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Original Research

Laboratory Production of Equine Embryos

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ABSTRACT

Assisted reproduction technologies (ART) are well developed in humans and cattle and are gaining momentum also in the equine industry because of the fact that the mare does not respond to superovulation but can donate large numbers of oocytes through ovum pick up (OPU). After collection, the oocytes can be fertilized by intracytoplasmic sperm injection (ICSI) using a variety of stallion semen samples, even of poor quality, and the resulting embryos can establish high pregnancy rates after cryopreservation and transfer. The discoveries that equine oocytes can be held at room temperature without loss of viability and that an increase in vitro maturation time can double the number of embryos produced are fueling the uptake of the OPU technique by several clinics that are shipping oocytes of their client's mares to specialized ICSI laboratories for embryo production and freezing. In this article, we present a retrospective analysis of 10 years of work at Avantea with a special focus on the last 3 years. Based on our data, an average production of 1.7 to 2 embryos per OPU-ICSI procedure can be obtained from warmblood donor mares with a pregnancy rate of 70% and a foaling rate in excess of 50%. OPU-ICSI offers the added value of freezing embryos that allows the development of embryo commercialization worldwide to the benefit of top horse breeders who are endorsing this technology as never before.

1. Introduction

Assisted reproduction technologies (ART) have now become a well established branch of reproductive medicine recognized with the award of the Nobel Prize for Medicine to Robert Edwards (https://www.nobelprize.org/prizes/medicine/2010/press-release/), one of the founding fathers and responsible for the birth of Louise Brown, the first human being conceived in a test tube [1]. Livestock species have for long been the model, together with the mouse, on which ART were developed and applied both in the human and animal fields. Now the human field has become the model for the animals, and techniques like intracytoplasmic sperm injection (ICSI) [2] or preimplantation genetic diagnosis (PGD) [3], first developed for infertile couples, are also applied to livestock including horses. Nevertheless in livestock as opposed to humans, for practical and sometimes physiological reasons, the oocytes are collected from the ovaries at the germinal vesicle stage and therefore require completion of maturation in vitro [4,5]. This adds an additional biological step that has to be accomplished in livestock as compared with humans.

In the horse, the use of ART has been severely limited by the difficulty to obtain successful in vitro fertilization, only reported sporadically with in vivo matured oocytes [6] despite the fact that in vitro maturation of oocytes was successful in generating viable embryos after transfer of in vitro matured oocytes into inseminated recipient mares [7]. Therefore, the main obstacles to the development of ART in the horse were considered the problem of zona hardening during maturation [8,9], the failure to have sperm capacitated that can consistently penetrate in vitro matured oocytes [10] and embryo culture to the blastocyst stage [11,12].

ICSI first reported in equine by Squires [13] and then made viable and reproducible by the use of the piezo-drill for sperm injection [14] has been the major breakthrough. ICSI superseded the

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unsuccessful conventional in vitro fertilization, and at the same time, it allows the use of semen of poor quality or available in very small amounts for embryo production [15].

Embryo culture to the blastocyst stage is an important requirement for the application of ART in clinical practice because it allows to freeze the embryos, therefore limiting the need for monitoring a large herd of recipients for fresh embryo transfer (ET), and also to perform nonsurgical transfer of early cleavage ICSI embryos to the uterus of recipient mares avoiding invasive procedures and respecting animal welfare. Over the years, several media have been used with variable degrees of success, indicating that embryos are quite tolerant to stress and can adapt to different in vitro conditions [12,16].

In vitro produced embryos in cattle have now doubled the number produced in vivo by superovulation and embryo flushing [17], confirming the interest and the value of ART to the cattle industry despite the fact that, contrary to the horse [18], superovulation is effective and it has been used in practice for several decades [19]. A similar trend is also reported in Europe for the equine and, in accordance with the same report of the International Embryo Transfer Society (IETS), in vitro produced ICSI embryos have overtaken in numbers those produced by embryo flushing [17].

In the early days of the application of equine ART in a clinical context, oviduct transfer of in vivo matured oocyte ("oocyte transfer") into inseminated recipient mares, or transfer of zygotes shortly after fertilization by ICSI, was used effectively by Carnevale [20]. Further developments of ICSI, embryo culture and cryopreservation have now shifted the interest in collecting immature oocytes by ovum pick up (OPU) from all antral follicles present on the ovaries, as it is carried out in cattle [21,22], resulting in a higher number of embryos produced per OPU session than oocyte transfer [23,24]. Another major breakthrough in the clinical application of OPU-ICSI has been the finding that equine oocytes can be stored at 22°C for 24 hours without any loss of viability [23,25]. Holding the oocytes for 24 hours allows the collection of the oocytes at the veterinary clinics and their shipment to a specialized laboratory with the necessary personnel, equipment and know-how for ICSI and embryo production including sexing and cryopreservation [23,26]. This technology, defined as OPU-ICSI, has now acquired a level of efficiency (in terms of number of embryos produced), flexibility (embryos can be produced at any time of the year also using poor quality/quantity semen of virtually any stallion) and consistency of pregnancy rates [27] that it is rapidly superseding (at least in Europe) conventional embryo flushing. More than ever before, this technology is rapidly taken up by the equine industry [28,29] as it opens new opportunities both for genetic improvement of equine populations and for the commercial potential offered by a reliable frozen embryo that can be marketed worldwide.

In this article, we present a retrospective analysis of in vitro embryo production of equine embryos after OPU-ICSI over several years in a clinical context and discuss our data in relation to the various biological steps involved and the practical implication to obtain successful pregnancies.

2. Materials and Methods

2.1. In Vitro Production of Equine Blastocysts

2.1.1. Oocyte Collection

In our OPU-ICSI program, the donor mares are ultrasound scanned transrectally 1 to 3 days before the OPU day to evaluate the number and size of the follicles present on the ovaries, considering the presence of at least eight to ten follicles of $\geq$ 1 cm of diameter, the threshold at which deciding to perform the OPU. However, in some old mares, sometimes this number is not available, and after a few weeks of monitoring, OPU will be performed in any case, even with only one suitable follicle present. This preliminary examination allows to select the donor mares in best ovarian conditions, and it is the routine protocol at Avantea. When mares are not residents in the clinic, we ask the client's veterinarian to do the scanning and report the number of follicles present. Usually, the interval between OPU on the same mare can range from 3 to 5 weeks and in some cases also 6 to 8 weeks for some donors. OPU is performed all the year around, regardless of the stage of the cycle or the season. If possible, we try to avoid OPU when large preovulatory follicles are present. Immature oocytes are collected by ultrasound-guided transvaginal aspiration of ovarian follicles $\geq$ 0.8 to 1 cm in diameter. Mares are prepared for OPU by sedation with detomidine hydrochloride (4 mg, i.e., 0.4 mL, three times or more during the procedure as required to have a good sedation) together with an epidural anesthesia (4–8 mL of 2% lidocaine). Antral follicles are punctured using a 12G double lumen needle mounted with a needle guide on a modified native endovaginal probe and repeatedly flushed eight to ten times with media (Euroflush, IMV, France) supplemented with 5 IU/mL of heparin (Heparin-Natrium 5000 UI/mL, B.Braun Melsungen AG, Germany) to prevent clotting of follicular fluid and blood [21]. The recovered flushing fluid is filtered with an embryo filter (EZ Way Filter, SPI, USA), and the residual fluid into the filter is searched to collect the oocytes. After two washes in Hepes synthetic oviductal fluid (H-SOF), each batch of oocytes is photographed before being transferred in maturation media with or without a preliminary period of holding at room temperature to synchronize maturation time with other batches of oocytes as required by the planning of laboratory activities.

2.1.2. In Vitro Maturation (IVM) of Oocytes, ICSI, and In Vitro Culture (IVC) of Embryos

Where not differently indicated, all reagents were purchased from Merck, Sigma-Aldrich division.

Immature oocytes recovered at Avantea are transferred directly to maturation media for 26 to 28 hours or kept in holding media at 22 to 24°C for 2 to 12 hours and then transferred to maturation media for 36 to 38 hours at 38°C with 5% CO_2 in air.

The immature oocytes recovered at remote clinics are shipped in holding medium H-SOF and are packaged in insulated boxes (www.sonoco.com) that maintain a temperature of 18 to 22°C during an average holding/transport time of 18 to 24 hours. At arrival, shipped oocytes are transferred immediately in maturation media for 26 to 30 hours. The IVM medium is a mixture (1:1) of Ham's F12 and Dulbecco's Modified Eagle's Medium with gonadotropins (Meropur, Ferring, 0.1 IU/mL of follicle-stimulating hormone and 0.1 IU/mL luteinizing hormone), epidermal growth factor (50 ng/mL) (PeproTech), 1 mM sodium pyruvate, 10% fetal calf serum (Gibco, Thermo Fisher), and insulin-transferrin-selenium supplement. After maturation, the oocytes are transferred to H-SOF supplemented with hyaluronidase (2.5 μg/mL) for 4 to 8 minutes and then mouth pipetted to remove cumulus cells. Denuded oocytes are returned to the maturation medium until the moment of ICSI. A small fraction of straw of frozen stallion semen is thawed in water at room temperature and prepared by density gradient centrifugation (45%–90% Redigrad, Amersham) for 20 minutes at 700g, or alternatively by swim-up procedure, followed by washing in Ca-free TALP for 5 minutes at 400g. Only very few stallions are prepared by swim-up because they do not survive density gradient centrifugation. After centrifugation, a modified SOF-IVF medium containing PHE (penicillamine, hypotaurine, and epinephrine) is used to resuspend the semen pellet [30]. Only metaphase II oocytes undergo ICSI that is performed on a Nikon inverted microscope equipped with a heated

plate (37.5°C), a Narishige micromanipulator and an Eppendorf PiezoXpert. Just before ICSI, the semen suspension is diluted 1:1 with 10% polyvinylpyrrolidone (PVP) (PVP 360) in H-SOF and dispensed in drops of 3 μL covered with mineral oil (M8410). Holding pipettes have inner diameter (ID) of 50 μm and outer diameter (OD) of 130 μM; injection pipettes have ID of 5 μM (Biomedical Instruments, Germany). Settings of the PiezoXpert are as follows: intensity 10 to 30 (depending on zona thickness and hardness), speed 10, and pulse infinite. Fluorinert (FC-770) is used for stabilizing the injection pipette. Piezo pulses are applied to immobilize selected morphologically normal and motile spermatozoa, and piezo-driven ICSI is performed cutting the zona, breaking the cell membrane with a few pulses, and releasing the sperm in the ooplasm. In vitro culture of injected metaphase II oocytes is carried out in a modified SOF-IVC medium supplemented with bovine serum albumin (BSA) and amino acids [30]. Two days after ICSI, cleavage is assessed and noncleaved embryos are removed. Approximately half of modified SOF-IVC medium is replaced on day 6 with the same medium supplemented with 10% of a mixture (1:1) of fetal calf serum and serum replacement (KnockOut Serum Replacement, Life Technologies). Blastocysts formation is assessed twice daily from day 6 to 9 (day 0 = day of ICSI), and embryos showing good morphology and a clear layer of aligned trophoblastic cells are selected for freezing (see 3.5 and Fig. 5).

2.1.3. Preimplantation Embryo Sexing

The biopsy procedure consists in the collection by gentle pipetting of a few cells that spontaneously herniate from the hole in the zona caused by the sperm injection pipette. Biopsies are collected from day 7 to day 9 post ICSI as soon as a minimal extrusion of trophoblast cells could be detected by observing the embryos at the stereomicroscope. After biopsy the embryos are put back into the culture or cryopreserved. Each biopsy (around 5 to 10 cells) is placed in a 0.5 mL microcentrifuge tube and frozen at −20°C. After thawing the sample, the content of each tube is treated with 20 mg/mL of proteinase K (Ambion, ThermoFisher Scientific, Milan, Italy) and incubated at 38°C for 30 minutes followed by 99°C for 10 minutes. The polymerase chain reaction (PCR) is performed targeting simultaneously the equine sex-determining region of the Y chromosome (eSRY) gene [31] and the constitutive glycoprotein alpha-galactosyltransferase 1 (GGTA1) gene. Duplex PCR is performed using LA Taq DNA Polymerase in accordance with the manufacturer's instructions (Takara Europe-Clontech, Saint-Germain-en-Laye, France) and the following conditions: 94° for 2 minutes followed by 40 cycles (94° for 30 seconds, 60° for 30 seconds, and 72° for 30 seconds) and final extension at 72° for 7 minutes. Lysated fibroblasts from male or female horses are used as positive controls. The products of PCR amplification are electrophoresed on agarose gels and analyzed and photographed using a UV transilluminator. The eSRY-specific primer pair amplifies a 429 bp product and the GGTA1 a 368 bp product.

2.1.4. Cryopreservation and Thawing of Equine In Vitro Produced (IVP) Blastocysts

IVP blastocysts are processed in accordance with the IETS manual, washed in H-SOF, and transferred into H-SOF containing 5% glycerol for 5 minutes followed by a step in 10% glycerol for 20 minutes at room temperature and loaded into 0.25 mL straws. The straws with the IVP blastocyst are placed into a precooled (−6.5°C) methanol bath programmable freezing machine (Bio-Cool IV, FTS Systems) with the following freezing curve: −6.5°C for 5 minutes, manual seeding, hold for 5 minutes, and then cooling (−0.5°C/minute) to −32°C before plunging the straws directly into liquid nitrogen (−196°C).

Thawing of the cryopreserved IVP blastocysts is carried out as follows: the straw is taken out of the liquid nitrogen and held at room temperature for 5 to 8 seconds before plunging into water at room temperature (22°C−24°C) until the thawing of the ice. The outside of the straw is dried with a tissue paper and the content is expelled into an empty dish. The IVP blastocyst is placed in H-SOF containing decreasing amounts of glycerol (8%, 6%, 4%, and 2%), each step for 5 minutes, and subsequently held in H-SOF until loading in a 0.25 mL straw that is inserted into a Cassou gun for transfer.

2.2. Embryo Transfer

Recipient mares are monitored by ultrasound examination to determine the day of ovulation. Occasionally, ovulation is induced/anticipated by the administration of human chorionic gonadotropin to reduce the number of scans or to avoid work on Sundays. Four to five days after ovulation (D 0: day of ovulation), the recipient is scanned to evaluate the presence of a normal corpus luteum. ET is performed after a mild sedation with detomidine using a speculum and Wilsher's forceps. Pregnancy diagnosis is performed 12 to 13 days after transfer (D+17) and then at D+30 and D+50.

3. Results

3.1. Complete Clinical Dataset From 2010 to 2019

In Fig. 1 we have provided an overview, covering a 10-year period, of the development and application of the OPU-ICSI technique in equine-assisted reproduction, in accordance with the data that we reported annually in the statistics of the European embryo technology association. During this period, the number of OPU-ICSI procedures increased at Avantea but also more OPU procedures were performed in external clinics and more clinics started to offer equine OPU services. Overall, 4,264 OPU procedures were performed at Avantea, and 2,466 were performed in external clinics that, after OPU, shipped the oocytes to Avantea for ICSI and embryo production, therefore reaching a total number of 6,730 ICSI procedures. Over the 10-year period, the number of donor mares treated for OPU at Avantea or at other clinics, and then referred to Avantea for ICSI, has been 2,459 and the number of stallions used for ICSI has been 619. In Fig. 1 are shown also the data of embryo production from all the ICSI procedures indicating the number of freezable/transferrable embryos (n.8,408) and the total number of embryos developed in culture up to day 9 after ICSI (n.11,800). The difference between the total number of embryos developed in culture and the number of freezable/transferable embryos represents those embryos (n.3,392) that were evaluated of lower quality and/or developmentally retarded and therefore were discarded/not frozen/not transferred as described at 3.5. The number of ICSI sessions, that corresponds to the sum of the OPU performed at Avantea and the OPU performed in the external clinics, was fairly stable in 2016 and 2017 (965 and 934) but increased sharply in 2018 (1,472) and again in 2019, reaching over 2,045 ICSI sessions, especially due to the OPU performed at the external clinics. The black column indicating the number of freezable embryos was almost equal to the ICSI column in 2017 indicating an average of about one embryo per ICSI. Later, in 2018, the black column exceeded the ICSI column therefore passing the threshold of one embryo per OPU and reaching two embryos per OPU in 2019.

In the following paragraphs, observations and comments on relevant aspects of the laboratory production of equine embryo are presented, with a focus on the period from 2017 to 2019.

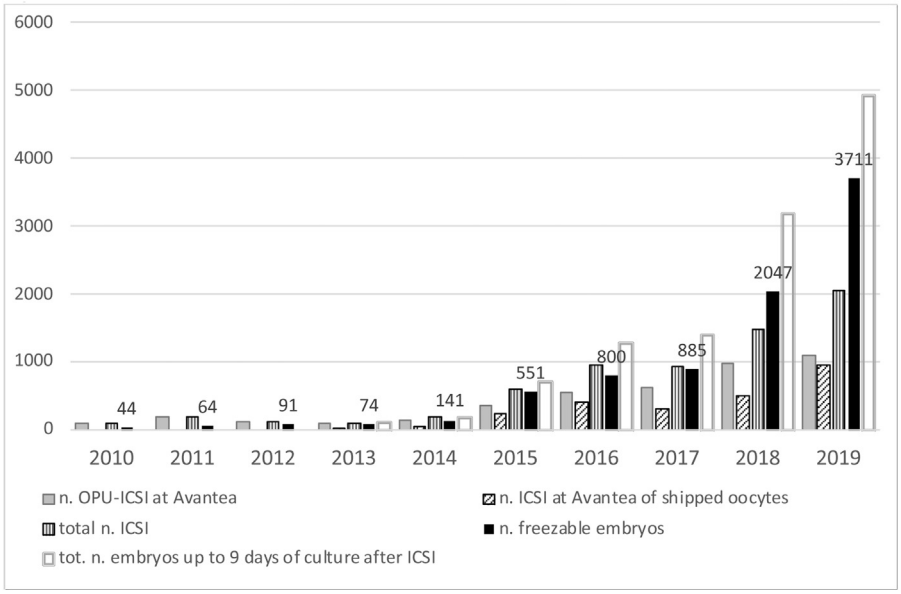

Fig. 1. Overview of the number of OPU procedures performed at Avantea and at external clinics, the number of ICSI procedures, including ICSI of shipped oocytes, and the number of embryos obtained over the last 10 years showing a progressive increase of the application of equine OPU-ICSI, especially from 2017 to 2019.

3.2. In Vitro Maturation Timing

Fig. 1 shows an increasing trend in the number of the ICSI procedures and embryos produced with the sharper increase from 2017 onwards. An important contribution to this improvement was the elongation of oocyte maturation time from 26–28 hours to 34–38 hours, as reported previously [32], that significantly benefited the overall efficiency of the procedures starting from 2018. Longer maturation time (26–30 hours of IVM) was also implemented for overnight shipped oocytes from external clinics. In Table 1 a comparison between clinical data sets of donors collected at Avantea recorded in 2017 (OPU followed by oocyte maturation for 26–28 hours) and another set recorded in 2018 (OPU followed by holding at 22°C–24°C for 2–12 hours and oocyte maturation for 36–38 hours) is shown. In both sets, the data are presented for two donor groups because we have already reported the observation of a breed effect comparing Warmblood donors with Arabian donors [15]. The data in Table 1 indicate a clear beneficial effect of the longer maturation time especially evident for the Arabian group of donors. The cleavage rate was improved significantly in the Arabian group (44.6% vs. 57.3%, $P < .05$, chi square test), whereas the number of freezable embryos and the percentage of total embryo development on day 9 increased in both groups (10.2% vs. 31.7 and 25.0% vs. 37.3% in Arabians and Warmbloods respectively, $P < .05$, chi square test). Therefore in 2018, we started to implement the longer maturation time more systematically also for shipped oocytes, and we registered an increase of the number of embryos developed per OPU-ICSI session from 0.9 freezable embryo in 2017 (885/934) to 1.8 (3711/2045) in 2019.

3.3. Breed Effect

In the 3 years, 2017 to 2019, Warmblood and Arabian donors represented most of the mares processed for OPU-ICSI at Avantea, being respectively 74% and 21% of the total. Therefore, the data are shown separately in Table 2 to highlight the differences between the two donor groups. A third group of the shipped oocytes was included in Table 2 representing mostly Warmblood donor mares. We observed that the number of oocytes collected per OPU was very similar between groups and across the 3 years. By contrast, the average cleavage rate was significantly higher for both Warmblood groups than that for Arabians (71.7%–73.6% vs. 52.9%) and also the embryo development rate followed the same trend (Warmblood (WB): 17.7%–19.2% vs. Arabian (AR): 9.8%). As expected, because of the elongation in maturation time, we observed an increasing rate of embryo development, within all donor groups: the average number of freezable embryos per OPU-ICSI increased from 0.9–1.2 to 1.7–2.1 for Warmblood donors and from 0.5 to 1.0 for Arabian donors, during the 3 years examined. Focusing on the two Warmblood groups, we confirmed, in this large dataset, that the maturation rate of shipped oocytes is lower than that of oocytes that are transferred to maturation soon after OPU (60.6% vs. 69.7%). This difference is due to a higher number of degenerated oocytes found in the shipped oocytes after the completion of maturation, as previously shown [32]. As a consequence, the rate of embryo development calculated on the total number of oocytes is lower for shipped oocytes versus freshly matured oocytes (17.7% vs. 19.2%) although it appears higher if calculated on the base of cleaved embryos after ICSI (40.9% vs. 37.5%).

Table 1
Effect of in vitro maturation time on maturation rate, cleavage, and embryo development in two donor groups/breeds.

Donor Group	IVM Time	n. OPU	n. Oocytes	n. M II Injected	% MII	n. Cleaved	Cleavage Rate %	n. Freezable Embryos	per OPU	Tot n. of Embryos	%/Oocytes	%/Cleaved
Arabian	IVM 26–28 hours	62	725	440	60,69 a	196	44,55 a	13	0.21	20	2,76 a	10,20 a
Arabian	Hold + IVM 36–38 hours	98	1,070	764	71,40 b	438	57,33 b	80	0.82	139	12,99 b	31,74 b
Warmblood	IVM 26–28 hours	161	2,059	1,339	65,03 A	981	73,26 A	195	1.21	245	11,90 A	24,97 A
Warmblood	Hold + IVM 36–38 hours	342	4,066	2,906	71,47 B	2057	70,78 A	460	1.35	768	18,89 B	37,34 B

Chi square $P < .05$. Numbers with different letters within columns and within donor groups are statistically different.

Table 2
OPU-ICSI at Avantea (warmblood (WB) and Arabian (AR) donors) and ICSI of oocytes shipped to Avantea from external clinics, period 2017 to 2019.

Donor Group	Year	n. OPU	n. Oocytes	n. M II Injected	% MII	n. Cleaved	Cleavage Rate %	n. Freezable Embryos	*per OPU*	Tot n. of Embryos	%/oocytes	%/cleaved
Avantea-WB	2017	382	4,689	3,185	67,92 a	2,189	68,73 a	455	1.19	758	16,17 a	34,63 a
Avantea-WB	2018	719	8,973	6,323	70,47 b	4,491	71,03 b	1,093	1.52	1,728	19,26 b	38,48 b
Avantea-WB	2019	879	12,155	8,462	69,62 b	6,552	77,43 c	1,831	2.08	2,476	20,37 c	37,79 b
Total		1,980	25,817	17,970	69,61 *	13,232	73,63 *	3,379		4,962	19,22 *	37,5 *
per OPU			13.04	9.08		6.68		1.71		2.51		
Avantea-AR	2017	185	2,264	1,490	65,81 A	654	43,89 A	85	0.46	146	6,45 A	22,32 A
Avantea-AR	2018	196	2,212	1,530	69,17 B	869	56,80 B	145	0.74	280	12,66 B	32,22 B
Avantea-AR	2019	174	2,518	1,676	66,56 AB	960	57,28 B	181	1.04	263	10,44 C	27,40 C
Total		555	6994	4696	67,14*	2483	52,87§	411		689	9,85§	27,75§
per OPU			12.60	8.46		4.47		0.74		1.24		
Shipped oocytes (@)	2017	322	3546	2113	59,59aa	1429	67,63aa	305	0.95	443	12,49aa	31,00aa
Shipped oocytes (@)	2018	502	5937	3551	59,81aa	2383	67,11aa	733	1.46	1054	17,75bb	44,23bb
Shipped oocytes (@)	2019	953	10738	6564	61,13aa	4962	75,59bb	1625	1.71	2088	19,44cc	42,08bb
Total		1777	20221	12228	60,47§	8774	71,75*	2663		3585	17,73#	40,86#
per OPU			11.38	6.88		4.94		1.50		2.02		

Chi square $P < .05$. Numbers with different letters within columns and within donor groups are statistically different. Different symbols (*,§,#) within columns indicate statistical difference in total MII rate, cleavage rate, % embryos/oocytes, and % embryos/cleaved among the three donor groups. (@) data from nine external clinics.

3.4. Multiple Embryo Outcome

Another aspect, referred to the same 3-year period and shown in Fig. 2, is the percentage of OPU-ICSI procedures that delivered one or more freezable embryos at the end of the culture period of maximum 9 days after ICSI. This dataset refers only to OPU-ICSI procedures performed at Avantea and is shown separately for Arabian and Warmblood donors because of the breed effect, as above. As expected from the data of Table 1, the percentage of successful procedures increased in both donor groups: from 28.6% to 56.9% for the Arabian group and from 60.2% to 75.6% for the Warmblood group. Within the Warmblood group only, we have analyzed in Fig. 3 the distribution of the successful procedures in accordance with the number of embryos produced per session, from one to over five embryos per OPU/ICSI procedure. We observed a progressive decrease of the procedures delivering one embryo (from 27% in 2017 to 23% in 2019) paralleled by increase of the procedures delivering multiple embryos. In particular, the data show that in 2019, over 30% of successful OPU-ICSI delivered three or more freezable embryos versus 15% in 2017.

3.5. Kinetics of Embryo Development

During the embryo culture period, we monitor the kinetics of embryo development by recording the number of embryos selected for freezing on day 6, 7, 8, and 9 of culture after ICSI. Again, this dataset refers only to OPU-ICSI procedures performed at Avantea, and data are shown separately for Arabian and Warmblood donors because of the breed effect. We observed that Warmblood embryos reached the stage suitable for freezing (uniform density and texture, defined layer of trophoblast cells, and 85%–100% viable embryonic mass, in accordance with grade 1 criteria of the IETS manual) more rapidly than Arabian embryos (Fig. 4). Of the 3,379 Warmblood embryos, 1.4% was frozen on day 6 (n.47), 32.0% on day 7 (n.1083), 60.2% on day 8 (n.2033), and 6.4% on day 9 (n.216), whereas of the 411 Arabian embryos, 0.5% (n.2) was frozen on day 6,

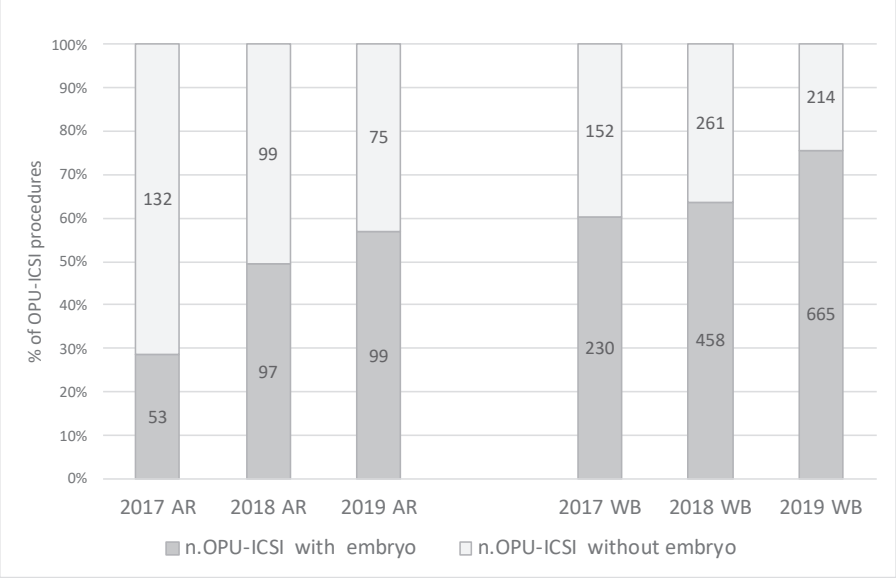

Fig. 2. Percentage of OPU-ICSI procedures performed at Avantea with or without embryo production. The darker columns show the increasing percentage of OPU-ICSI procedures producing at least one embryo over the period 2017–2019, for Arabian donors (AR) and for Warmblood donors (WB). The numbers within the columns indicate the OPU-ICSI procedures performed in each year per donor group.

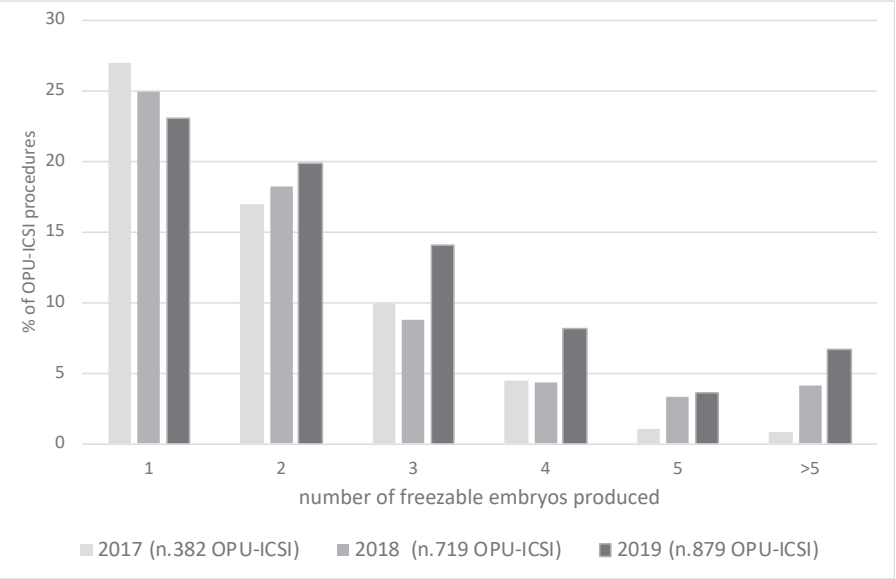

Fig. 3. Percentage of OPU-ICSI procedures of Warmblood donors with positive outcome of one or more embryos produced for the three years 2017–2019. The percentage of OPU-ICSI procedures with no embryos produced can be extrapolated from Fig. 2.

10.7% (n.44) was frozen on day 7, 59.1% was frozen on day 8 (n.243), and 29.7% was frozen on day 9 (n.122). Examples of embryos selected for freezing and discarded embryos are shown in Fig. 5.

3.6. Pregnancy Rate Following Embryo Transfer

The ET data are shown in Fig. 6 and refer to the transfers carried out only at Avantea for the 3-year period from 2017 to 2019, divided between Warmblood and Arabian embryos and for pregnancy rate at day 17, 30, and 50 after ovulation. The data of Warmblood donors refer to 760 ETs, and pregnancy rate was very similar in the three subsequent ET seasons, averaging 70.9% ± 1.9 at 17 days, 63.2% ± 1.8 at 30 days, and 58.6% ± 1.3 at 50 days of gestation. The data of Arabian donors refer to 248 ET, and pregnancy rates were 64.5% ± 3.6 at 17 days, 53.4% ± 3.4 at 30 days, and 43.5% ± 1.3% at 50 days of gestation. The same ET data of the 3-year period are presented in Fig. 7 divided per donor group and per day of embryo freezing (d7, d8, and d9) to highlight differences in pregnancy rate not only between embryos of the different donor group but also of different speed of development in vitro. Results indicate a similar pregnancy rate at day 17 after ET (69%–73 %) for embryos frozen on day 7 and 8 of both donor groups and a lower pregnancy rate (55%–62%) for embryos frozen on day 9, but statistically significant only for the Arabian group. At day 50 of gestation, embryos frozen on day 7 and 8 of the Warmblood group maintain a 60% pregnancy rate, whereas embryos frozen on day 9 undergo more losses reaching a pregnancy rate of 38%. In the Arabian group, the pregnancy rate at 50 days of gestation decreases to 46%–49% for embryos frozen on day 7 and 8, with a tendency to further decrease to 32% for embryos frozen on day 9.

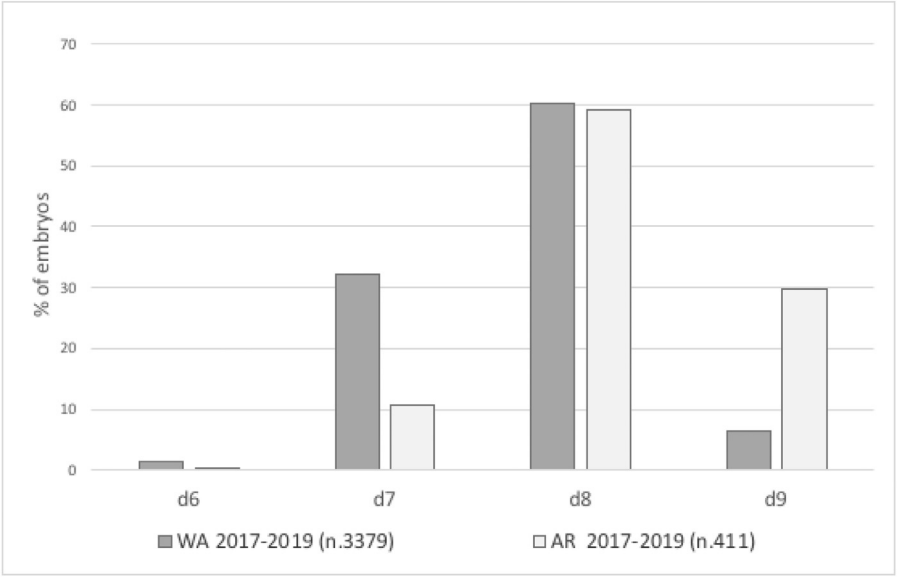

Fig. 4. Kinetics of development of embryos obtained from OPU-ICSI procedures on Warmblood (WB) and Arabian (AR) donors, shown as percentage of embryos selected for freezing on day 6, 7, 8, and 9 of culture after ICSI.

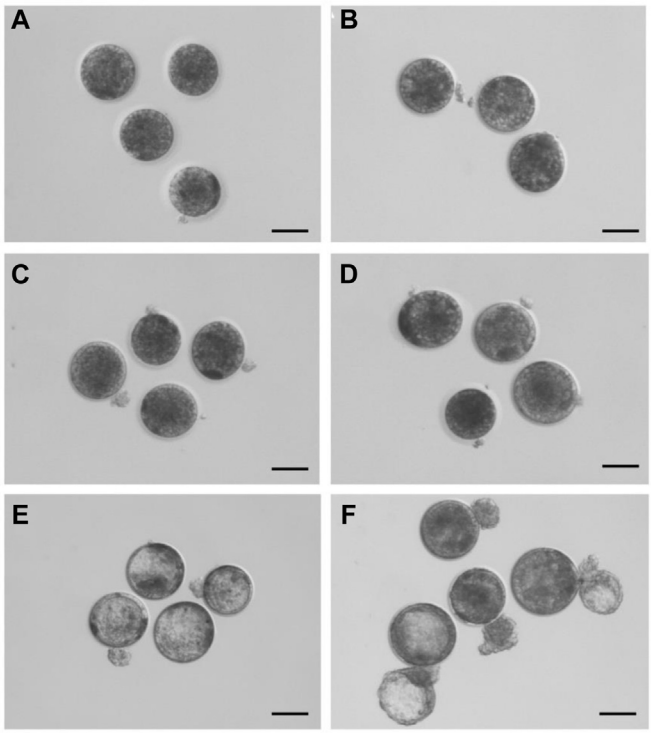

Fig. 5. A–D: embryos derived from four OPU-ICSI procedures and selected for freezing at day 7 (A) and day 8 (B, C, D) after ICSI. E: embryos not selected for freezing for low number of cells and irregular texture at day 8. F: embryos at day 11 of culture after ICSI that were not selected for freezing at day 7–8 but continued to develop and changed morphology over time. Bar 100 μm.

3.7. Embryo Sexing

During 2019, 250 embryos were biopsied before freezing to determine the sex. Fifteen of the 250 biopsies could not be amplified by PCR, or the result was uncertain (6%). Of the remaining 235 embryo biopsies, 126 (53.6%) were diagnosed male and 109 (46.4%) female. Thirty-three sexed embryos (4 d8 WB + 15 d8 AR + 14 d9 AR) were thawed and transferred in recipient mares giving rise to 19 pregnancies (57.6%), a result very similar to the pregnancy rate obtained with d8/d9 Arabian embryos that represented most of those transferred. This result, although based on a small number of ET, indicates that the sexing procedure of OPU-ICSI equine embryos does not affect embryo survival and the pregnancy rate after ET.

3.8. Monthly Distribution of OPU-ICSI During the Period September 2018 to July 2019

The number of monthly OPU and ICSI procedures over almost a year period is shown in Fig. 8 for Avantea and, as cumulative data, for external clinics. The most intense period is the late autumn to early spring both for Avantea and for all the external clinics. In late spring, there is a rapid drop of the OPU at external clinics, whereas for Avantea there is a more constant activity throughout the year. This difference is due to the start of the breeding season with conventional artificial insemination/embryo flushing/transfer that are the core activities of the external clinics, whereas Avantea performs only OPU-ICSI throughout the year, with the exception of August when no OPU-ICSI is performed.

4. Discussion

In this article, we have reviewed how OPU-ICSI has developed in our laboratory over the last few years. Our laboratory has become the center of reference for clinics and practitioners wanting to provide to their clients the most innovative ART service to produce embryos and foals. Over the years, the number of procedures performed has grown exponentially and what is most notable is the rapid growth of shipped oocytes that in 2019 were sent by nine clinics and equaled the number collected at our clinic (Fig. 1). Over the years, we have also witnessed an increase of the number of embryos produced per OPU-ICSI session, as we started from less than one embryo to reach over one embryo for Arabian donors and over two embryos for Warmblood donors. In Arabian mares, we also observed a slight delay in embryo development with more embryos frozen on day 9 and transferred in recipient mares,

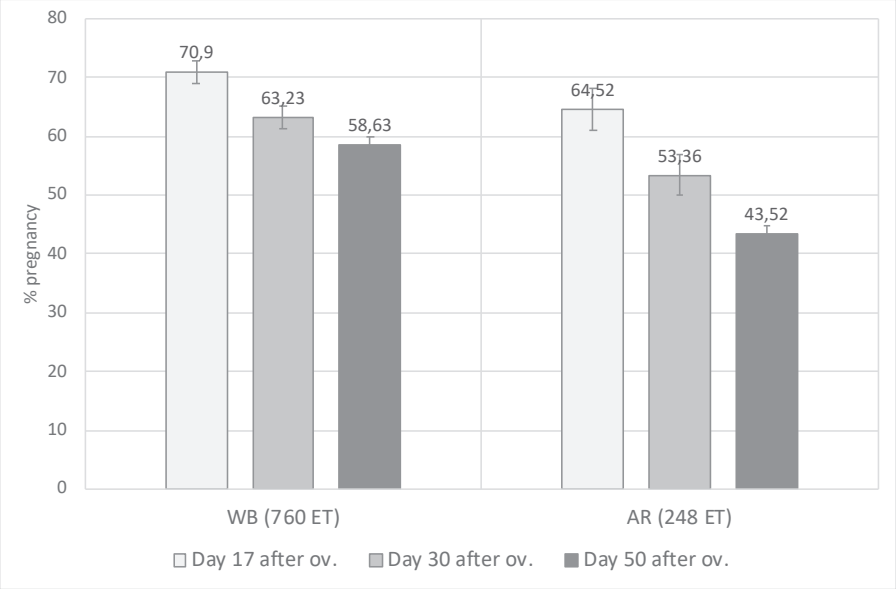

Fig. 6. Pregnancy rate after ET of warmblood and Arabian OPU-ICSI frozen-thawed embryos determined at 17, 30, and 50 days after ovulation. Cumulative data of 3 ET seasons 2017–2019.

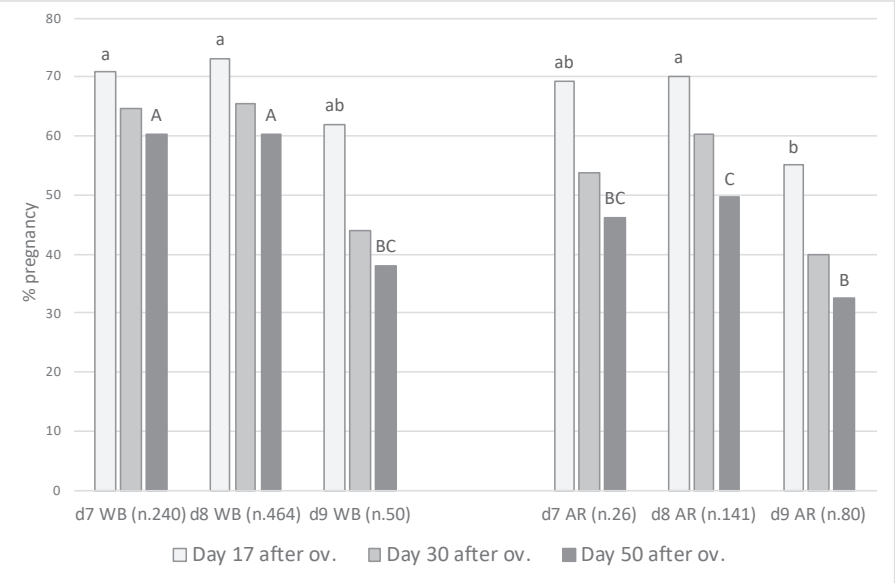

Fig. 7. Pregnancy rate after ET of Warmblood and Arabian OPU-ICSI embryos frozen at day 7, 8, and 9 of culture after ICSI. Cumulative data of three ET seasons at 17, 30, and 50 days after ovulation. (chi square $P < .05$). Columns with different letters within series "day 17 after ovulation" and "day 50 after ovulation" are different.

therefore lowering the overall pregnancy rate of Arabian embryos compared with Warmblood. This is the reason why we do not freeze or transfer embryos developing after D9 although this fraction of late embryos amounts to almost 30% of the total embryos developed in culture.

The recent improvement in the number of embryos produced is the result of several changes introduced in the protocols (increase maturation time, different piezo drill, etc) [32] and by refinements of the whole procedure by better planning, timing, and management of all the steps involved from in vitro maturation to ICSI, embryo culture, cryopreservation, and ET. Interestingly, more embryos produced meant also less OPU sessions that do not provide any embryo and more sessions where multiple embryos are produced. Similar outcomes and improvements have been recorded for shipped oocytes as previously reported [23].

The peak of our activity is during the nonbreeding season because it is the period in which our OPU-ICSI activity overlaps with the OPU of the external clinics that are not so busy doing inseminations and flushing as during the reproductive season. Instead, at Avantea, the OPU-ICSI procedures continue all year around with similar results, even during the breeding season with a slow down during the summer for reasons not related to the donor mares but for statutory holidays of the personnel and routine cleaning/sterilization of the equipment.

Almost all in vitro produced ICSI embryos are cryopreserved for later transfer or commercialization. Pregnancy rates after thawing and transfer are extremely high both compared with those of in vitro produced bovine embryos or with those of in vivo produced flushed equine embryos that are very difficult to cryopreserve, and results are available only for small numbers [33]. Therefore, it is

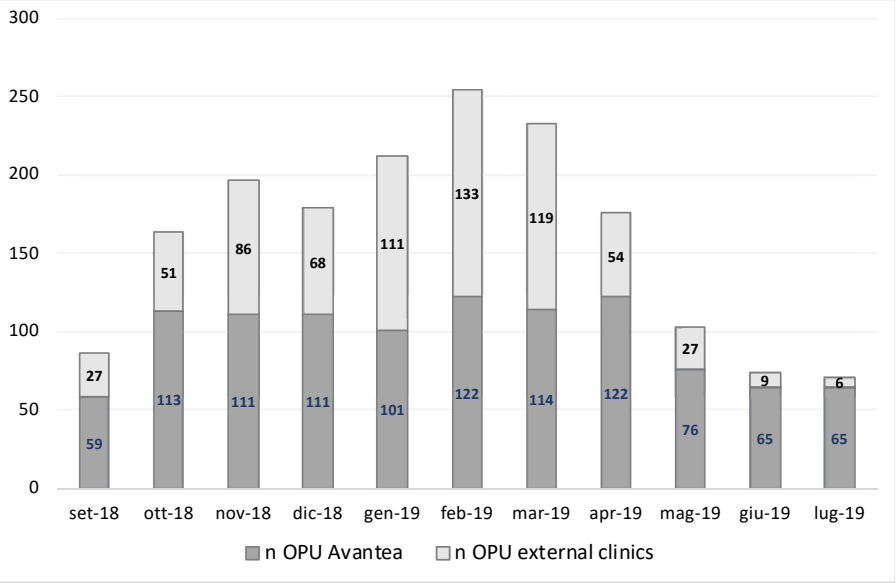

Fig. 8. Period September 2018–July 2019: number of OPU at Avantea and at external clinics shipping oocytes to Avantea for ICSI and embryo culture. The sum of the lighter and darker columns represents the number of ICSI sessions performed at Avantea during the period.

inappropriate to compare in vitro produced frozen-thawed ICSI embryos with fresh in vivo flushed embryos and there is no real comparison with in vivo frozen embryos because of the poor performance of such embryos. The high survival to freezing-thawing of ICSI embryos is most likely due to the limited size (180–220 μM) and incomplete capsule formation that allow an efficient penetration of the cryoprotectant.

It is clear that in vitro produced ICSI embryos are slower in development and therefore require a younger uterine environment and achieve a higher pregnancy rate when transferred in recipient mares D+4 after ovulation [34]. This need for asynchrony might be also helpful to recover from the thawing procedure. Although it is unfair comparing pregnancies derived from fresh in vivo produced embryos with those established by frozen-thawed in vitro embryos, Cuervo-Arango et al [34] observed a higher pregnancy loss for in vitro frozen-thawed embryos compared to fresh in vivo large embryos but a lower pregnancy loss compared to in vivo fresh small embryos. This suggests that the quality of the embryos rather than the technique applied is responsible for a successful pregnancy as we showed for embryos of different donor groups (Warmblood vs. Arabian) and/or of day of development at the time of freezing/transfer.

As reported in the human field, a consequence of ART [35], also seen in the horse, is an increase of monozygotic twin pregnancies up to 1.6% [36] that is however lower than the 2.2% reported in human studies. Apparently, this risk factor is not associated with the micromanipulation of the zona pellucida, the culture media, or the freezing of the embryo but with the extended culture to the blastocyst stage [35]. Monozygotic twins are diagnosed later during pregnancy in recipient mares, that is between 25 to 30 days and cannot be reduced as it is performed with twin pregnancies originating from two different embryos.

In addition to the production of embryos, this technology allows also to perform PGD collecting a few trophoblast cells that herniate through the ICSI opening during blastocyst expansion. We currently use these biopsies to diagnose the sex of the embryo, if requested by the client, but we also set up a test for the warmblood fragile foal syndrome type 1 (WFFST1) (Barandalla et al, this issue), and other preimplantation screening protocols can be developed.

5. Conclusions

OPU-ICSI is giving a strong impulse to the application of embryo technologies with impact at several levels of the horse industry. First, more embryos are produced from the very best donor mares and stallions, therefore increasing the genetic value of the population and the level of sporting performances. Second, the possibility of producing embryos at any time of the year gives extreme planning flexibility especially for mares that are in sport but also for the breeding programs of broodmares. Third, the availability of frozen embryos, even of known sex if desired, allows to plan in advance the production of foals and also the marketing of the genetics through auctions worldwide. Finally, based on our data, an average production of 1.7 to 2 embryos per OPU-ICSI procedure can be expected from warmblood donor mares, which is more than twice reported with flushing in vivo embryos [37], with a pregnancy rate of 70% and a foaling rate in excess of 50%. Therefore, it is no surprise that the equine industry has been so fast in endorsing these technologies.

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