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A retrospective comparison of the efficiency of different assisted reproductive techniques in the horse, emphasizing the impact of maternal age

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ABSTRACT

Advancing maternal age is known to negatively affect fertility in the horse. This age-related decrease in fertility has been linked primarily to reduced oocyte quality rather than to impaired uterine function. In the past decade, the use of ovum pick-up (OPU) and ICSI to produce foals has rapidly gaining popularity amongst sport horse breeders. However, it is not yet known how maternal age influences the efficiency of a commercial OPU-ICSI program and whether the age effect is similar to that observed for other ART in the horse. To answer this question, reproductive records of 289 mares bred by natural mating (NM), 328 mares bred by AI, 205 embryo donor mares (AI-EF-ET), and 473 mares submitted for OPU-ICSI and ET were analyzed retrospectively using a regression model to investigate the effects of maternal age and breeding technique on the likelihood of producing a viable pregnancy. The reproductive efficiency (quantified as the proportion of mares that yielded at least one Day 45 pregnancy) of the different breeding techniques NM, AI, AI-EF-ET and OPU-ICSI-ET was 63.3, 43.9, 45.8 and 37.4%, respectively ($P < 0.05$). However, the frequent production of multiple embryos per ICSI session (up to 10 embryos in one attempt), makes OPU-ICSI-ET as effective as AI-EF-ET when measured in terms of the mean number of Day 45 pregnant recipients per donor mare. Increasing maternal age was associated with a reduction ($P < 0.05$) in the reproductive efficiency of all breeding techniques (NM, AI, AI-EF-ET) except OPU-ICSI-ET ($P > 0.05$). In the OPU-ICSI-ET group, increasing maternal age was associated with a lower number of follicles aspirated and oocytes recovered per mare. Nevertheless, the percentage of blastocysts per injected oocyte, and post-ET likelihoods of pregnancy and pregnancy loss were not influenced by the age of the oocyte donor mare ($P > 0.05$).

1. Introduction

Since the earliest scientific report of artificial insemination (AI) in horses in 1898 [1], the horse breeding industry has witnessed a slow but steady development of other assisted reproductive techniques (ART), from AI to cloning; for various reasons, uptake into commercial practice has lagged behind other domestic species like cattle. Nevertheless, currently AI is the most common form of breeding in most types of domestic horse, excepting the Thoroughbred, where ARTs of any form remain banned by the Thoroughbred registration authorities [2], and some of the native pony breeds. Embryo recovery by uterine flushing 7–9 days after ovulation, followed by embryo transfer (ET) into a synchronized recipient mare is the ART used most commonly to generate more than one offspring per year per mare and/or to bypass the need for own gestation in mares that are not able to carry a foal to term, for reproductive, medical or competitive (sport) reasons [3].

In the last decade, *in vitro* production of embryos by ovum pick up (OPU) followed by ICSI, has become popular amongst sport horse veterinarians and breeders, and led to the development of several equine ICSI laboratories offering commercial OPU-ICSI programs in different parts of the world [4]. OPU-ICSI was initially considered primarily for sub-fertile, often aged, mares that are unable to conceive or produce a viable embryo by more standard breeding methods (natural mating or AI) due to oviduct blockage, chronic uterine infection or fibrosis, irreparable damage to the reproductive tract or infertility of unknown origin, as well as

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for stallion sub-fertility (low sperm output, low numbers of normal sperm, idiopathic infertility). However, there is also an increasing demand from horse breeders to use OPU-ICSI in reproductively normal, fertile mares to enable more efficient use of limited and costly frozen/thawed semen from valuable stallions [5]. Other ARTs such as oocyte transfer (OT) after aspiration of an oocyte from the pre-ovulatory follicle of donor mares are also performed commercially in Argentina [6] and the USA [7]. In Europe, however, OT is not widespread not least because breeding techniques requiring surgery are banned in several countries, for welfare reasons.

It is difficult to compare the reproductive efficiency of embryo flushing (EF) and OPU-ICSI-ET between studies, not least because the horse populations and indications may differ markedly. Furthermore, the expertise of the operator performing the ET [8] and the reproductive management of the recipient [9] have a significant impact on the final success of the ART. In contrast, within a given veterinary clinic, where the reproductive management of mares and expertise of personnel is less variable, it should be possible to usefully compare the efficiency of each technique to give a more accurate prognosis or prediction of the chance of obtaining a viable pregnancy, and thereby assist a horse owner in choosing between techniques.

The detrimental effect of advancing maternal age on fertility is a well-known and gradual phenomenon in the horse [10–14] and is primarily a function of a markedly higher incidence of early embryonic loss between Days 2–14 after fertilization in aged compared to younger mares with little or no difference in the percentage of oocytes that actually gets fertilized initially [15,16]. Nevertheless, studies involving the transfer of early stage embryos from old mares to the oviduct or uterus of younger mares suggests that main factor underlying the age-related reduction in fertility is reduced oocyte quality [14] rather than declining uterine function [15–18]. Since the likelihood of sub-fertility increases with age [15], the population of oocyte donor mares enrolled in a commercial OPU-ICSI program is likely to be more skewed towards advanced age compared to donors for embryo flushing. Theoretically, it follows that the quality of embryos produced by OPU-ICSI from aged mares should be of lower than that of embryos from younger mares, given that the dogma is that age primarily affects oocyte quality; however, early reports suggest that this might not be the case [19]. The latter study compared oocyte developmental competence between young (4–9 years) and old (≥20 years) mares following ICSI and, surprisingly, found no significant differences in likelihood of cleavage, blastocyst formation or pregnancy after transfer of ICSI embryos produced from oocytes from young versus old mares [19]. However, the reported study used oocytes recovered from pre-ovulatory follicles which were therefore partially matured *in vivo*, while the total number of mares used was too low to draw definitive conclusions. It is therefore not clear how mare age influences the reproductive efficiency of a commercial OPU-ICSI program based on the aspiration of immature follicles, not least because aged mares are known to have a lower antral follicle count [20].

The objectives of this study were 1) to compare the reproductive efficiency of three different ARTs (AI, AI-EF-ET and OPU-ICSI-ET) performed in a single veterinary clinic and 2) to determine the influence of maternal age on the likelihood of producing a viable pregnancy via each ART. We hypothesized that advancing maternal age would be associated with a reduced reproductive efficiency in all ARTs except OPU-ICSI-ET. It was also assumed that advancing maternal age would be associated with a lower number of aspirated follicles and recovered oocytes per OPU session, but that there would be no effect on oocyte/embryo developmental competence following ICSI and ET.

2. Materials and methods

2.1. Animals and experimental groups

Retrospective reproductive records of mares admitted to the Department of Equine Sciences, Utrecht University during the years 2015–2018, for either 1) artificial insemination (AI; n = 328 mares); 2) AI followed by embryo flushing (EF) 8–9 days after ovulation and immediate embryo transfer (ET) into a synchronized recipient (AI-EF-ET; n = 205 mares); and 3) Ovum pick-up (OPU) of immature follicles, followed by *in vitro* maturation (IVM) of oocytes, intracytoplasmic sperm injection (ICSI), *in vitro* culture (IVC) and cryopreservation of embryos and ET into recipients (OPU-ICSI-ET; n = 473 mares).

Most of the mares included in the study were warmbloods (show jumping or dressage mares): 87.0, 96.6 and 99.4% for the AI, AI-EF-ET and OPU-ICSI-ET groups, respectively. The rest included Friesian, Standardbred, Arabian, Cobs and Quarter-horse breeds. To minimize the effects of individual fertility, mares were only included in the study once (i.e. the first visit during the study period): the number of mares is therefore equal to the number of estrous cycles or OPU sessions. All procedures (AI, EF, OPU and ET) were performed in the same clinic and by the same team of veterinary surgeons, except IVM, ICSI, IVC and cryopreservation of embryos which were performed at an external laboratory in Italy (Avantea, Cremona). A fourth group of mares (Thoroughbred) bred by natural mating (NM) was used as a reference value for reproductive efficiency, for comparison with the three ARTs. The NM group consisted of first cycle covers of 289 (the number of mares also equaled the number of estrous cycles) National Hunt Thoroughbred mares bred by one of five different stallions of proven fertility (historical breeding records of 62–68% first cycle pregnancy rate) in a single stud farm during three consecutive breeding seasons (2016–2018) in England, UK, and managed by a different veterinary surgeon (John R. Newcombe) to those in charge of AI, EF, OPU and ET.

The mares of the OPU-ICSI-ET group were presented for clinical IVF for one of three major reasons: 1) the mare was actively participating in high level sporting competition, and it was therefore not desirable that she carry a foal to term; 2) the mare had a previous history of subfertility or infertility (due for example to advanced age, recalcitrant uterine infection, or infertility of unexplained origin); 3) in the majority of cases, for efficient use of sperm of limited availability (e.g. from deceased stallions or from semen doses of high cost).

Recipients mares used in the study (AI-EF-ET and OPU-ICSI-ET groups) were mainly (>90%) warmbloods with a mean age of 7.3 ± 3.2 years old (3–12 years old).

2.2. Reproductive procedures

2.2.1. Artificial insemination (AI)

The protocol for AI varied depending on the type of semen used for each mare. For inseminations with cooled-transported semen, ovulation was induced with 1500 IU of hCG (Chorulon, MSD Animal Health, The Netherlands) or 200 μg buserelin (Suprefact, Sanofi-Aventis, Italy), depending on the history of refractoriness to hCG [21], when the mare was in estrus and 24–32 h before the expected time of AI. A commercial dose (volume of 15 mL) of cooled sperm collected and processed approximately 8 h before AI, was inseminated into the uterine body. Ovulation was confirmed ultrasonographically the next morning. Mares that did not ovulate within 48 h of AI were re-inseminated.

For mares inseminated with frozen semen, the protocol varied depending on the number of straws available for each dose. The

mean number of straws per dose in the studied period was 3.0 ± 2.4 (range 1–8 straws per dose). For inseminations with 1–2 straws, ovulation in estrous mares was induced with hCG or buserelin on the evening of Day 0 (at 22:00 and 18:00 h, respectively), and mares were examined at 8:00 and 16:00 h of Day 1, and at 8:00 and 12:00 h of Day 2. Mares were inseminated immediately after ovulation was first detected. Most mares ovulated between 8:00 and 12:00 h of Day 2. When more than 2 straws were available for AI, half of the dose was inseminated at 16:00 h (32 h after induction of ovulation: pre-ovulatory AI), and the second half immediately after ovulation was detected, usually by 8:00 h the next morning (post-ovulatory AI). All straws were thawed by immersion for 30 s in a water bath at 37 °C. Deep horn intrauterine AI was performed with a long flexible catheter into the tip of the horn ipsilateral to the ovary containing the preovulatory or freshly ovulated follicle.

Regardless of the type of semen used for AI, all mares were examined within 24 h after the last AI for signs of mating-induced endometritis. Oxytocin and uterine lavage with Lactated Ringer's solution were used accordingly. In mares that were to remain pregnant (AI), pregnancy diagnosis was performed 14–18 days after ovulation, and re-confirmed at 45 days after ovulation.

2.2.2. Embryo flushing (EF)

The uterus of embryo donor mares was flushed using 3 × 1 L of a commercial Lactated Ringer's solution (Baxter Nederland BV, Utrecht, The Netherlands) supplemented with 0.5% v:v fetal calf serum via a closed system with an in-line embryo filter on Day 8 or 9 after ovulation. Embryos were located using a dissecting microscope (Olympus SZ60, Olympus Nederland B·V., Leiderdorp, NL) and, after washing, were held in holding medium (Syngro; Bioniche Animal Health INC, Athens, GA, USA) at room temperature between 30 min and 2 h before being transferred.

2.2.3. Ovum pick-up (OPU)

The majority of oocyte donor mares were checked for antral follicle count by a referring veterinarian at a distant location. The recommendation was for mares to have at least 15 to 20 follicles>1 cm in diameter prior to OPU. However, this was not always possible, as some mares never reach that number of follicles. Out patients were admitted to the clinic on the day of OPU. For these mares, the total time spent in the clinic varied between 2 and 3 h. A small percentage of mares were resident at the clinic for several weeks or months and were scheduled for OPU whenever the number of antral follicles was considered suitable (i.e. > 15–20 follicles), regardless of reproductive phase (diestrus, estrus or anestrus).

Peri-operative antibiotic treatment, analgesia and sedation were initiated by administering broad spectrum intravenous antibiotics (gentamycin and benzyl penicillin), flunixin-meglumine, detomidine hydrochloride and butorphanol tartrate immediately prior to the OPU procedure. Caudal epidural anesthesia was induced with 7–8 mL of 2% lidocaine hydrochloride. After aseptic scrubbing of the perineum, a urinary catheter was inserted. OPU was performed by transvaginal ultrasound guided follicle aspiration via a 12G double lumen needle attached via a collection bottle to a vacuum pump. All antral follicles larger than 3 mm were punctured, follicular fluid was aspirated, and each follicle was flushed 8 to 10 times with 0.5–5 mL (depending on follicle size) embryo flushing medium supplemented with heparin sodium (20 IU/mL) and prewarmed to 37 °C. Follicular fluid and flushing media were collected into sterile 50 mL conical tubes maintained at 37 °C. The collection effluent was poured through a sterile 70 μm filter immediately after the end of the OPU procedure. The contents of the filter were emptied into a sterile petri dish. Subsequently, oocytes were identified using a stereomicroscope, washed three times, transferred into a cryovial containing modified Hepes buffered Synthetic Oviductal Fluid (mH-SOF) [22,23], and shipped overnight in a polystyrene box designed for transporting organs for transplantation (ChillTherm, Sonoco Thermosafe Ltd, Arlington Heights, IL, USA), at 22 °C to a dedicated equine ICSI laboratory (Avantea, Italy) for *in vitro* maturation (IVM), ICSI, *in vitro* culture (IVC) of embryos, and embryo cryopreservation.

2.2.4. IVM, ICSI, IVC and embryo cryopreservation

In vitro maturation of oocytes, ICSI and IVC to produce blastocysts was performed as described by Colleoni and others [24] with one of 107 different stallions chosen by the mare's owner. Blastocysts were identified 6–8 days after ICSI and cryopreserved by slow freezing in 10% glycerol [25], and sent back to Utrecht University in liquid nitrogen for ET. Blastocysts that developed on day 9 or later were discarded. In brief, embryos were taken from culture to H-SOF medium to cool to room temperature for 30 min. Then embryos were put in 5% glycerol H-SOF solution for 5 min and then in 10% solution for 20 min before being loaded in 0.25 mL straws. The straw was sealed and placed in alcohol bath at −6.5 °C for 5 min. Then the straw was seeded by touching it with a pair of tweezers previously immersed in liquid nitrogen. Freezing was continued at a rate of −0.5 °C until it reached −35 °C, and finally plunged into liquid nitrogen.

2.2.5. Embryo transfer (ET)

Recipient mares in estrus were scanned daily for diagnosis of ovulation. Day 4–9 and Day 3–5 recipients (Day 0 = Day of ovulation) were used for transfer of *in vivo* recovered and *in vitro* produced (IVP) embryos, respectively. The choice of recipient's day after ovulation at the time of ET for *in vivo* recovered (Days 4–9) or *in vitro* produced embryos (Days 3–5) reflected the use of available evidence on the optimal window of embryo-uterine synchrony [26]; in this respect, ICSI blastocysts have been shown to be less advanced in development than flushed embryos [27] and to 'behave' like day 6 flushed embryos [5]. Potential recipient mares were examined by transrectal ultrasonography to confirm the absence of endometrial edema, free intrauterine fluid and the presence of uterine tone, a CL and a tight cervix. If considered suitable for ET, recipient mares were restrained in stocks and sedated with 4 mg of intravenous detomidine hydrochloride, regardless of weight. Next, the perineum was thoroughly scrubbed with a povidone iodine solution and, after rinsing with clean water, the cutaneous-mucosal border at the entrance into the vestibule was disinfected with a chlorhexidine gluconate-alcohol ketonatus solution (Spervasept forte; Spervital, Toldijk, The Netherlands). No further routine pre- or post-ET supportive treatment was administered to recipient mares. Embryos were transferred transcervically with the aid of a cervical forceps and vaginal speculum [8]. The first pregnancy diagnosis was performed 4- and 7-days post-ET for *in vivo* and IVP embryos, respectively, as those were the anticipated times at which an embryonic vesicle of 5–10 mm in diameter was expected to be present in the uterus of pregnant recipients following ET of each type of embryo [26]. An additional pregnancy diagnosis was performed at Day 45 of embryo development. IVP embryos were thawed in a water bath at room temperature for 20 s and then placed in mH-SOF solution with decreasing percentages of glycerol (8, 6, 4 and 2%) for 5 min each, and finally, held in mH-SOF medium before ET (all embryos were transferred within 20 min of thawing).

2.2.6. Natural mating (NM)

Thoroughbred mares were mated according to previous records of follicle growth, preovulatory follicle size, and day of the cycle (if this was known). Ovulation inducing drugs were not used

routinely, and only to ensure that the interval from mating to ovulation was less than 5 days. Therefore, if a mare had not ovulated within 48–72 h of mating, she was treated with 200 μg buserelin (Suprefact, Sanofi-Aventis, UK) subcutaneously to ensure ovulation during the following 48 h. Every mare received an intrauterine instillation of 12 mL of an antibiotic solution containing 1800 mg of procaine penicillin (6 mL of Depocillin 250 mL; MSD Animal Health) and 900 mg of framycetin (6 mL of Framomycin 15%; Novartis Animal Health, Cambridge, UK) between 4 and 48 h after mating. In addition, all mares were treated with 25 IU of oxytocin intravenously (2.5 mL of Oxytocin Leo; Leo Animal Health Laboratories, Aylesbury, UK) 24 h after the intrauterine antibiotic treatment. Pregnancy diagnosis was performed at 12–14 days and again at 45 days post-ovulation to confirm on-going pregnancy. Twin reduction was carried out manually, if possible, at the first pregnancy diagnosis.

2.3. End points and statistical analyses

At least one Day 12–18 pregnancy: number of mares pregnant (NM and AI) by Day 18, or number of donor mares (AI-EF-ET and OPU-ICSI-ET) that yielded at least one Day 12 pregnant recipient, divided by the total number of mares in the treatment group (%).

At least one Day 45 pregnancy: number of mares pregnant (NM and AI) at Day 45, and number of donor mares (AI-EF-ET and OPU-ICSI-ET) that yielded at least one Day 45 pregnant recipient, divided by the total number of mares in the treatment group (%). This is the main parameter of reproductive efficiency in this study.

Post-ET Day 12 pregnancy: number of pregnant recipient mares 4 or 7 days after ET divided by the total number of transfers of flushed or IVP embryos, respectively (%).

Early embryonic loss (EEL): Number of pregnancy losses between Day 12–18 and Day 45 divided by the total number of Day 12–18 pregnancies.

For OPU-ICSI-ET the following extra end points were calculated:

Oocyte recovery: number of oocytes recovered after OPU, divided by the total number of follicles aspirated (%).

Maturation percentage: number of injected oocytes (MII) divided by the number of recovered oocytes (including the oocytes that degenerated or failed to -mature (%).

Cleavage percentage: number of cleaved zygotes by day 3 after ICSI divided by the total number of injected (MII) oocytes (%).

Blastocysts per injected or recovered oocyte: number of blastocysts produced 6–9 days after ICSI divided by the total number of injected (MII) or recovered oocytes, respectively.

No ICSI: number of mares submitted for OPU in which ICSI was not performed because of the absence of MII oocytes, divided by the total number of mares (%).

Day 12/45 pregnancy per ET: number of Day 12 or Day 45 pregnant recipients divided by the number of ETs, respectively (%). For donor mares with more than one ET, a weighted average was calculated. For example, a donor mare with 4 ETs (originating from a single OPU attempt) which resulted in three Day 12 and two Day 45 pregnancies, had a Day 12 and Day 45 per ET pregnancy percentage of 75 and 50%, respectively. The same system was used to calculate the incidence of early embryonic loss (EEL).

Mean Day 45 pregnant recipients: total number of Day 45 pregnant recipients divided by the number of donor mares.

Excess IVP embryos: number of non-transferred IVP embryos during the studied period which were still stored in liquid nitrogen for future ET. All mares included in the OPU-ICSI-ET group with a successful OPU-ICSI session had at least 1 embryo transferred.

All data were analyzed using the statistical package Systat13 (Software Systat13 Inc. San Jose, CA, USA). Linear association between breeding technique groups and continuous or dichotomous variables was tested using analysis of variance or chi-squared tests, respectively. For each breeding technique group, a logistic regression model was used to calculate odds ratios for a Day 45 pregnancy, using age (as sequential data), type of semen used (cooled vs. frozen) and number of inseminations per estrus (for the AI and AI-EF-ET groups only), and mare status (foaling vs non-foaling) as explanatory variables. The stallion ID and the year of study were included in the models as random effects. To examine the potential effect of maternal age on reproductive efficiency of mares from the OPU-ICSI-ET group, linear or logistic regression models were created to account for continuous or dichotomous dependent variables, respectively, using maternal age (sequential data) and the number of injected oocytes per mare as independent variables. Sequential data is expressed as mean ± standard deviation. To aid clarity of presentation, in the result tables maternal age is grouped into four categories (1–7, 8 to 13, 14 to 19 and ≥20 years old). However, age was always entered into the regression models as years old, i.e. as continuous data. Statistical significance was set at $p < 0.05$. A p value between ≤0.1 and >0.05 was used to refer to an effect that approached significance (tendency).

3. Results

The mares in the OPU-ICSI-ET group were older (median age of 12 years old; $P = 0.02$) than the mares in the other groups (median ages of 10, 11 and 9 for the NM, AI and AI-EF-ET groups, respectively), this was partly explained by a larger percentage of mares of 20 years or more in this group (Table 1). The age distribution of mares in the different breeding technique groups is shown in Fig. 1. The number of mares, percentage of foaling mares, number of different stallions, type of semen used and incidence of multiple ovulation (MO) for the four groups is shown in Table 2.

The type of semen used to inseminate mares (frozen *vs*. cooled) had an impact on the odds of achieving at least one Day 45 pregnancy for the AI group (39.2 vs. 49.6%, respectively; $P = 0.03$; Odds: 0.612) but not for the AI-EF-ET group (42.5 vs. 48.6%, respectively; $P > 0.1$). The number of inseminations per estrus using frozen semen: single (post-ovulation) versus twice (pre- and post-ovulation) had no significant effect on the percentage of mares with at least one Day 45 pregnancy for the AI group (36.0 vs. 41.9%) or the AI-EF-ET group (42.6 vs. 42.5%), respectively. The number of injected oocytes (MII) did significantly ($P < 0.0001$; Odds: 0.836) affect the percentage of OPU-ICSI sessions that resulted in at least one Day 45 pregnancy.

Table 3 shows several reproductive efficiency indicators for the four breeding techniques investigated. The breeding technique with the highest ($P < 0.001$) reproductive efficiency was NM (63.3% chance of having at least one Day 45 pregnancy per attempt). By contrast, the OPU-ICSI-ET group had the lowest reproductive efficiency (37.4%; $P < 0.05$) which compared unfavorably to AI and AI-EF-ET (43.9 and 45.8%, respectively). However, the multiple blastocysts produced per attempt from some of the embryo and oocyte donors increased the overall outcome to 0.53 ± 0.6 and 0.50 ± 0.7 Day 45 pregnancies per donor mare for the AI-EF-ET and OPU-ICSI-ET groups, respectively (Table 3). Moreover, a surplus of IVP embryos ($n = 83$) was maintained in liquid nitrogen in the OPU-ICSI-ET group, and remained non-transferred during the study period.

The age of the mare was negatively associated ($P < 0.05$) with reproductive efficiency (percentage of mares with at least one Day 45 pregnancy) in all breeding techniques except OPU-ICSI-ET ($P > 0.1$; Table 4). The number of embryos per ovulation in mares from the AI-EF-ET group decreased with increasing maternal age ($P = 0.028$), whereas maternal age did not affect ($P > 0.1$) the number of blastocysts per injected oocyte in the OPU-ICSI group (Fig. 2). A more thorough analysis of the effect of age on fertility

Table 1
Mare age distribution across the breeding technique groups.

Breeding technique	Median age	Min-max age	Mare distribution per age group (years)			
			1 to 7 (%)	8 to 13 (%)	14 to 19 (%)	≥20 (%)
NM	10[a]	3–23	83 (28.7)	113 (39.1)	87 (30.1)	6 (2.1)
AI	11[a]	3–24	87 (26.5)	128 (39.1)	91 (27.7)	22 (6.7)
AI-EF-ET	9[b]	2–23	79 (38.5)	66 (32.2)	43 (21.0)	17 (8.3)
OPU-ICSI-ET	12[c]	1–29	131 (27.7)	136 (28.7)	132 (27.9)	74 (15.7)

NM: natural mating; AI: artificial insemination; EF: Embryo flushing; ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection. Different letters (a,b,c) indicate a significant different ($P < 0.05$) in median age.

parameters in oocyte donor mares (OPU-ICSI-ET) is shown in Table 5. Increasing maternal age was associated ($P < 0.05$) with a lower antral follicle count (number of aspirated follicles), lower number of recovered oocytes, reduced oocyte recovery efficiency but a higher maturation percentage. On the other hand, the post-ICSI cleavage percentage, the blastocysts per injected or recovered oocyte, and the Day 12 and 45 pregnancy per ET and the embryonic loss incidences were not influenced by maternal age ($P > 0.05$; Table 5). Although increasing maternal age tended to be associated with a decrease in the number of blastocysts per mare ($P = 0.08$), the percentage of mares with at least one blastocyst ($P = 0.07$), and the number of attempts that resulted in at least one Day 12 ($P = 0.1$) or Day 45 ($P = 0.07$) pregnant recipient per mare, when the regression models were adjusted for the number of oocytes injected per mare, the tendencies disappeared ($P > 0.1$; Table 5).

The influence of the stallion on the reproductive efficiency is difficult to assess as stallions were often used in only 1 or 2 different breeding techniques. However, a single straw breeding from two stallions was used in all three ARTs, so fertility data for these stallions could be compared descriptively (Table 6).

There was a year effect ($P = 0.03$) on the number of blastocysts per mares in the OPU-ICSI-ET group but not ($P > 0.1$) in the AI-EF-ET group (Table 7). The mean number of blastocysts per OPU-ICSI session increased with calendar year.

4. Discussion

Natural mating was the breeding system that resulted in the highest reproductive efficiency of the techniques compared in this study. Possible factors accounting for the higher reproductive efficiency in the NM group may include: 1) increased percentage of foaling mares within the population, since foaling mares are known to exhibit higher fertility than barren mares [11,28]; 2) use of a high

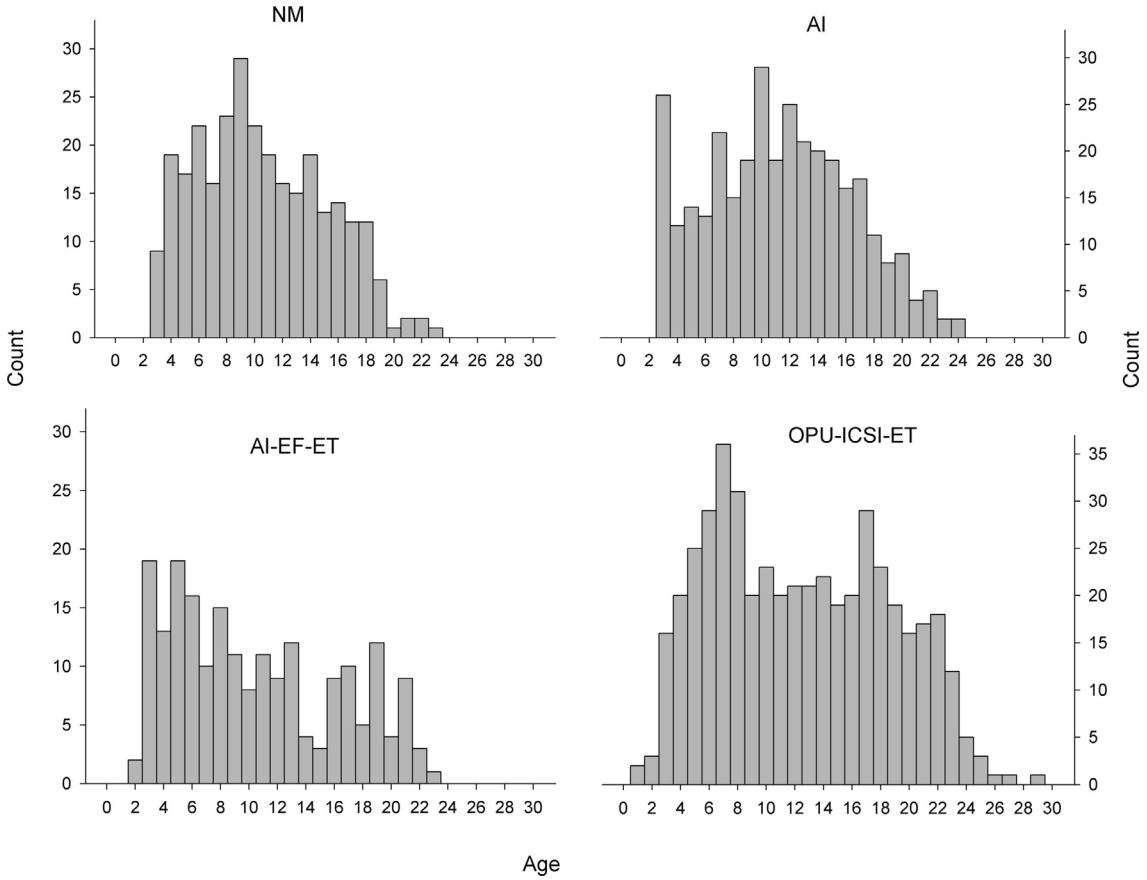

Fig. 1. Histograms of age (years old) distribution of mares from different breeding techniques: natural mating (NM), artificial insemination (AI), embryo flushing and embryo transfer (EF-ET), ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI).

Table 2
Characteristics of mares and semen used in the different breeding technique groups.

Breeding technique	Number of mares	Foaling mares (%)	MO rate (%)	Stallions (n)	Frozen semen (%)	Cooled semen (%)
NM	289	50.2	26.3	5	–	–
AI	328	24.4	20.1	192	55.2	44.8
AI-EF-ET	205	8.8	37.6	97	45.8	54.2
OPU-ICSI-ET	473	0.4	–	107	100.0	0.0

NM: natural mating; AI: artificial insemination; EF: Embryo flushing; ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection; MO: multiple ovulation.

Table 3
Effect of breeding technique on reproductive efficiency.

End point	NM	AI	AI-EF-ET	OPU-ICSI-ET
Donor mares with at least one blastocyst (%)	–	–	112/205 (54.6)	260/473 (55.0)
Total number of blastocysts (Mean per donor mare)	–	–	132 (0.64 ± 0.6^{a})	502 (1.1 ± 1.4^{b})
At least one Day 12–18 pregnancy per mare (%)	195/289 (67.5^{a})	153/328 (46.6^{b})	98/205 (47.8^{b})	191/473 (40.4^{b})
At least one Day 45 pregnancy per mare (%)	183/289 (63.3^{a})	144/328 (43.9^{b})	94/205 (45.8^{b})	176/473 (37.4^{c})
Post-ET Day 12 pregnancy (%)	–	–	114/132 (86.4^{a})	270/419 (64.4^{b})
Day 12 pregnant recipients (Mean per donor mare)	–	–	114 (0.56 ± 0.7)	270 (0.57 ± 0.8)
Day 45 pregnant recipients (Mean per donor mare)	–	–	109 (0.53 ± 0.6)	235 (0.50 ± 0.7)
Pregnancy loss by Day 45 (%)	12/195 (6.1^{a})	9/153 (5.9^{a})	5/114 (4.4^{a})	35/270 (13.0^{b})
Excess IVP embryos in N_2	–	–	–	83

NM: natural mating; AI: artificial insemination; EF: Embryo flushing; ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection; IVP: *in vitro* produced; N_2: liquid nitrogen. Within row, different letters (a,b,c) indicate a significant difference ($P < 0.05$) between breeding techniques.

Table 4
Effect of mare's age and breeding technique on reproductive efficiency.

Breeding technique	At least one Day 45 pregnancy per mare (%)					
	Age groups (years)				P value	Odds
	1 to 7	8 to 13	14 to 19	≥20		
NM	59/83	81/113	40/87	1/6	0.0001	1.15
	71.1	73.4	45.9	16.7		
AI	46/87	65/134	29/82	4/25	0.0005	1.09
	52.9	48.5	35.4	16.0		
AI-EF-ET	40/79	32/66	16/43	6/17	0.04	1.07
	50.6	48.5	37.2	35.3		
OPU-ICSI-ET	50/131	55/136	48/132	23/74	0.07	1.03
	38.2	40.4	36.4	31.1		

NM: natural mating; AI: artificial insemination; EF: Embryo flushing; ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection. The P value and odds ratios show the effect of maternal age (as sequential data) on the reproductive efficiency.

quantity (whole ejaculate) of non-processed sperm (natural mating), since fresh semen has been shown to remain viable in the mares' reproductive tract for longer than frozen [28] or cooled-transported semen [29]. Moreover, the total number of sperm in a processed semen dose (i.e. frozen or cooled semen) varies widely across studs, and is influenced by stallion popularity (cooled semen) and semen agent policy (number of frozen semen straws per dose). In the AI and AI-EF-ET groups, half of the mares were inseminated with 3 or fewer straws of frozen semen. A large field study in Argentina [30] using fresh semen from stallions standing at the same ET center as Polo Argentino donor mares reported 68.3% positive embryo flushes and 0.81 embryos per mare, compared to the 54.6% positive flushes and 0.64 embryos per mare in the current study (AI-EF-ET). This confirms the influence of using adequate quantities of non-processed semen to achieve maximal reproductive efficiency. In this respect, a retrospective study at the University clinic in Pisa (Italy) also reported a significantly higher embryo recovery per ovulation in mares inseminated with fresh semen (0.46) than in mares inseminated with either frozen (0.31) or cooled (0.35) semen [31].

The reproductive efficiency of mares in the AI and AI-EF-ET groups was similar (43.9 and 45.8% of mares yielded at least one Day 45 pregnancy), which highlights the high success of embryo flushing and transfer techniques when using good quality, synchronized recipient mares. In short, the wastage of embryos (either during the uterine flushing or transcervical transfer procedures) must have been minimal for the reproductive efficiency to equal that of mares left to stay pregnant. On the other hand, it is worth noting that the population of mares in the AI-EF-ET group was slightly biased towards a younger age and higher multiple ovulation rate than the AI group. which could have contributed positively to reproductive efficiency [12,30]. Some clients also decided whether to submit a mare for embryo flushing or to allow her to stay pregnant based on the number of preovulatory follicles/ovulations at the time of AI. Furthermore, in some cases the choice of whether to use frozen semen from a specific stallion (generally more expensive and involving single straw AI) as opposed to cooled semen of a less costly stallion depended on the characteristics of the embryo donor mare (intrinsic fertility and age) and optimal progression of the estrous cycle [32,33]. These additional criteria in some embryo donor mares might explain, at least in part, the lack of a difference in the reproductive efficiency between mares used for ET compared to the AI group.

The breeding technique that resulted in the lowest percentage of donor mares with at least one Day 45 pregnancy (37.4%) was OPU-ICSI-ET. Despite a similar percentage of mares with at least one blastocyst (per EF or OPU-ICSI session), the overall efficiency of OPU-ICSI-ET was slightly lower than AI-EF-ET as a result of a lower post-ET pregnancy and higher EEL incidence. However, it must be borne in mind that the population of oocyte donor mares was much older than the rest, with many having been barren for several years due to persistent uterine infections or simply because of advanced age. Furthermore, some of the stallions used for ICSI were already dead, with very limited availability of semen, and others had an *in vivo* fertility close to zero. In fact, 21 mares were submitted for ICSI with sperm from a stallion whose semen is considered infertile for *in vivo* AI (the semen owner only provides semen doses for ICSI, as the fertility after AI in the past years was not acceptable). OPU-ICSI in these mares yielded 1.21 embryos per mare, of which 45% resulted in at least one Day 45 pregnancy (data not shown), which is comparable to the average results with other stallions without

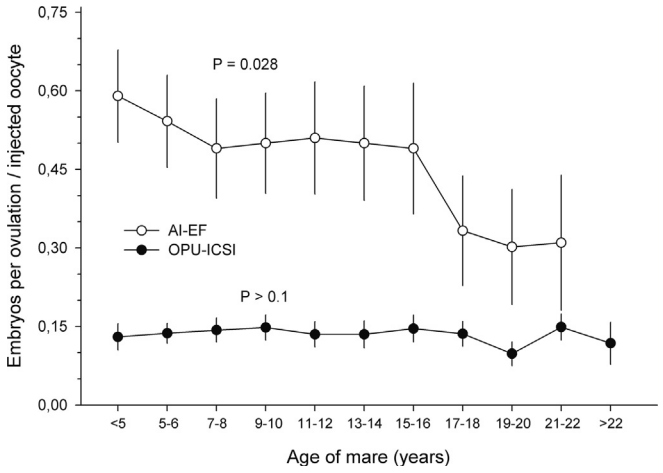

Fig. 2. Mean blastocysts per ovulation (AI-EF: mares submitted for artificial insemination and embryo flushing) or per injected oocytes (OPU-ICSI: mares submitted for ovum pick-up and intracytoplasmic sperm injection) ± standard deviation in mares of different ages. The effect of age was calculated as a continuous variable. For clarity, the means are depicted for age groups of 2 years.

in vivo fertility problems. This observation and the data shown in Table 6 confirm that the *in vivo* fertility of a given stallion does not necessarily correlate with the fertility observed after ICSI.

In addition, the OPU-ICSI procedure resulted in more embryos per mare than AI-EF (often 3 to 4 embryos and up to 10 embryos in one mare), such that the mean number of Day 45 pregnant recipients per mare was similar in the two groups. Furthermore, a number of IVP embryos were not transferred, as the owners did not want to have more pregnancies, or there were insufficient recipients available. Had these embryos been transferred during the studied period, the mean number of Day 45 pregnancies per mare would have been even higher for the OPU-ICSI group. It should be noted that only ICSI-produced embryos (and not *in vivo* recovered embryos) were cryopreserved in this study. The cryopreservation of *in vivo* recovered embryos has been classically associated with a reduced post-thawed pregnancy rate [34]. The main factor responsible for the low post-thaw viability of cryopreserved embryos, however, appears to be the large blastocoel cavity of flushed embryos at the time cryopreservation. In fact, cryopreserved embryos larger than 300 μm have a lower chance of producing a viable pregnancy than smaller embryos [34]. In contrast, ICSI-produced blastocysts are cryopreserved at a smaller diameter (all ICSI embryos in this study were smaller than 160 μm at the time of cryopreservation). A recent report showed no difference in foaling rates between recipients receiving non-cryopreserved versus cryopreserved (by vitrification) ICSI-produced embryos [35]. Therefore, it seems that cryopreservation of ICSI-produced embryos has little or no detrimental effect on post-ET viability.

Overall, the OPU-ICSI procedure is a relatively new breeding technique with considerable potential; however, there is still room for improvement, as evidenced by the gradual but continuing increase in the number of embryos produced per procedure since the start of the OPU-ICSI clinical program. In contrast, the reproductive efficiency of AI-EF-ET has plateaued, in that the number of embryos per flushing remained constant over the studied period. On the other hand, the equipment and level of technical expertise of IVF staff required to achieve reasonable reproductive efficiency, is high.

Table 6
Interaction between stallion and breeding technique on reproductive efficiency.

Breeding technique	Variable	Stallion ID	
		A	B
AI (frozen semen)	Number of mares	31	13
	At least one D45 pregnancy (%)	32.2	53.8
AI-EF-ET	Number of mares	15	10
	Embryos per ovulation	0.37 ± 0.4	0.58 ± 0.5
	Embryos per mare	0.47 ± 0.5	1.0 ± 0.8
	At least one D45 pregnancy (%)	33.3	60.0
OPU-ICSI-ET	Number of mares	47	28
	Blastocysts per injected oocyte	0.15 ± 0.2	0.10 ± 0.1
	Embryos per mare	1.3 ± 1.6	0.8 ± 0.9
	At least one D45 pregnancy (%)	42.5	39.3
	Excess of IVP embryos in N_2	11	2

AI: artificial insemination; EF: Embryo flushing; ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection; IVP: *in vitro* produced; N2: liquid nitrogen.

Table 5
Effect of age on different reproductive efficiency indicators in mares submitted for OPU-ICSI

OPU-ICSI parameter	Age of mare (years)				P value	Adjusted P value
	1 to 7	8 to 13	14 to 19	≥20		
n	131	136	132	74	–	–
Antral follicle count	23.9 ± 8.6	26.8 ± 12.1	25.1 ± 9.7	20.9 ± 7.9	0.04	–
Recovered oocytes	13.7 ± 6.6	14.7 ± 6.2	13.5 ± 7.1	9.7 ± 4.7	0.002	–
Oocyte recovery (%)	56.5 ± 14.7	56.4 ± 15.6	54.1 ± 16.7	48.1 ± 16.1	0.001	–
Maturation (%)	57.1 ± 18.1	54.3 ± 16.3	57.3 ± 17.5	66.7 ± 20.1	0.001	–
Cleavage (%)	67.3 ± 21.3	72.4 ± 19.1	68.9 ± 20.2	67.3 ± 20.6	NS	–
Blastocysts per recovered oocyte	0.08 ± 0.1	0.08 ± 0.1	0.08 ± 0.1	0.09 ± 0.1	NS	–
Blastocysts per injected oocyte	0.135 ± 0.2	0.141 ± 0.1	0.127 ± 0.2	0.137 ± 0.2	NS	–
Blastocysts per mare	1.0 ± 1.3	1.3 ± 1.6	1.0 ± 1.3	0.8 ± 1.1	0.08	0.29
No ICSI (%)	1.1	1.7	0.0	1.1	NS	–
Mares with no embryo (%)	44.3	39.7	48.5	50.0	0.1	0.32
Mares with 1 embryo (%)	26.7	30.8	24.3	28.4	NS	–
Mares with 2 embryos (%)	16.8	14.0	13.6	12.2	NS	–
Mares with ≥3 embryos (%)	12.2	15.5	13.6	9.4	NS	–
At least one D12 pregnancy (%)	41.0	43.0	39.0	36.0	0.1	0.47
At least one D45 pregnancy (%)	38.2	40.4	36.4	31.1	0.07	0.34
D12 pregnancy per ET (%)	64.5 ± 43	60.2 ± 43	68.6 ± 42.5	66.4 ± 44	NS	–
D45 pregnancy per ET (%)	57.7 ± 44	52.0 ± 42	62.0 ± 44	57.0 ± 47	NS	–
EEL (%)	9.3 ± 25	12.1 ± 26	8.6 ± 27	11.1 ± 32	NS	–
Mean D45 pregnant recipients	0.50 ± 0.7	0.52 ± 0.7	0.47 ± 0.6	0.49 ± 0.6	NS	–
Excess IVP embryos in N_2	15	35	29	4	–	–

ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection; EEL: early embryonic loss by Day 45 of pregnancy; N_2: liquid nitrogen; NS: not statistically significant. The adjusted P value took into account the number of injected oocytes per mare in the regression model.

Table 7
Effect of year on the number of blastocysts per donor mare.

Breeding technique	Blastocysts per mare				P value
	2015	2016	2017	2018	
AI-EF-ET (n)	–	0.67 ± 0.6 (n = 63)	0.61 ± 0.5 (n = 71)	0.66 ± 0.6 (n = 71)	NS
OPU-ICSI-ET (n)	0.96 ± 1.2 (n = 122)	0.93 ± 1.1 (164)	1.11 ± 1.6 (n = 93)	1.38 ± 1.8 (n = 94)	0.03

AI: artificial insemination; EF: Embryo flushing; ET: Embryo transfer; OPU: ovum pick-up; ICSI: intracytoplasmic sperm injection; NS: not statistically significant. The number of mares is shown in brackets (n).

Conversely, not every OPU clinic requires a specialized IVF laboratory on site, since overnight shipment of oocytes from remote locations has been shown to yield similar results to non-shipped oocytes [36]. The downside of shipping, however, is the possibility of the shipment being delayed or lost in transit.

Finally, it is important to highlight that OPU is an invasive procedure, that is not free of risk for the mare's health. Some of the side effects observed in mares treated in our program include minor rectal bleeds/abrasions, vaginal and abdominal hemorrhage, ventral edema, fever, dullness, pain and/or anorexia lasting from 12 h to several days, others have reported rectal tears, peritonitis, and ovarian abscesses [37,38]. In short, clients of sport mares should be warned that the horse may not be fit for training or competition for several days, or in occasional cases even weeks, after undergoing the OPU procedure.

The early embryo loss in the NM, AI and AI-EF-ET groups between Days 12–18 and 45 (<7%) is similar to the figures reported recently for others studies with larger data sets [13,39]. The breeding technique that resulted in the highest EEL was OPU-ICSI-ET. This is in agreement with other studies in horses [40,41] and cattle, suggesting that IVP embryos are of lower quality than *in vivo* derived embryos/pregnancies [42].

Increasing maternal age had the strongest negative association with reproductive efficiency in mares bred to stay pregnant (NM and AI groups). This association is well documented and is thought to be due primarily to a reduction in oocyte quality, and possibly a suboptimal oviductal environment [14–16] rather than reduced uterine quality [17,18] in aged mares. However, uterine causes presumably also contribute to the reduction in fertility in aged mares, since the decrease in reproductive efficiency in embryo donor mares (AI-EF-ET) was less pronounced than that in the NM and AI groups.

Despite the strong evidence of reduced quality of oocytes from aged mares after *in vivo* fertilization [14–16,18], the findings of the current study show no association between maternal age and oocyte developmental competence (ability of oocytes to mature, be fertilized, cleave and develop into a transferable blastocyst) or embryo quality (post-ET pregnancy and EEL) in the OPU-ICSI-ET group. These results are in agreement with a previous study in which ICSI was performed following aspiration of pre-ovulatory follicles [19]. The only obvious effect of increasing maternal age on reproductive efficiency of mares in the OPU-ICSI-ET group was lower number of antral follicles. This phenomenon has previously been documented in the horse [20] and women [43]. The reduction in antral follicle count in aged mares results in fewer oocytes being recovered per OPU, and therefore fewer oocytes injected and fewer embryos per OPU-ICSI session. Nevertheless, the quality of the resulting embryos was similar to that of embryos from younger mares, just as reported for women [43]. Another study investigating effects on human embryo quality [44] found an association between maternal age and the percentage of zygotes with gross aneuploidy (≥3 pronuclei). Even though this association was significant, the percentage of zygotes with ≥3 pronuclei only increased from 5.1 to 4.4% in women aged 18–24 and 25–29 years old to 4.9 and 6.8% in women aged 35–39 and 40–46, respectively. By contrast, the maturation, fertilization (zygotes with 2 pronuclei) and cleavage rates were unaffected by maternal age [44].

A limitation of the current study is that the follow up of pregnancies only continued up to Day 45 of pregnancy. Therefore, it cannot be ruled out that embryos with aneuploidies from older mares died later in pregnancy, during the fetal stage. On the other hand, previous studies have shown that reduced fertility ascribed to oocyte factors in older mares resulted primarily in a high pregnancy loss (>60%) between Days 2 and 14 of pregnancy [15,16]. Furthermore, a recent study involving a large number of pregnancies [13] showed that the proportion of pregnancy losses that occurred during the fetal stage (between Day 43 and foaling) as compared to the whole gestation (Day 15 to foaling) was larger in young mares (69 and 57% for mares aged 2–4 and 5–10, respectively) than in older mares (50 and 52% for mares aged 11–19 and > 19, respectively). Overall, it appears unlikely that the lack of follow up of pregnancies to term masked a major effect of maternal age on the reproductive efficiency of OPU-ICSI.

In conclusion, AI and AI-EF-ET are slightly more efficient than OPU-ICSI-ET in producing at least one viable pregnancy per donor mare, under the conditions reported in the current study. However, the frequent production of multiple embryos per ICSI session, makes the OPU-ICSI-ET as effective as the AI-EF-ET when measured in terms of the mean number of Day 45 pregnant recipients per mare. Moreover, increasing maternal age was associated with a reduction in the reproductive efficiency of all breeding techniques except OPU-ICSI-ET. Therefore, it is reasonable to advise horse owners that the breeding technique of choice for advanced age mares is OPU-ICSI. Furthermore, continuing improvements in the ability of IVF laboratories to produce not only a higher percentage of embryos per injected oocyte but also embryos with improved viability, along with the possibility to obtain and cryopreserve surplus IVP embryos all year round, and the opportunity to use very limited amounts of semen, even from stallions that are not fertile using more conventional breeding techniques, will likely make OPU-ICSI-ET a more efficient and desirable ART in the near future.

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