, he did not report the success rates of each step of the process obtained in the field [27].

Conclusion

In summary, the technique of equine embryos collection, handling and transferring is not so difficult in itself. For the EET practitioners, each step must be done with rigor ensuring compliance with the sterility of handling, as well as, the monitoring of the donor mares must be extremely rigorous and precise, just like that of the recipient mares. To have a recipient mare at the right stage of her oestrous cycle, EET practitioners have to follow a large number of mares for each donor, so practitioner have to be ready to spend a lot of time there. If EET practitioner is ready to do that, embryo transfer can be a tremendously rewarding part of equine practice and if he strives for excellence in each step, success will follow. Therefore, EET practitioner has also to keep in mind that there is an European regulation and sometimes rules specific to each breed about equine embryo transfer.

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