Praca oryginalna *Original paper*

The first equine blastocyst in Poland cultured after ICSI of oocytes derived by TVA-OPU*

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Received 02.02.2026 *Accepted 18.02.2026*

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Summary

The aim of this study was to demonstrate the feasibility of obtaining equine embryos developed to the blastocyst stage using oocyte retrieval by transvaginal ultrasound-guided ovum pick-up (OPU-TVA) and fertilization by intracytoplasmic sperm injection (ICSI) within a fully implemented domestic laboratory setting, where such procedures have not previously been successfully completed, as a basis for further optimization and standardization of *in vitro* equine embryo production.

Materials and methods: Two OPU sessions were performed in five warmblood mares under continuous veterinary care. Oocytes were retrieved using transvaginal ultrasound-guided ovum pick-up (OPU-TVA), followed by *in vitro* maturation (IVM) and fertilization by intracytoplasmic sperm injection (ICSI) using frozen stallion semen. Embryos were cultured under sequential conditions to the blastocyst stage, and embryonic development was monitored. The embryo that reached the early blastocyst stage was cryopreserved by vitrification.

Results: In two OPU sessions, five warmblood mares yielded 25 oocytes. Of these, 16 oocytes (64%) were morphologically competent and subjected to ICSI. Following in vitro maturation for 24-26 h, 15 of 24 oocytes (62.5%) reached metaphase II, while the single oocyte cultured for 35 h also matured. Post-ICSI cleavage occurred in 12 oocytes, leading to one morula and one blastocyst, with the blastocyst originating from the 35 h IVM oocyte. These findings highlight variability in oocyte developmental competence between mares and the impact of IVM duration on embryo development.

Conclusions: The production of the first mare blastocyst in Poland marks a major milestone in national equine reproductive biotechnology, demonstrating the effectiveness of the OPU-ICSI procedure. This achievement enables breeders to access advanced assisted reproductive technologies domestically, reducing the need for transport of animals or genetic material, and lays the groundwork for the development and commercialization of *in vitro* reproduction programs in Poland. Continued research and optimization of oocyte recovery and ICSI outcomes are needed to increase the efficiency and reproducibility of the method.

Keywords: OPU, ICSI, blastocyst, horse, reproductive biotechnology, horse breeding

Assisted reproductive techniques (ART) play an increasingly important role in equine breeding, enabling accelerated genetic progress and more efficient use of the genetic potential of animals with high breeding value. Techniques routinely used in horses include insemination with fresh and frozen semen, embryo transfer (ET), and semen cryopreservation. In recent years, however, dynamic development has been observed in the field of *in vitro* embryo production (IVP), including oocyte retrieval by ovum pick-up (OPU) and fertilization via intracytoplasmic sperm injection (ICSI) (6, 10).

Despite the widespread application of conventional ART techniques, their effectiveness in horses remains

* This study was supported by the Polish Ministry of Science and Higher Education under the "Implementation PhD" program (doctoral project: Assessment of mare oocyte competence for ICSI using molecular markers – donor diagnostics and embryo characterization). Total funding: 374,800.06 PLN. Agreement signed in December 2025.

significantly limited by the species-specific reproductive physiology of the mare. This species is characterized by single ovulation per oestrous cycle, a poor response to superovulatory stimulation, and ovarian anatomy that limits the occurrence of multiple ovulations, which in practice restricts embryo recovery to one embryo per cycle (1, 2, 10). In addition, conventional embryo transfer requires maintaining recipient mare herds and precise control of oestrous cycles, generating substantial costs and logistical challenges (20). Repeated uterine flushings may further reduce mare fertility, and embryos recovered on days 7-8 of development exhibit limited tolerance to cryopreservation compared with earlier embryonic stages (2, 6, 10).

In response to these limitations, the combination of OPU and ICSI has gained increasing clinical and breeding significance, as it allows retrieval of multiple oocytes from a single mare regardless of the stage of the oestrous cycle and enables the use of semen with reduced quality parameters, including frozen semen or semen available in very limited quantities (3, 6, 10). This approach is particularly applicable in mares with ovulatory disorders, intensively used sport mares, and cases in which conventional embryo recovery techniques have failed (5, 18). An additional advantage of OPU-ICSI is the possibility of cryopreserving the obtained embryos without immediate transfer, eliminating the need to maintain a permanent recipient herd and increasing the organizational flexibility of breeding programs (7, 16).

A key milestone in assessing the effectiveness of IVP procedures in horses is the achievement of the blastocyst stage, which represents a critical point in embryonic development determining the embryo's implantation potential, cryopreservation tolerance, and post-transfer viability. Equine embryogenesis is characterized by high sensitivity to *in vitro* environmental conditions, and efficient blastocyst production remains one of the greatest biological challenges in assisted reproduction of this species (10). Therefore, the presence of embryos developed to the blastocyst stage is considered a primary indicator of OPU-ICSI procedure quality and of the developmental competence of in vitro-produced oocytes and embryos.

To date, the available scientific literature contains no published reports describing the production of a mare blastocyst using the OPU-ICSI approach in Poland. Previous attempts at *in vitro* equine embryo production using ICSI have been undertaken in Poland; however, these efforts did not result in embryo development to the blastocyst stage, most likely due to biological constraints and technical challenges inherent to the equine species (11, 12).

The aim of this study was to demonstrate the feasibility of producing equine embryos developed to the blastocyst stage in Poland using oocyte retrieval by transvaginal ovum pick-up (TVA-OPU) followed by fertilization via ICSI, thereby providing a foundation for the further optimization and standardization of *in vitro* equine embryo production.

Material and methods

All procedures involving animals were conducted in accordance with the guidelines for the care and use of experimental animals and were approved by the I Local Ethical Committee for Animal Experiments in Kraków (Resolution No. 101-2025, dated 18 December 2025). Experiments were performed at the Institute of Animal Production – National Research Institute, Kraków, Poland, and at the Arabian Horse Stud Farm Maciej Jerzy Malinowski, Jasieniec, Poland.

Warmblood donor mares belonging to the donor herd were qualified for TVA-OPU sessions according to the standard protocol of the Mavet Equine Breeding Center. In total, five warmblood mares aged between 16 and 20 years were used in two OPU sessions.

As part of the admission procedure, all mares were screened for infectious diseases, including *Taylorella equigenitalis* and equine infectious anemia. Only mares confirmed free from these diseases were accepted into the donor herd.

OPU were conducted during the transitional period preceding the physiological breeding season. This period is characterized by the presence of multiple medium-sized follicles (polyfollicular ovarian pattern) in the absence of a dominant preovulatory follicle. The transitional phase was intentionally selected, as it is associated with enhanced follicular recruitment and may increase the number of aspiratable follicles per session.

All donor mares were maintained under continuous veterinary supervision and showed no clinical signs of general or reproductive disorders. The mares were monitored by transrectal ultrasonography at least once per week, with examination frequency adjusted to their reproductive status. When required, hormonal stimulation was applied to optimize ovarian activity.

Final qualification for the OPU procedure was based on the simultaneous fulfillment of the following criteria: the absence of a corpus luteum and a dominant follicle, and the presence of at least 12 ovarian follicles with a diameter of approximately 10 mm.

To optimize ovarian status and meet the qualification criteria, the estrous cycle stage of the mares was managed by pharmacological modulation of luteal activity when indicated. Three days prior to the scheduled OPU procedure, selected mares received prostaglandin $F_2\alpha$ in the form of cloprostenol (Genestran®, Livisto), 37.5 µg per mare, corresponding to approximately 0.075 µg/kg for a 500-kg mare, administered intramuscularly) to induce luteolysis and promote an early proestrus stage. However, prostaglandin $F_2\alpha$ was administered only when its use was not expected to increase the likelihood of estrus occurring on the day of OPU; therefore, in mares in which $PGF_2\alpha$ treatment could potentially induce estrus at the time of the procedure, the hormone was withheld.

On the day of OPU, a final transrectal ultrasonographic examination was performed to confirm the absence of luteal

structures and dominant follicles and to determine the definitive number of follicles qualified for aspiration.

Immediately prior to OPU, the mares underwent routine aseptic preparation, including restraint in stocks, tail bandaging, rectal evacuation, and thorough washing and disinfection of the perineal area. Premedication consisted of detomidine (0.01-0.02 mg/kg b.w., administered intravenously) combined with butorphanol (0.01-0.02 mg/kg b.w., intravenously). Acepromazine was administered at a dose of 0.02-0.03 mg/kg b.w., intravenously or intramuscularly, depending on the individual mare's response and level of sedation required. Additional boluses of detomidine and butorphanol were given as needed to maintain adequate sedation and analgesia. Flunixin meglumine (1.0 mg/kg b.w., intravenously) was administered when indicated for additional analgesic and anti-inflammatory support.

In cases of excessive rectal wall tension, hyoscine butylbromide (0.3 mg/kg b.w., intravenously) combined with metamizole sodium (20-25 mg/kg b.w., intravenously) was administered to achieve smooth muscle relaxation and additional analgesia.

The OPU sessions were performed under ultrasonographic guidance using a 7.5 MHz microconvex probe mounted in a transvaginal probe holder equipped with a guide for a dual-channel aspiration needle. In the first OPU session, a Dramiński Blue ultrasound system was used in combination with a Dramiński equine OPU gun, a dual-lumen aspiration needle (Minitube), and an equine OPU vacuum pump (Minitube). In the second OPU session, the procedure was performed using IMV Technologies equipment, including an Exapad Mini ultrasound system, an equine OPU gun (IMV Technologies), a dual-lumen aspiration needle (WTA), and a WTA equine OPU vacuum pump. Follicular aspiration was conducted using the vacuum pump, with simultaneous flushing and recovery of oocytes using a commercial PVA-based OPU medium (Equiplus, Minitube), in accordance with the transvaginal aspiration (TVA) procedure as described by Sánchez et al. (17).

Oocytes were recovered from the aspirated follicular fluid using embryological filters (EmSafe, Minitube). The aspirated fluid was passed through the filter and subsequently rinsed with Equiplus medium to obtain a clear solution. The filter was examined under a stereomicroscope (OptaTech) at 20 × magnification to identify the presence of oocytes. Recovered oocytes were washed and maintained in H-SOF (HEPES-buffered Synthetic Oviduct Fluid) medium, which allows short-term storage and transport outside the incubator while maintaining stable pH under atmospheric conditions. Oocytes were transferred into sterile Eppendorf tubes and transported in a thermally insulated container equipped with temperature-stabilizing elements, maintaining a range of 20-22°C, from the Mavet Equine Breeding Center to the laboratory of the National Research Institute of Animal Production in Balice, over a distance of approximately 300 km.

After transport, the oocytes were introduced into *in vitro* culture conditions approximately 17-20 hours after completion of OPU. *In vitro* maturation (IVM) was conducted for approximately 24-26 or 35 hours in Equine IVM Medium (Stroebech) supplemented with 5% FBS (Sigma Aldrich F2442), in an incubator maintained at 38.5°C with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Upon completion of maturation, oocytes were assessed for maturity based on morphological criteria, including cumulus expansion, cytoplasmic appearance, and first polar body extrusion, as described previously (8, 19).

Prior to fertilization by ICSI, oocytes underwent denudation to remove the surrounding cumulus cells, allowing evaluation of nuclear status and proper execution of microinjection. Only oocytes at the metaphase II (MII) stage, confirmed by the presence of the first polar body, were selected for fertilization.

Fertilization was performed by intracytoplasmic sperm injection (ICSI) using frozen-thawed ejaculated semen from a warmblood stallion. Prior to microinjection, spermatozoa were processed using established laboratory protocols to enable selection of single, morphologically normal cells, suitable for injection and to facilitate immobilization of the selected sperm.

Briefly, frozen semen straws were thawed in a 37°C water bath for 30-60 s and immediately diluted in an appropriate handling medium (e.g., HEPES-buffered medium supplemented with albumin or commercial equine semen handling media) to reduce cryoprotectant concentration. Spermatozoa were separated from seminal plasma, extender, and debris by density gradient or colloid centrifugation (e.g., single-layer colloid) according to manufacturer instructions, or alternatively by centrifugation and swim-up, depending on semen quality. The resulting sperm pellet was washed in fresh handling medium and adjusted to a low concentration for micromanipulation.

For single-sperm selection, processed sperm suspension was mixed with polyvinylpyrrolidone (PVP) solution (commonly 7-10% PVP in handling medium) and placed as microdroplets under mineral oil on an ICSI dish. Individual, progressively motile spermatozoa with normal morphology were selected at the microscope and mechanically immobilized by disrupting the tail prior to aspiration into the injection pipette, as previously described in equine ICSI protocols (14, 15).

Microinjection was performed using a RI micromanipulator mounted on Nikon inverted microscope, in a droplet of ICSI medium (Stroebech) supplemented with 5% FBS covered with mineral oil. For decreasing the motility of sperm for ICSI, equine PVP medium (Stroebech) was used.

Following ICSI, oocytes were transferred to Equine One Step IVC Medium (Stroebech) supplemented with 5% FBS (Sigma-Aldrich, F2442) and incubated at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Embryo culture was conducted in a sequential system, with media composition adjusted to successive stages of embryonic development, while minimizing handling and exposure to temperature and pH fluctuations. Embryonic development was monitored daily until the blastocyst stage.

The resulting blastocyst was cryopreserved by vitrification on day 9 of development, following established laboratory procedures routinely applied in equine embryology. Vitrification was performed using a commercial vitrification kit (Stroebech, Denmark), according to the manufacturer's instructions and established equine vitrification protocols (9, 13).

Results and discussion

The mares included in the study were between 16 and 20 years of age. The inclusion of older mares was intentional, as OPU–ICSI is most frequently applied in clinical practice in aged, subfertile, or high-genetic-value mares that are unable to produce embryos efficiently through conventional embryo transfer. Advanced maternal age in mares is associated with reduced oocyte competence, increased incidence of chromosomal abnormalities, mitochondrial dysfunction, and early embryonic death (EED). Therefore, the present model reflects a realistic clinical population rather than an optimal experimental cohort. The possible impact of increased sperm DNA fragmentation on reduced embryo developmental competence should also be considered.

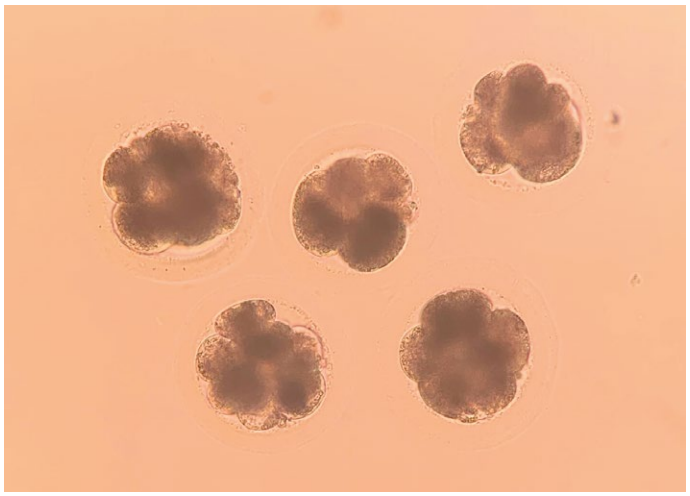

Fig. 1. 6-8 cells equine embryos

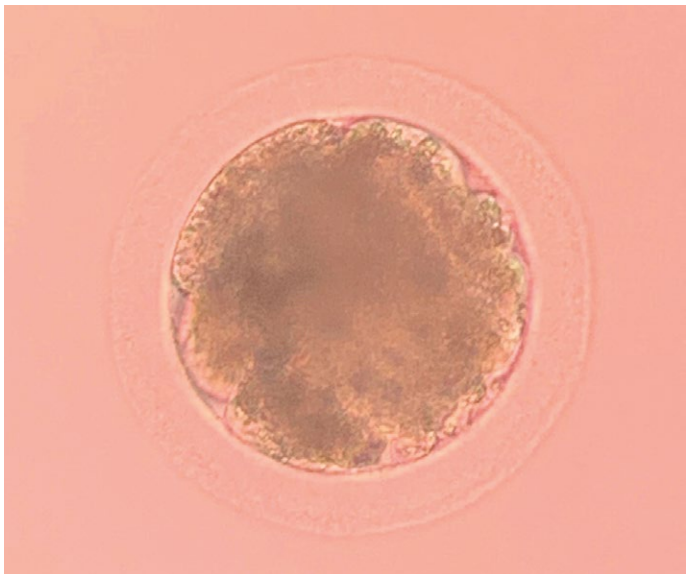

Fig. 2. 4-day morula stage

It is well established that developmental competence declines with age, which may partly explain the limited blastocyst yield (observed in these studies (8, 10)).

Within the two OPU sessions, a total of five warmblood mares were included, yielding 25 oocytes. Oocytes were subjected to in vitro maturation (IVM) for 24-26 hours (n = 24) or 35 hours (n = 1). After IVM, 15 of 24 oocytes (62.5%) from the 24-26 h group reached metaphase II (MII) and were considered suitable for ICSI, whereas the single oocyte cultured for 35 hours also matured to MII.

Following ICSI, injected oocytes were cultured under standard conditions and monitored for embryonic development. Cleavage was observed in 73.3% (12/18) of the oocytes from the 24-26 h IVM group, with development progressing to the 8-cell and morula stages; however, no blastocyst formation was recorded in this group. In contrast, the single oocyte matured for 35 hours underwent cleavage, progressed through the morula stage, and successfully developed into a blastocyst on the post-ICSI day indicated in Table 1.

During the post-ICSI culture, individual blastomeres were subjected to micromanipulation to remove the zona pellucida, after which embryos were cryopreserved at –80°C for further analyses. Notably, the blastocyst observed originated from mare no. 2, highlighting the variability in oocyte developmental competence between mares and the influence of follicular characteristics and IVM duration. Representative images of embryos at different developmental stages are presented in Figures 1-3.

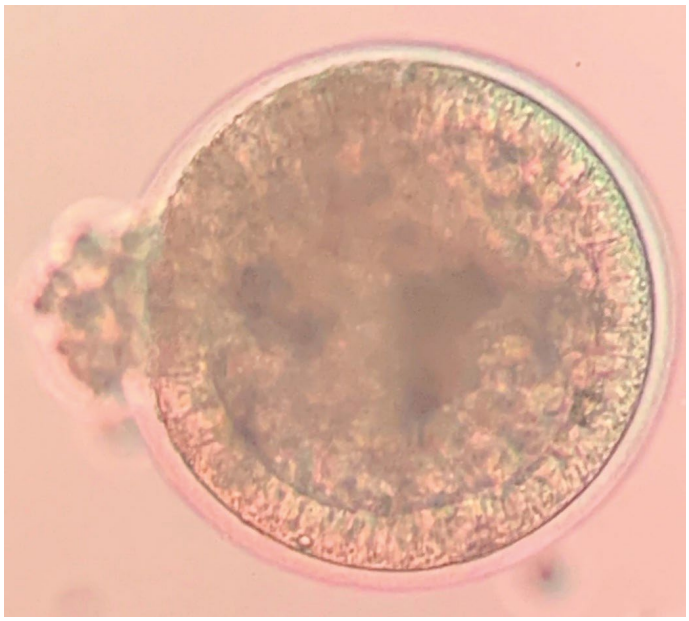

Fig. 3. Hatching blastocyt: trophectoderm starting to herniate through zona pellucida

Tab. 1. *In vitro* maturation (IVM) outcomes of oocytes and embryo development

IVM time	n. of oocytes	n. of MII injected (%)	Cleavage embryos (%)	8-cells stage (%)	Morula (%)	Blastocyst (%)
24-26 hours	24	15 (62.5)	11 (73.3)	11 (73.3)	1 (6.7)	0 (0)
35 hours	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)

Overall, these data demonstrate that, of the 25 recovered oocytes, 16 (64%) were considered morphologically competent and injected via ICSI, resulting in 12 cleaved embryos, one morula, and one blastocyst (Tab. 1).

Due to the limited sample size and the pilot nature of the study, conclusions regarding efficiency should be interpreted cautiously, and further studies including a larger number of mares and comprehensive gamete quality assessment are warranted.

The observed maturation rate (62.5%) and cleavage rate (73.3%) are within the ranges reported in previous equine OPU–ICSI studies, where MII rates typically vary between 55-75% and cleavage rates between 65-80%, depending on mare, follicular status, and laboratory conditions (7, 12, 18). However, the blastocyst yield in the present study (1/16 injected oocytes) was lower than that reported by established commercial programs, in which blastocyst rates of 20-40% per injected oocyte have been described. This discrepancy may be related to the limited sample size, the early stage of laboratory implementation, and individual mare variability.

Given the historically limited efficiency of conventional *in vitro* fertilization (IVF) in horses, intracytoplasmic sperm injection (ICSI), originally developed in human medicine, was adapted for equine reproduction and became the dominant technique in equine *in vitro* embryo production. Although recent studies have reported improved fertilization and blastocyst rates following conventional IVF, ICSI remains the most widely applied method in clinical practice.

The first report of pregnancy following ICSI in horses originated from the United States (19). In subsequent years, advances in *in vitro* oocyte maturation (IVM) control, the use of piezo-assisted micromanipulation, and improved culture media significantly enhanced outcomes achieved with this method. It was demonstrated that the combination of OPU and ICSI can result in stable blastulation rates, high transfer success, and the birth of live foals (7). More recent reviews emphasize that this technique has become a standard approach for high-value mares, with commercial OPU-ICSI programs operating successfully worldwide (12).

Compared with data published by Spacek and Carnevale (18) and other clinical OPU-ICSI programs, the present results confirm that acceptable cleavage rates can be achieved even during the initial implementation phase of the technique. Nevertheless, the absence of additional blastocysts highlights the need for further optimization of IVM duration, culture media composition, and sperm preparation protocols, as suggested by previous authors.

The equine blastocyst created by combining TVA-OPU and ICSI techniques in our laboratory is, to the best of our knowledge, the first one produced in its entirety in Poland and represents a significant milestone in equine reproductive biotechnology in our country. Blastocyst formation is one of the key indicators of the efficiency of the OPU-ICSI procedure, confirming the proper execution of the entire process, from oocyte retrieval through ICSI fertilization to early embryonic development. This accomplishment not only demonstrates technical competence but also places Polish laboratories on par with leading international centres in equine-assisted reproduction.

Equine ICSI remains a highly specialized and technologically demanding technique, with only a limited number of laboratories worldwide capable of performing it routinely (10, 12). Experts estimate that, due to the need for specialized equipment and highly trained personnel, only about a dozen dedicated equine ICSI laboratories operate globally. Although this number is gradually increasing as commercial interest and technological accessibility grow, the technique remains confined to a small community of advanced centers, highlighting the rarity and significance of successful blastocyst production. In Europe, only a handful of established facilities currently offer equine ICSI.

Within this highly specialized landscape, the successful development of the first Polish mare blastocyst not only demonstrates technical competence but also represents a milestone in national reproductive capacity. This achievement enables breeders to access advanced assisted reproductive technologies domestically, reducing the logistical and financial burdens associated with transporting mares or oocytes abroad.

Furthermore, this achievement opens opportunities for the further development and commercialization of reproductive procedures in Poland and increases competitiveness at the European level. At the same time, the observed variability in oocyte quality and developmental dynamics underscores the need for continued research and protocol refinement to increase recovery rates of high-quality oocytes and the number of viable ICSI-derived embryos (10, 12, 18).

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