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Factors affecting the likelihood of pregnancy and embryonic loss after transfer of cryopreserved in vitro produced equine embryos

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Summary

Background: In vitro embryo production (IVEP) is increasingly popular but data assessing the outcome of transferred embryos are scarce.
Objectives: To determine the likelihood of pregnancy and embryonic loss after transfer of frozen-thawed IVP embryos and identify factors influencing success.
Study design: Retrospective clinical study.
Methods: Blastocysts (n = 261) were produced from immature oocytes of Warmblood mares (n = 116) by Intracytoplasmic Sperm Injection (ICSI) and in vitro culture, and cryopreserved. Thawed IVP embryos were transferred into recipient mares on day 4, 5 or 6 after ovulation. The influence of donor mare (age, reproductive history), recipient mare (age, reproductive status, management; in-house vs. outpatient, day post-ovulation), embryo (interval from ICSI to blastocyst formation) and management factors (season when ovum pickup was performed, year and method of transfer) on likelihood of pregnancy and embryonic loss was examined, and the developmental stage of the IVP embryo at the time of transfer was estimated.
Results: The percentage of mares pregnant 7–10, 23 and 37 days after transfer was 56% (147/261), 49% (129/261), and 48% (124/261), respectively. Development of IVP embryos after transfer equated to day 5 or 6 in vivo embryos. With the exception of year of transfer, none of the factors had an impact on the likelihood of pregnancy or embryonic loss. Nevertheless, the likelihood of pregnancy tended to be lower for IVP embryos from infertile mares or when embryos were transferred into recipient mares on day 6 after ovulation rather than on day 4 or 5. Finally, the diameter of the embryonic vesicle 7 days post transfer was lower for pregnancies that were lost compared to those that were maintained.
Main limitations: Small sample size in some of the donor and recipient mare categories.
Conclusions: Cryopreserved IVP embryos should be transferred into recipient mares on day 4 or 5 after ovulation and a slower rate of post transfer vesicle expansion indicates a higher risk of subsequent embryonic loss

The Summary is available in Portuguese – see Supporting Information

Keywords: horse; in vitro embryo production; pregnancy; embryonic loss; intracytoplasmic sperm injection

Introduction

In vitro production of equine embryos (IVEP) by intracytoplasmic sperm injection (ICSI) is a complex process offered by only a few laboratories worldwide. During the last 4–5 years, there has been a marked increase in interest in IVEP among sport horse breeders for reasons including the efficient use of scarce or expensive frozen semen, the production of foals from subfertile stallions and mares [1] and the possibility of performing Ovum PickUp (OPU) year-round at remote locations as an outpatient procedure [2]. Moreover, improved in vitro maturation and culture media have increased the success of IVEP, which has also contributed significantly to the increased popularity [3]. Currently, almost 60% of OPU-ICSI procedures yield one or more blastocysts, with an average of 1.8 blastocysts per successful session [4]. As IVEP is frequently performed outside the physiological breeding season and regularly results in multiple embryos per OPU session, IVP embryos are commonly cryopreserved either by vitrification [5] or slow freezing [6]. Cryopreservation of embryos offers several advantages; valuable genetics can be distributed worldwide and recipient mares can be used more efficiently [7]. Nevertheless, little has been published about the likelihood of pregnancy and embryonic loss after transfer of a large number of frozen-thawed IVP embryos.

Beyond determining the likelihood of pregnancy and embryonic loss, identifying factors that affect these outcomes can be of great value to equine practitioners when making a treatment plan and managing client expectations. Several embryonic (embryo size, developmental stage, age and quality), donor mare (age, fertility status) and recipient mare (age, day post-ovulation) factors have been reported to influence the likelihood of pregnancy for flushed equine embryos [8, 9]. Although it is tempting to speculate that the same factors will determine the likelihood of pregnancy for IVP embryos, there are distinct differences between in vivo and in vitro embryos. In particular, the rate of in vitro embryo development varies markedly among IVP embryos, which can take 6–9 days to reach the blastocyst stage [10]: however, it is not known whether the likelihood of pregnancy or embryonic loss is influenced by the rate of in vitro embryo development. Furthermore, although early embryo development is slower in vitro than in vivo, it is not clear to what in vivo developmental age an IVP embryo is equivalent when it reaches the blastocyst stage. Based on morphological characteristics, IVP blastocysts have been proposed to correspond most closely to day 5 in vivo embryos or those that are 1 or 2 days delayed in development [11] and it is therefore recommended to transfer IVP embryos into recipient mares with a corpus luteum that is 4, 5 or 6 days old [12]. Nevertheless, because discrepancies in donor-recipient synchrony for flushed, in vivo embryos are less well tolerated in recipient mares that ovulated before rather than after the donor mare [9], we speculate that IVP embryos might benefit from a less advanced uterine environment. Finally, because IVEP can be performed year-round and older mares are often over-represented in clinical IVEP programs, it is imperative to assess the impact of both mare age and time of year at OPU on the likelihood of success. Therefore, the objectives of this study were to determine the

percentages of mares that became and remained pregnant after receiving a frozen-thawed IVP embryo and to identify factors affecting the likelihood of pregnancy and embryonic loss.

Materials and methods

In vitro embryos (n = 261) were produced from privately owned Warmblood mares (n = 116) presented to the clinical OPU program of the University Clinic for Horses at Utrecht University. On admission, the age (in years) and reproductive history of the donor mare and the month in which OPU was performed were recorded. The fertility of the donor mares was evaluated either by reviewing clinical records or through a thorough anamnesis (owner or referring veterinarian). Donor mares were classified as fertile, subfertile, infertile or unknown depending on the history of pregnancy or embryo recovery during previous breeding seasons (fertile – the mare became pregnant or at least one embryo was recovered within threeattempts; subfertile – the mare conceived or an embryo was flushed within 4–6 attempts; infertile – the mare failed to conceive or deliver an embryo within six attempts or two consecutive breeding seasons; unknown – maiden mares or mares without a reproductive history). The meteorological seasons spring, summer, autumn, and winter were, respectively defined as 1 March – 1 May, 1 June – 1 August, 1 September – 1 November and 1 December – 1 February.

Ovum-pick up

Peri-operative antibiosis, analgesia and sedation were initiated by administering gentamycin-sulphate[a] (6.6 mg/kg i.v), benzyl penicillin sodium[a] (20,000 IU/kg i.v.), meloxicam[b] (0.6 mg/kg i.v) or flunixin meglumine[a] (1.1 mg/kg i.v.), detomidine hydrochloride[a] (0.01 mg/kg i.v.) and butorphanol tartrate[c] (0.01 mg/kg) immediately prior to the OPU procedure. Epidural anaesthesia was induced using 8 mL of lidocaine hydrochloride[d] . After antiseptic scrubbing of the perineum, the vagina was cleaned by flushing with sterile phosphate buffered saline via a speculum, and a urinary catheter was inserted. OPU was performed by transvaginal ultrasound guided follicle aspiration via a 12G double lumen needle. All follicles larger than 5 mm were punctured, follicular fluid was aspirated, and each follicle was flushed 8–10 times with commercial embryo flushing medium[e] supplemented with heparin (20,000 I.E./L)[f] . Follicular fluid and flushing medium was collected into sterile 50 mL conical tubes and poured through a sterile 70 μm filter. The contents of the filter were emptied into a sterile Petri dish containing flushing medium. Subsequently, oocytes were identified using a stereomicroscope, washed three times, transferred into a cryovial containing modified Hepes Synthetic Oviductal Fluid (mH-SOF), and shipped overnight in a polystyrene box designed for transporting organs for transplantation at 22°C to Avantea, Italy for in vitro maturation (IVM), ICSI, in vitro culture (IVC), and cryopreservation.

IVM, ICSI, and in-vitro culture (IVC)

IVM of oocytes, ICSI and IVC to produce blastocysts was performed as described by Colleoni *et al.* [13]. All chemicals were purchased from Sigma[g] unless specified differently. Briefly, shipped oocytes were washed in modified HEPES-buffered SOF medium and subsequently transferred into IVM medium composed of a 50:50 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 supplemented with 10% fetal calf serum, insulin, transferrin, sodium selenite, epidermal growth factor (50 ng/mL), 1 mmol/L sodium pyruvate, follicle stimulating hormone (0.1 IU/mL) and luteinising hormone (0.1 IU/mL) (HMG)[h] . Oocytes were incubated in IVM medium at 38.5°C and in a humidified atmosphere of 5% CO2-in-air for 24 h. After IVM, oocytes were incubated for 5 min in HEPES-buffered SOF with hyaluronidase (2.5 μg/mL) before pipetting in mH-SOF to denude them of their cumulus cells. Oocytes with a first polar body were selected for ICSI.

ICSI was performed using frozen-thawed semen from a stallion selected by the mare owner. A small piece of a 0.5 mL straw was cut-off and thawed in a water bath at 38°C. Approximately 100–150 μL of thawed semen was gently pipetted under 900 μL TALP and the tubes were incubated upright at 38.5°C for 1 h. The upper 800 μL was then diluted with 4 mL of TALP and centrifuged for 10 min (400 ***g***). Alternatively, a 45–90% Percoll density gradient was used to select viable spermatozoa as described by Colleoni [13]. The supernatant was removed and the pellet was resuspended in modified SOF-IVF medium supplemented with PHE (penicillamine, hypotaurine, epinephrine) [14].

ICSI was performed using a piezo drill[i] . Motile, grossly morphologically normal spermatozoa were immobilised using a piezo pulse and a single spermatozoon was injected into the cytoplasm of each metaphase II oocyte. Injected oocytes were cultured in 4-well dishes in modified SOF-IVC medium supplemented with amino acids and BSA [14]. Cleavage was assessed 2 days after ICSI, and non-cleaved oocytes were removed. On days 4 and 6 of IVC, half of the culture medium was replaced. In vitro blastocyst formation was assessed daily on days 6–9 (Day of ICSI = day 0). Blastocysts were cryopreserved and the time required to reach the blastocyst stage (ICSI – blastocyst formation interval) was categorised into two groups: ≤7 and ≥8 days.

Embryo cryopreservation and thawing

Blastocysts were washed with mH-SOF medium at room temperature and transferred to 5% glycerol in mH-SOF for 5 min. Next, embryos were transferred into mH-SOF containing 10% glycerol for 20 min, which included loading the embryo into a 0.25 mL straw and sealing the open end of the straw. The straws were loaded into a freezing machine set at −6.5°C and the cryopreservation protocol was as follows: cooling by exposure to −6.5°C for 5 min, manual seeding followed by holding at −6.5°C for 5 min, followed by cooling to −32°C at −0.5°C/min, after which straws were plunged into liquid nitrogen. Straws were stored at −196°C in liquid nitrogen.

At the time of embryo transfer (ET), the straw containing the embryo was removed from the liquid nitrogen, held in air for 8 s, and then plunged into a 22–24°C water bath for 30 s. The straw was cleaned with an ethanol-soaked tissue and the sealed end of the straw was removed. The contents of the straw were expelled into a Petri dish. The embryo was washed in 8, 6, 4 and 2% glycerol in mH-SOF for 5 min each before being transferred to mH-SOF medium. The thawed embryo was inspected and its size noted before it was loaded into a 0.5 mL straw for ET.

Reproductive management of recipient mares

Recipient mares were housed, and stage of oestrous cycle monitored by transrectal ultrasonography, either at Utrecht University (in-house recipient mares: n = 170) or at the client's premises (outpatient recipient mares: n = 91) across two successive breeding seasons. Towards the end of the follicular phase, transrectal ultrasonography was performed daily to determine the day of ovulation. Outpatient recipient mares were transported to Utrecht University on the day of ET and left immediately after transfer. The following data were recorded for each recipient mare; age, reproductive status (maiden, foaling, barren), day post-ovulation (day 4, 5 or 6), and method of transfer (manual vs. Wilsher forceps) and year of transfer (2015, 2016). Recipient mares were sedated with detomidine hydrochloride[a] (0.067 mg/kg i.v.) and the perineal area was cleaned with betadine scrub[j] and a chlorhexidine gluconate alcohol solution (Spervasept-forte)[k] . Thawed embryos were transferred using a Cassou gun[e] either manually, or using a Polansky speculum and Wilsher forceps. Pregnancy detection was performed 7 days after ET in in-house recipient mares and the size of the embryonic vesicle (n = 65) was recorded. The size of the embryonic vesicle (n = 49) from ongoing pregnancies (but not for lost pregnancies) 7 days after transfer was compared to reference ranges for embryonic vesicle diameter from conventional pregnancies at 10, 11, 12, 13 and 14 days of gestation [15] to estimate the equivalent developmental age of the IVP embryo at the time of transfer (ultrasonographic vesicle age minus 7 days = developmental age of IVP embryo at time of ET). Subsequent pregnancy examinations in in-house recipient mares were performed 23 and 37 days after ET. Pregnancy examinations in outside mares were performed at 10, 23, and 37 days after ET. If abnormal development or impending early embryonic loss [16] were suspected during transrectal ultrasonography, examinations were performed more frequently to determine the period of pregnancy loss more accurately.

Data analysis

Multivariable logistic regression was used to examine the influence of the donor mare (age and reproductive history), recipient mare (age,

reproductive status, management; in-house vs. outpatient, day post-ovulation), embryo (ICSI-blastocyst formation interval) and management (season when OPU was performed; spring–summer–autumn–winter, year of transfer: year 1 vs. year 2, and method of transfer; manually vs. Wilsher technique) on the likelihood of pregnancy and embryonic loss after transfer of an IVP embryo. We did not use P values as they are problematic in stepwise regression and can give rise to irreproducible results [17]. Deviance residuals were used for model checking and model reduction was done using a backward stepwise method based on Akaike's Information Criterion [17, 18]. The final model for the likelihood of pregnancy contained year of transfer, day post-ovulation and age of the recipient mare as variables while the final model for the likelihood of embryonic loss contained the interval ICSI – blastocyst formation and management of the recipient mare. The 95% profile (log–) likelihood intervals were calculated for the odds ratios; 95% profile (log–) likelihood intervals differ from other confidence intervals in that their calculation is based on the profile (log) likelihood but their interpretation is the same as other confidence intervals. A Student's *t* test was used to examine whether embryonic vesicle size 7 days after ET differed between ongoing pregnancies and pregnancies that were lost. Statistical analyses were performed using R version 3.4.0.

Results

The percentage of recipient mares found to be pregnant 7–10, 23 and 37 days after transfer of 261 IVP embryos was 56% (147/261), 49% (129/261) and 48% (124/261), respectively. The diameter of IVP embryos prior to transfer ranged from 128 to 160 μm. The mean (±s.d.) diameter of the embryonic vesicle (n = 65) 7 days after ET to in-house recipient mares was 8.1 (±2.9) mm with a range of 2–16 mm. Despite this great variation in embryonic vesicle size 7 days after transfer, the majority corresponded to a day 12 (33%) or 13 (49%) pregnancy and, therefore, most IVP embryos were equivalent to day 5 or 6 in vivo embryos at the time of ET, and therefore are behind in development (Table 1).

Even though the proportion of pregnancies was lower for embryos transferred on day 6 after ovulation and for embryos produced from infertile mares (Table 2), only year of transfer had an impact on the likelihood of pregnancy. The odds of obtaining a pregnancy was lower in 2016 (53%) than in 2015 (65%) (odds ratio 0.45, 95% profile (log–) likelihood interval: 0.23, 0.84). Neither the age nor the previous fertility of the donor mare had an influence on the likelihood of pregnancy, despite an apparently lower incidence of pregnancy for IVP embryos produced from infertile compared to fertile and subfertile donor mares or mares with an unknown reproductive history. In addition, the odds of obtaining a pregnancy was lower for embryos transferred on day 6 after ovulation (35%) than on day 4 (63%) or 5 (58%) after ovulation (odds ratio 0.4, 95% profile (log–) likelihood interval: 0.13, 1.13), although the 95% profile (log–) likelihood interval contained the number one. None of the other recipient mares (age, reproductive status and management; in-house vs. outpatient), embryo (ICSI-blastocyst formation interval) or management factors (season when OPU was performed and method of transfer) influenced the likelihood of pregnancy.

Embryonic loss by day 42 of gestation was 16% (23/147) and was almost 3.6 times higher (78% 18/23) during early (up to day 28) compared to late (day 28–42) embryonic development (22%, 5/23). Anembryonic vesicles accounted for 26% (6/23) of all embryonic losses, while another 26% (6/23) of the losses occurred before the time at which the embryo proper should be detectable (day 21 of gestation). The mean diameter (±s.d.) of the embryonic vesicle 7 days post transfer was lower for pregnancies that were lost (n = 16; 6.6 ± 2.2 mm) compared to ongoing pregnancies (n = 49; 8.6 ± 3.0 mm). Finally, embryonic loss was not influenced by any of the examined donor mare, recipient mare, embryo or management factors. Nevertheless, the incidence of embryonic loss was higher for an ICSI-blastocyst formation interval of ≥8 days (19.8%, 17/86) than for an interval of ≤7 days (10%, 6/60), (odds ratio 2.2, 95% profile (log–) likelihood interval: 0.8, 7.2).

TABLE 1: Frequency distribution of embryonic vesicle size (<3, 3–5.8, 5.8–8.6, 8.6–13.4, >13.4 mm) 7 days after ET, excluding vesicles that were lost before day 42. Vesicle sizes of <3, 3–5.8, 5.8–8.6, 8.6–13.4, >13.4 mm were considered to equate to days 10, 11, 12, 13 and 14 of pregnancy, respectively [15]. These reference ranges were generated for embryonic vesicle size of conventional pregnancies. The developmental age of an IVP embryo at the time of transfer was estimated using the size of the embryonic vesicle 7 days after ET (developmental age of IVP embryo at transfer = day of gestation assumed from embryonic vesicle size minus 7 days)

Embryonic vesicle size 7 days post transfer	Effective age of embryo at transfer (based on embryonic vesicle size 7 days later)	Number of pregnancies (frequency in %)
<3 mm	3 days	n = 1 (2)
3–5.8 mm	4 days	n = 6 (12)
5.8–8.6 mm	5 days	n = 16 (33)
8.6–13.4 mm	6 days	n = 24 (49)
>13.4 mm	7 days	n = 2 (4)

Discussion

This is the first study to report likelihoods of pregnancy and embryonic loss after transfer of a substantial number of IVP embryos cryopreserved by slow freezing and to examine factors affecting the likelihood of pregnancy and embryonic loss. While 56% of recipients were pregnant at the first post transfer examination, this had declined to 48% by day 42 of gestation, representing embryo losses of 16%. Apart from year of transfer, none of the factors examined had a significant impact on the likelihood of pregnancy.

The 56% of mares recorded as pregnant after transfer of an IVP embryo cryopreserved by slow freezing is comparable to the 59% reported for vitrified IVP embryos in a smaller study [5]. Interestingly, it appears that in vitro production per se has a more marked influence on the likelihood of pregnancy for IVP embryos than cryopreservation. In this respect, McCue *et al.* reported that the likelihood of pregnancy is almost 20% lower for

TABLE 2: Likelihood of pregnancy after transfer of frozen-thawed equine IVP embryos in relation to donor mare, recipient mare, embryo and management factors. Different superscripts within a factor group indicate differences in the likelihood of pregnancy

Influence factor			Proportion of successful transfers
Donor mare	Fertility	Infertile	41%[a] (7/17)
		Subfertile	60%[a] (40/67)
		Fertile	57%[a] (63/111)
		Unknown	56%[a] (37/66)
Recipient mare	Day post-ovulation	Day 4	63%[a] (12/19)
		Day 5	58%[a] (126/217)
		Day 6	35%[a] (6/17)
	Reproductive status	Maiden	58%[a] (36/62)
		Foaling	56%[a] (30/54)
		Barren	56%[a] (81/145)
	Management	Inside	57%[a] (97/170)
		Outside	55%[a] (50/91)
Embryo	ICSI-blastocyst formation Interval	≤7 days	60%[a] (60/100)
		≥8 days	54%[a] (87/161)
Management	Year of transfer	Year 1	65%[a] (43/66)
		Year 2	53%[b] (104/195)
	Season	Spring	52%[a] (46/88)
		Summer	61%[a] (14/23)
		Autumn	58%[a] (48/83)
		Winter	58%[a] (39/67)
	Method of transfer	Wilsher	53%[a] (38/72)
		Manual	58%[a] (109/189)

freshly transferred equine IVP embryos than for in vivo developed, flushed embryos [19]. IVP embryos are probably less susceptible to cryopreservation-induced damage because they are small and do not have a confluent embryonic capsule at the time of blastocyst formation [2, 11]. These morphological characteristics of IVP embryos are considered critical to avoid freezing-induced damage [20]. The incidence of pregnancy dropped to 48% by day 42 of gestation, indicating an incidence of embryonic loss (16%) somewhat higher than for flushed embryos [16]. The incidence of embryonic loss in our study was similar to the 21% reported previously for IVP embryos [21], where Hinrichs attributed this relatively high percentage to the effects of in vitro culture of embryos [22]. However different laboratory culture conditions and embryo selection criteria might also affect the mean quality of the embryos and consequently the incidence of pregnancy loss. Disregarding the embryos that failed to result in a detectable pregnancy, the majority of pregnancy losses occurred during the early embryonic period; more specifically before detection of the embryo proper or a heartbeat.

Besides reporting likelihoods of pregnancy and embryonic loss, this study examined factors affecting the likelihood of pregnancy and embryonic loss for cryopreserved IVP embryos. Surprisingly, the proportion of pregnant recipient mares was higher in 2015 than 2016. It is possible that our study did not include other important influence factors or the different factors within a specific year had a cumulative effect on likelihood of pregnancy. With the exception of year of transfer, none of the examined factors significantly influenced the likelihood of pregnancy. Nevertheless, apparent differences in the likelihood of pregnancy were observed for differences in previous fertility of the donor mare and day post-ovulation of the recipient mare. The proportion of pregnancy appeared to be lower for IVP embryos produced from infertile mares, or when IVP embryos were transferred into recipient mares on day 6 post-ovulation. It is likely that the small sample size in both of the poorly successful categories (relative low number of in-vitro embryos transferred from infertile mares or in recipient mares on day 6 after ovulation) limited the statistical power to identify differences. The lower likelihood of pregnancy for IVP embryos from infertile mares could be a reflection of poor embryo quality, possibly due to poor intrinsic oocyte quality but larger studies are required to confirm this result. In addition, there are several other factors that could influence the fertility of a mare in clinical practice but those factors were not taken into account in our study. Therefore, some care should be taken in interpreting these results. The poor results for IVP embryos transferred into recipient mares on day 6 after ovulation presumably reflects excessive asynchrony between the uterine environment and the effective developmental age of the embryo. For conventional ET, it has been reported that recipient mares can ovulate between 1 day before and 4 days after the donor mare without affecting the likelihood of pregnancy [9]. If a similar window of synchrony applies to IVP embryos, it would be safer to select recipient mares with a less advanced uterine environment (day 4–5), not least because the exact effective developmental age of an IVP embryo is not known at the time of transfer. From a morphological standpoint, equine IVP embryos are thought tobest resemble day 5 in vivo embryos [11]. Indeed, our results indicate that the majority of IVP embryos develop as if they were 5 or 6 days old at the time of transfer or are 1 or 2 days behind in development assuming that the growth rate of in vitro and in vivo embryos after embryo transfer is similar. Nonetheless, some IVP embryos behaved as if they were less developed (day 4 or even younger) and presumably required a less advanced uterine environment. In short, it is plausible that the uterine environment of a recipient mare on day 6 after ovulation is too advanced for a proportion of IVP embryos, and therefore compromises establishment of pregnancy. As a result, it is concluded that, to offer an optimal chance of pregnancy, IVP embryos should be transferred into recipients on day 4 or 5 post-ovulation.

Although none of the factors examined significantly influenced the likelihood of embryonic loss; the proportion of embryonic loss was almost twice as high for embryos that developed slowly (≥8 days) into a blastocyst after ICSI than for embryos that developed quickly (≤7 days). In this respect, a slower rate of in vitro embryo development could reflect diminished embryo quality or viability, but a larger sample size is needed to confirm this hypothesis. In addition, the rate of embryonic vesicle expansion between transfer and first pregnancy diagnosis may help predict subsequent pregnancy loss. The variation in embryonic vesicle size at the first pregnancy detection indicates differences in either the effective developmental age of the IVP embryo at the time of transfer or in embryonic vesicle growth immediately following transfer. Thus, a small embryonic vesicle size at first pregnancy detection could indicate a heightened risk of embryonic loss if, and only if, it results from reduced post transfer embryonic growth. This hypothesis is supported by the finding that the embryonic vesicle at first pregnancy diagnosis was significantly smaller for pregnancies that were lost than those that were maintained.

Overall, our study showed that the likelihood of pregnancy and embryonic loss after transferring frozen-thawed equine IVP embryos is 56 and 16%, respectively. The majority of IVP embryos develop as if they were 5 or 6 days old at the time of ET. With the exception of year of transfer, the likelihood of pregnancy was not statistically influenced by any of the factors examined. Nevertheless, the likelihood of pregnancy appeared to be lower for IVP embryos from infertile mares, or when embryos were transferred into recipient mares on day 6 post-ovulation. Finally, a small embryonic vesicle at the first pregnancy diagnosis could indicate a heightened risk of embryonic loss.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Research ethics committee oversight not required by this journal: retrospective analysis of clinical data. Explicit owner informed consent for inclusion of animals in this study was not stated.

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Authorship

All authors have critically reviewed the manuscript and approved the manuscript for submission. A. Claes, J. Cuervo-Arango, M. Beitsma, C. Deelen and T. Stout were involved in all aspects while J. Van den Broek was involved in study design, data analysis and interpretation and reviewed the manuscript. C. Galli, S. Colleoni and G. Lazzari were involved in data analysis, prepration of the manuscript and also reviewed the manuscript.

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[c]Intervet International BV, Boxmeer, the Netherlands.
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[e]IMV Technologies, Leeuwarden, the Netherlands.
[f]LEO Pharma B.V., Amsterdam, the Netherlands.
[g]Sigma, Milan, Italy.
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Supporting Information

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Summary in Portuguese.