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Success rate in a clinical equine *in vitro* embryo production program

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ABSTRACT

In vitro embryo production (IVEP) via Ovum Pick-Up (OPU) and Intracytoplasmic Sperm Injection (ICSI) has become a popular breeding technique in Warmblood mares because of the high success rate and several practical advantages. IVEP offers a solution for a variety of reproductive issues including, but not limited to, sub-fertility in stallions or mares, poor quality or scarce frozen semen, difficulty in synchronizing donor and recipient mares, and inefficient use of recipient mares. In 515 OPU-ICSI sessions performed in our facility in 2021, a mean of 25.9 antral follicles were aspirated yielding an average 13.8 immature oocytes, which were shipped overnight to a specialized ICSI laboratory (Avantea). One or more blastocysts (range: 0–13 blastocysts) were produced from 78% of procedures with a mean of 2.12 blastocysts per session; the likelihood of pregnancy after transfer of a cryopreserved thawed IVP blastocysts in 2021 (n = 781) was 77.7%. Several donor mare, recipient mare, stallion and embryonic factors influence the likelihood of producing an *in vitro* blastocyst or achieving pregnancy. Approximately 60% of the transferred IVP blastocysts yield a foal; moreover, neither gestation length nor the health of foals is noticeably influenced by IVEP. On the other hand, a skewed sex ratio towards colts is apparent among IVEP foals resulting from day 7 but not day 8 embryos, suggesting that male embryos develop more rapidly *in vitro*. Although serious complications after OPU are uncommon, owners should be aware of their existence, because some complications can be life-threating.

1. Introduction

In vitro embryo production (IVEP) via Ovum Pick-Up (OPU) and Intracytoplasmic Sperm Injection (ICSI) is an advanced breeding technique that has seen an enormous increase in popularity in sport horse breeding over the last 5 years, largely because of improvements in oocyte recovery and blastocyst production rates [1,2]. IVEP is popular among breeders of Warmblood mares because of the high success rate and a number of advantages over other breeding techniques. In particular, OPU-ICSI allows breeders to produce foals from stallions and mares with various fertility problems that other breeding techniques cannot remedy [3]. Furthermore, IVEP can be performed at any stage of the estrous cycle or season [4], and it is the only breeding technique that can be applied in the case of a mare that dies suddenly or needs to be euthanized due to a fracture, colic, etc. [5]. Another important advantage is that hundreds of IVP blastocysts can be produced from a single straw of frozen semen, because ICSI allows semen to be used very efficiently. Finally, IVP horse embryos tolerate cryopreservation better than large *in vivo*, flushed embryos probably as a result of their physical characteristics, including their small size and minimal blastocoele fluid content, and the absence of a confluent embryonic capsule [6]. The ability to cryopreserve IVP embryos with negligible loss of viability has allowed the development of an international trade in embryos and enables much more efficient use of recipient mares. In particular, recipient mares can be used early in the season and their cycle does not need to be synchronized with that of the donor mare. Last but not least, the overall success of IVEP appears to be similar or to exceed that of traditional embryo transfer and allows the possibility of producing one or more embryos from different stallions in a single treatment session [1].

The production of *in vitro* equine blastocysts involves numerous steps including recovery of immature oocytes by transvaginal aspiration of antral follicles, maturation of immature oocytes, ICSI, and culture of embryos to the blastocyst stage [7]. The last step is the most challenging and rate limiting step of the entire IVEP process [8]. A substantial amount of expertise is required to obtain consistently excellent results with regard to the production of *in vitro* blastocysts of high quality. It is common practice to freeze IVP blastocysts by either slow freezing or vitrification, not least

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because IVEP is often done outside the physiological breeding season and frequently results in multiple embryos. An important contributor to the growth in IVEP, is that oocytes can be collected at different locations and shipped overnight at room temperature in an appropriate holding medium (modified synthetic oviductal fluid or embryo holding media) to a laboratory where *in vitro* maturation of oocytes, ICSI and embryo culture can be performed [9,10].

2. Results of IVEP through OPU and ICSI in 2021

Between January 10th and October 20th, 2021, a total of 515 OPU sessions were performed at our facility, predominantly on Warmblood mares. On average, 25.9 antral follicles (range: 5–89) were punctured, aspirated and flushed 6 to 10 times with commercial oocyte recovery medium. A mean of 13.8 oocytes (range 1–48) were recovered per mare, resulting in an average oocyte recovery rate of 53%. All oocytes were shipped overnight at room temperature (20–22 °C) in modified synthetic oviductal fluid [11] to an ICSI laboratory (Avantea, Italy). The average maturation rate for oocytes shipped to the ICSI laboratory was 59%. The age of the donor mare was an important factor affecting the success of OPU. The number of follicles aspirated and oocytes recovered is significantly lower in mares above 20 years of age [12]. This age-related decline in the antral follicle number is likely the result of a depleted follicular reserve [13]. Surprisingly, the maturation rate of oocytes is significantly higher for aged mares, which largely compensates for the reduced number of oocytes recovered [12]. The success of equine *in vitro* embryo production has improved significantly over the years. Approximately 78% of the OPU-ICSI sessions (n = 515) performed in 2021 yielded one or more blastocysts, compared to only 48% (n = 309) in 2016. Furthermore, multiple IVP blastocysts (range 0–13 embryos) can be obtained per OPU-ICSI session with a mean of 2.12 embryos per session in 2021 (Fig. 1). In contrast, the embryo recovery rate after artificial insemination and embryo flushing in Warmblood mares is typically around 50–65% and the number of recovered embryos very rarely exceeds 2 embryos [14]. Thus, for most mares, the success rate of embryo production by IVEP exceeds that of traditional embryo transfer; on the other hand, IVEP is more invasive and costly.

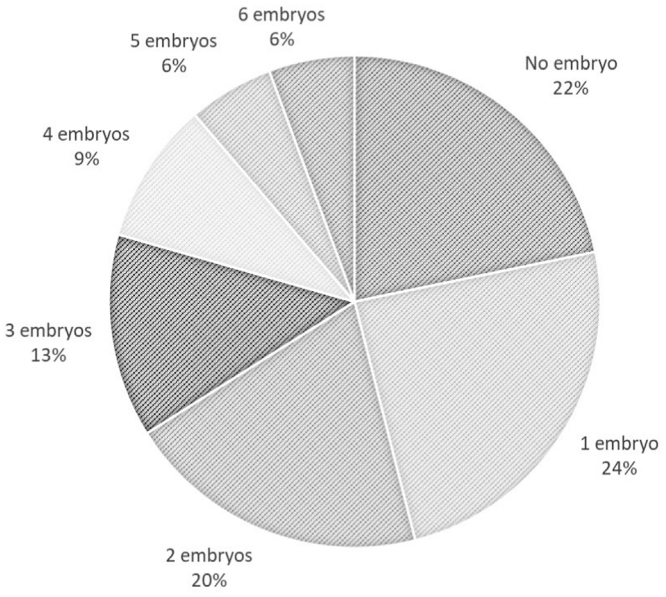

Fig. 1. The likelihood of producing multiple IVP embryos in a single OPU session (n = 515 OPU-ICSI sessions).

There are several mare and stallion factors that influence the production of IVP blastocysts. The two most important mare factors affecting the success of *in vitro* blastocyst production are the identity of the mare and the number of oocytes recovered [15]. The success of IVEP is highly repeatable within mares. Donor mares that are successful (i.e. produce at least 1 embryo) in the first OPU-ICSI session are more likely to be successful in a subsequent OPU-ICSI session than mares that failed to produce an embryo and *vice versa*. In unsuccessful donor mares, it is recommended to delay performing a second OPU-ICSI session until the mare has more antral follicles than at the first attempt in order to increase the number of oocytes recovered. Alternatively, changing the stallion used for ICSI can improve the likelihood of success in mares that previously failed to yield embryos [15]. As mentioned earlier, a donor mare should have sufficient antral follicles prior to OPU, because the likelihood of success is also influenced by the number of follicles aspirated and oocytes recovered [4,15]. The percentage of successful OPU-ICSI sessions increases proportionally with the number of aspirated follicles until reaching a plateau after 20 aspirated follicles [15]. The success of *in vitro* embryo production is also influenced by the breed of the donor mare and stallion. Although the number of oocytes recovered is similar between Warmblood and Arabian mares, the cleavage and blastocyst rates are significantly lower for the Arabian horse oocytes [16]. With respect to the age of the donor mare, even though the antral follicle count and the number of oocytes recovered is significantly lower in aged mares, the cleavage and blastocyst rate is not reduced, suggesting that the developmental competence of oocytes from aged mares is not compromised. However, the total number of IVP blastocysts does tend to be lower for aged mares, although this is predominantly a reflection of the reduced number of oocytes recovered [12].

The stallion also has an effect on the success of *in vitro* blastocyst production by ICSI. Nevertheless, the variation in the blastocyst rate between individual horses used for at least 4 ICSI sessions was much less between stallions than between mares [15]. Besides the variation in blastocyst rate between individual stallions, there can also be considerable variation in blastocyst rate between different batches of frozen semen from the same stallion. This could be the result of the age of the stallion, characteristics of a particular ejaculate, or an effect of the freezing centre or different conditions under which the semen was processed, frozen and stored [16]. In particular, it is important that stallion spermatozoa are motile after thawing, because ICSI with immotile spermatozoa is unlikely to result in production of blastocysts, irrespective of whether an exogenous oocyte activation medium is used [17]. Furthermore, although it has been reported that re-frozen semen can be used without compromising the blastocyst rate, provided that motile spermatozoa are present after thawing [18], the authors do not recommend using refrozen of Warmblood stallion semen for ICSI because the IVEP success rates within our clinical program are very low; only 27% of the ICSI procedures (n = 15) performed using re-frozen semen from 5 different stallions resulted into the production of one embryo, and none resulted into the production of multiple embryos. The poor success after using re-frozen semen is evident in both a low cleavage (21%) and blastocyst (4%) rate. Similarly, although cryopreserved sex-sorted semen has not been used in our program, other studies have shown that the cleavage rate was low (20–30%) when sex-sorted semen was used for ICSI [19,20]. Despite the low cleavage rate, the quality of embryos produced using sex-sorted semen does not appear to be compromised because they can yield a pregnancy after transfer [19] and develop into live, healthy foals [20]. It is generally accepted that advanced semen processing techniques such as swim up and/or density gradient centrifugation should be applied to obtain a high quality semen

sample for ICSI. Choi et al. [17] demonstrated that a combination of swim up and density gradient significantly improved the cleavage and blastocyst rates for re-frozen semen with low fertility via ICSI [17]. Finally, *in vitro* fertility of a stallion is not a simple reflection of *in vivo* fertility because semen from sub- or infertile stallions can be used to produce blastocysts, although the cleavage and blastocyst rates after ICSI of abattoir oocytes is significantly lower for infertile than fertile stallions [16]. Conversely, it is important to remember that using semen of apparently good quality (motility and morphology) from a fertile stallion is no guarantee for success in an IVEP program [16].

3. Likelihood of pregnancy

In 2021, the likelihood of pregnancy 7–10 days after transfer of a frozen-thawed IVP equine embryo (n = 781) was 77.7%. Although the likelihood of pregnancy was lower and more variable during the first two years of our clinical program (2015–16), it has remained stable at above 70% over the last 5 years. Interestingly, there is considerable variation in embryonic vesicle size one week after transfer (2–16 mm). This could indicate differences in effective embryonic developmental age at the time transfer. On the other hand, a reduced embryonic growth rate in the period after transfer could also result in a small embryonic vesicle which, in turn, could indicate a higher risk of subsequent early pregnancy loss [21]. Unless the embryonic vesicle is unusually small (less than 4 mm), pregnant recipient mares are usually not examined again until 28–30 days of gestation (taking day of transfer as day 4) to confirm pregnancy and to rule out monozygotic twins or triplets. These monozygotic multiple pregnancies are characterized by a single embryonic vesicle in which two or more embryo propers develop; they cannot be detected before the appearance of the embryo proper. Although the incidence of monozygotic twins after transfer of a single IVP embryo is low (1.6%), detection prior to development of the endometrial cups is crucial, because of the poor outcome irrespective of whether an attempt to reduce the twin to a singleton is attempted or not [22].

Several donor mare, recipient mare and embryonic factors influence the likelihood of pregnancy and foaling. First, the pregnancy outcome at day 50 of gestation is approximately 15% lower in Arabian than Warmblood mares [1]. Interestingly, in contrast to other breeding techniques, the likelihood of pregnancy does not appear to be influenced by the age of the donor mare. IVP blastocysts from aged mares are as likely to result into a pregnancy after transfer as IVP blastocysts from younger mares [12]. Currently, almost all cryopreserved IVP equine embryos in our program are transferred into recipient mares on day 4 after ovulation (day of ovulation = day 0), and a small percentage of embryos are transferred into recipient mares on day 3 after ovulation. Previously, we showed that a more advanced uterine environment has a negative impact on the likelihood of pregnancy. The pregnancy rate was significantly lower when IVP embryos were transferred into recipient mares on day 5 (57%) or day 6 (42%) after ovulation compared to day 3 (71%) or day 4 (74%). Furthermore, the embryonic vesicle was significantly larger one week after transfer when IVP embryos were transferred on day 4 compared to day 5 or day 6 after ovulation. Besides the number of days after ovulation, the recipient's number of corpora lutea (CL) on day 3 after ovulation tended to affect the percentage of ongoing pregnancies; it tended to be higher in day 3 recipient mares with 2 CLs than those with 1 CL. In addition, the embryonic vesicle was significantly larger one week after transfer in day 3 recipient mares with 2 corpora lutea compared to those with 1 corpus luteum [23].

The likelihood of pregnancy is also influenced by the speed of *in vitro* embryo development [1,24,25]. In general, embryos are cultured *in vitro* for 6–9 days after ICSI before they reach the blastocyst stage at which they can be either transferred or cryopreserved by slow-freezing or vitrification [1]. The likelihood of pregnancy is similar for IVP blastocysts cryopreserved on day 7 and day 8 (69–73%) but lower for day 9 IVP blastocysts (55–62%). Furthermore, pregnancy loss appears to be higher for day 9 than for day 7 or 8 IVP blastocysts [1]. Thus, slow *in vitro* embryo development is associated with a lower likelihood of pregnancy and a higher incidence of embryonic loss.

4. Likelihood of foaling

The production of equine blastocysts *in vitro* does not appear to influence the length of gestation (339 days) which is comparable to the gestation length of mares bred by other artificial breeding techniques [24]. Approximately 15% of IVEP pregnancies are lost between one week after embryo transfer and foaling [24] such that, currently, approximately 60% of cryopreserved IVP equine embryos will result into a live foal after transfer. Scientific data regarding the health of foals produced via ICSI are rather scarce, likely because of the difficulty in obtaining large sets of data from clinical programs. A study from Texas A&M, however, reported that foals produced by ICSI had a longer upper hindlimb whereas in other respects their general health, weight and height was not significantly different to those of foals produced by natural breeding [26]. Similarly, we recently reported that 98.4% of the foals (n = 193) born after transfer of cryopreserved-thawed equine IVP blastocysts survived and were healthy. In short, IVEP does not appear to have any adverse effects on the health of newborn foals [24]. At present, however, it is not known if there are any effects on health or performance later in life.

The sex ratio (male:female) of foals born from our clinical IVEP program during the first 3 years was skewed towards males since 61% of the foals were colts and 39% were fillies. One of the factors that influenced this skewed sex ratio was the speed of *in vitro* embryo development with a higher likelihood of obtaining a colt after transfer of a day 7 than a day 8 IVP blastocyst. More specifically, 71% of foals were colts and 29% were fillies after transfer of day 7 IVP blastocysts (n = 79) whereas transfer of day 8 IVP blastocysts (n = 114) resulted into 54% colts and 46% fillies. Thus, it appears that using our system male equine embryos develop more rapidly *in vitro* than female embryos [24]. It is well known that *in vitro* culture conditions and media can have a significant impact on embryo quality, pregnancy outcome and in some species the sex ratio. Whether similar findings can also be detected in other equine IVEP systems remains to be determined.

5. Disadvantages of OPU

There is no doubt that OPU is more invasive than traditional embryo transfer. Nevertheless, the complication rate appears to be low, especially if the procedure is performed by experienced veterinarians. It is however crucial that mare owners are aware of the potential complications becomes some of these complications can be life-threatening. Prophylactic antibiotics were administrated in all donor mares prior to the OPU procedure. Despite proper sedation and administration of an anti-spasmolytic drug, there is always a risk of causing a rectal tear since the ovary has to be grasped, manipulated caudally and medially towards the head of the transvaginal ultrasound probe, and held firmly via the rectum throughout the procedure. A study from Texas A&M reported that some rectal bleeding occurred in 16% of 153 OPU sessions, although none of the mares displayed clinical symptoms afterwards [27]. The incidence of rectal bleeding in the last year of our clinical OPU program was 0.4% (n = 2/515 OPU sessions) and both were limited

to mucosal damage. The authors strongly believe that the incidence of rectal damage is influenced by the experience of the veterinarians performing the OPU; rectal bleeding/trauma is more common during the training period of inexperienced veterinarians who experience more difficulty in adequately grasping and manipulating the ovary into position and holding it for the whole procedure; repeatedly dropping and regrasping the ovary can lead to damage to the rectal wall.

Another potential complication of transvaginal follicle aspiration is vaginal or intra-abdominal haemorrhage caused by accidently puncturing a blood vessel during the procedure [28]. Furthermore, if the needle damages a blood vessel in the broad ligament of the uterus, or the uterine artery is damaged by tension on the ligament, a hematoma can form within the broad ligament [28]. To date, the authors have encountered 3 mares that suffered severe intra-abdominal haemorrhage and developed clinical signs of acute blood loss (colic, pale mucus membranes, weak pulse, elevated heart and respiratory rates, and a subsequent drop in the packed cell volume) either immediately after or the day after OPU. All 3 mares survived after hospitalization and conservative treatment. A small remnant of the hematoma could still be detected in one of the mares 1 year later. Nevertheless, it is difficult to accurately estimate the incidence of internal haemorrhage as minor bleeding often goes unnoticed. Velez et al. showed the number of red blood cells in peritoneal fluid were increased 3 days after OPU in all of 6 mares monitored, indicating that each OPU session is associated with some form of intra-abdominal bleeding [27].

There is also a small risk of mares developing a peritonitis after transvaginal follicle aspiration. To reduce this risk, the perineal region of donor mares should be cleaned thoroughly prior to the OPU procedure and care should be taken not to puncture either intestine or rectum when the needle is introduced into the peritoneum and used to puncture the ovary. To the authors knowledge, only one of the OPU donor mares from our program was diagnosed with a peritonitis after OPU, she subsequently developed acute laminitis. Although this clinical peritonitis appears to be an uncommon complication, a previous study showed that the number of white blood cells in peritoneal fluid was slightly increased at 3 days post-OPU in 2 of 6 mares suggesting that a reasonable percentage of mares may develop a sub-clinical peritonitis after OPU that spontaneously resolves. Finally, an ovarian abscess can also form as a consequence of OPU, even though the incidence of ovarian abscessation appears to be very low (<0.5%) [27].

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