Development of the first equine blastocyst produced by conventional IVF and in vitro culture in Europe resulting in the birth of a foal

Ontwikkeling van de eerste equine blastocyst geproduceerd door conventionele IVF en in-vitrocultuur in Europa resulterend in de geboorte van een veulen

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ABSTRACT

In this case report, the production of the first equine blastocyst using conventional in vitro fertilization (IVF) and in vitro embryo culture methods in Europe is described. A healthy foal was born after transfer of a blastocyst using a complete in vitro production process, including in vitro oocyte maturation, in vitro sperm capacitation, fertilization in vitro and culture to the blastocyst stage in vitro. Oocytes were recovered from ovaries of slaughtered mares. After in vitro maturation, the oocytes were fertilized by conventional IVF and cultured for nine days. One embryo reached the blastocyst stage and was vitrified. After the selection of a suitable recipient mare that had ovulated four days earlier, the blastocyst was thawed and transferred. Five days after embryo transfer, a single embryonic vesicle was detected by transrectal ultrasonography. After a normal pregnancy of 323 days, a healthy colt was born. Parentage testing via microsatellite genotyping confirmed that the recipient was excluded as the foal's dam and that the stallion whose semen was used, qualified as the sire.

SAMENVATTING

In de voorliggende casuïstiek wordt de productie van de eerste equine blastocyst met behulp van conventionele in-vitrofertilisatie (IVF) in Europa beschreven. Een gezond veulen werd geboren na transplantatie van een blastocyst die volledig in vitro geproduceerd werd, inclusief in-vitro-eicelrijping, in-vitrospermacapacitatie, in-vitrofertilisatie en in-vitro-embryocultuur. Eicellen werden gecollecteerd uit slachthuismateriaal. Na in-vitrorijping werden de eicellen bevrucht met behulp van conventionele IVF en gedurende negen dagen verder gekweekt in het labo. Eén embryo bereikte het blastocyststadium en werd vervolgens ingevroren. Na de selectie van een draagmerrie, vier dagen na haar ovulatie, werd de blastocyst ontdooid en overgeplaatst. Vijf dagen na de embryotransplantatie werd één vruchtblaasje gediagnosticeerd via transrectale echografie. Na een normale dracht van 323 dagen werd een gezond veulen geboren. Afstammingscontrole via microsatellietgenotypering bevestigde dat de draagmerrie was uitgesloten als moeder van het veulen en dat de hengst, die gebruikt werd als spermadonor voor IVF, inderdaad de vader was.

INTRODUCTION

Advancements in reproductive technologies have revolutionized horse breeding. In vitro production (IVP) of embryos is proving to be a valuable tool for addressing fertility problems in both mares and stallions. Until now, the main method for producing equine in vitro embryos has been intracytoplasmic sperm injection (ICSI) (Stout, 2020; Morris, 2018), a process involving the injection of a single sperm

into the cytoplasm of a mature oocyte. This technique requires expensive equipment, such as a high-quality microscope and micromanipulator, along with the skill and expertise of the persons who use these tools, and relies on the subjective selection of the fertilizing sperm. Conventional in vitro fertilization (IVF) could represent a more accessible option for producing in vitro embryos without the use of a micromanipulator and could provide a more physiological selection of spermatozoa. Nevertheless, until recently, conventional IVF had limited success in horses as compared to other species (Leemans et al., 2016). Although two foals produced by IVF followed by surgical transfer of the fertilized oocyte to the oviduct were born in the 1990s (Bézard, 1992; Palmer et al., 1991), the IVF protocol was not reproducible over the next three decades. The main reason for the failure of IVF in horses appeared to be the inability of stallion spermatozoa to capacitate under in vitro conditions (Leemans et al., 2019; Tremoleda et al., 2004). Sperm capacitation refers to the membrane changes and intracellular changes (protein tyrosine phosphorylation, increased intracellular pH and calcium ion concentration) that lead to hyperactivated motility and the acrosome reaction, which are required for fertilization of the oocyte (Yanagimachi, 1994).

Recently, a revolutionary discovery has been made at the University of Pennsylvania School of Veterinary Medicine, at the laboratory of Katrin Hinrich (Kennett Square, USA) (Felix et al., 2022): by combining a metal chelator (penicillamine), an amino acid (hypotaurine) and a catecholamine (epinephrine) in the sperm incubation medium, sperm motility could be maintained for an extended period of time, up to 24 hours. This longer incubation period enabled equine spermatozoa to become capacitated, and to successfully achieve IVF when exposed to oocytes. This innovative approach has led to the development of a reliable and effective procedure for equine IVF. However, to date, no other research laboratory has reported success in reproducing this complex protocol. In this case report, the development of the first equine blastocyst produced by conventional IVF and in vitro embryo culture in Europe is described.

MATERIALS AND METHODS

All procedures were approved by the Ethical Committee 2021-094, Ghent University. Unless otherwise stated, the IVF procedure was carried out in accordance with the protocol established by doctor Katrin Hinrichs' laboratory (Felix et al., 2022).

Oocyte collection

Ovaries obtained from slaughtered mares were used to collect oocytes. They were placed on a warmed surface (38°C) and follicles larger than 5 mm were aspirated and scraped using a 18-G needle connected to a vacuum pump set at -100 mm Hg, followed by rinsing with prewarmed (38°C) flushing medium (Equiplus, Minitube, Tiefenbach, Germany). Oocytes were located via microscopic evaluation of the collected medium and were subsequently cultured for thirty hours in maturation medium (Medium 199 with Earl's salts (Gibco, Fisher Scientific, Merelbeke, Belgium) containing 10% (v/v) FBS (Gibco), supplemented with a combination of 9.4 µg/mL follicle stimulating hormone, and 1.88 µg/mL luteinising hormone (Stimufol, Reprobiol, Ouffet, Belgium). Maturation was carried out in groups of 15 to 20 cumulus-oocyte complexes (COCs) in 500 µL of maturation medium, placed under oil (CooperSurgical, Venlo, the Netherlands) and maintained at 38.2°C in an environment containing 5% CO_2 in ambient air.

Semen collection

Semen was collected from two stallions on a nearby farm and was immediately extended at a 1:1 (v:v) ratio with a milk protein-based extender (INRA96, IMV Technologies, France). Within twenty minutes of collection, the extended semen was processed in the authors' laboratory. Briefly, the extended semen was placed under 1 mL of pre-warmed G-MOPS (Vitrolife, Londerzeel, Belgium) and incubated at a 45° angle at 38°C in air for twenty minutes. The resulting supernatant was collected, transferred to a 1.5 mL Eppendorf tube and centrifuged at 700 x *g* for five minutes at room temperature. The sperm pellet was resuspended in 495 µL of Hanks Balanced Salt Solution with calcium and magnesium. After a second centrifugation at 700 x g for five minutes at room temperature, 30 µL of the sperm pellet was combined with an equal volume of a modified Tyrode's Albumin Lactate Pyruvate-based media (TALP-R) (Felix et al., 2022), that was previously equilibrated in 5% CO_2 in air. The sperm concentration was measured and adjusted to 10 x 10^6 per mL. Five µL of the sperm suspension was added to 45 µL droplets of fertilization medium containing penicillamine, hypotaurine, and epinephrine (PHE) in a modified TALP medium (FERT-TALP-PHE) (Felix et al., 2022) to obtain a final sperm concentration of 1 x 10^6 per mL in a 50 µL FERT-TALP-PHE droplet. The sperm underwent a twenty-hour incubation period in these droplets at 38°C in a humidified atmosphere comprising 5% CO_2 in air.

Oocyte – sperm co-incubation, zygote culture and embryo development

The end of the pre-incubation period was timed to coincide with the end of the thirty-hour incubation period for oocyte maturation. After the sperm pre-incubation period, COCs were removed from the maturation medium and rinsed by washing in four

100-µL droplets of FERT-TALP medium without PHE. One to three COCs were added to each 50-µL FT-PHE droplet containing the pre-incubated sperm for three hours of co-incubation. After this period, one to three COCs were transferred to 15-µL droplets of DMEM-F12 with 10% fetal calf serum and incubated at 38.5°C in an environment containing 5% CO_2, 5% O_2, and 90% N_2. Unlike the commercial human embryo culture medium (Global medium, LGGG-050, LifeGlobal, Guilford, CT, USA) supplemented with 10% fetal calf serum used in doctor Katrin Hinrichs' laboratory, the authors of the present case opted for the embryo culture medium that was well established in their commercial ICSI laboratory.

After 36 hours of presumed zygote culture, the cleaved presumptive embryos were denuded from their cumulus cells and transferred individually to new 5-µL droplets of DMEM-F12 with 10% fetal calf serum. They were cultured under the same conditions as were the zygotes. On Day 5, embryos were similarly transferred to droplets of fresh culture medium and cultured in these droplets until assessed for blastocyst formation. Selected blastocysts were vitrified individually in a minimal volume on a Cryolock (Irvine Scientific, Santa Ana, CA, USA) at room temperature using a commercial vitrification medium (Equine VIT-Kit, Minitube, Tiefenbach, Germany) as per manufacturer's instructions.

Embryo transfer

The mare selected as recipient was housed at the Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University. This nulliparous American Quarter Horse – type mare, weighing 450 kg, was three years old. The mare underwent daily monitoring during estrus using transrectal ultrasonography until ovulation was detected. Embryo transfer was performed using the Wilsher technique (Wilsher and Allen, 2004), four days after ovulation.

Embryo warming was performed by plunging the Cryolock directly into 3.5 mL of embryo holding medium (Emcare, Spervital, the Netherlands) at 38.2°C. After 5 minutes, the embryo was washed four times in the same medium and loaded into a 500-µL straw for transfer (Papas et al., 2023).

RESULTS

Three attempts were performed. In the first attempt, eight oocytes were retrieved, matured and co-incubated with sperm of stallion 1. Although one presumed zygote appeared to cleave, it did not reach the blastocyst stage. In the second attempt, the sperm of stallion 2 was processed; no sperm motility was observed after twenty hours of incubation. For the third attempt, semen of the stallion used in the first trial was processed. A total of 31 oocytes were submitted to maturation, then co-incubated with sperm. Seven presumptive zygotes cleaved 36 hours after fertilization. At Day 9, one blastocyst was recognized and vitrified (Figure 1).

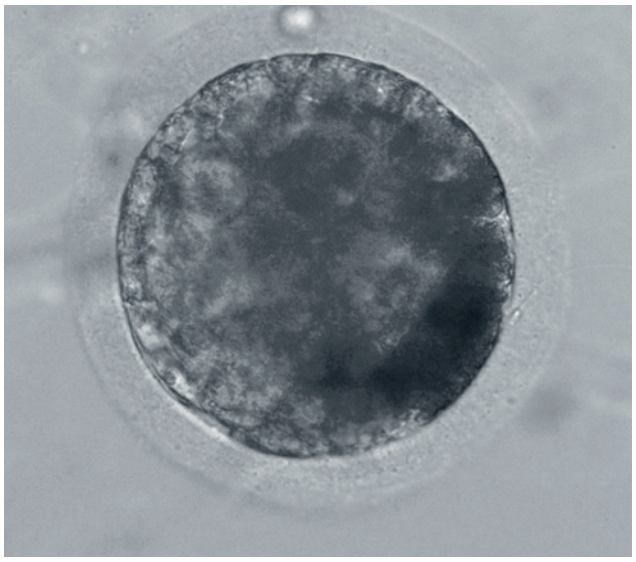

Figure 1. Equine IVF blastocyst (Day 9).

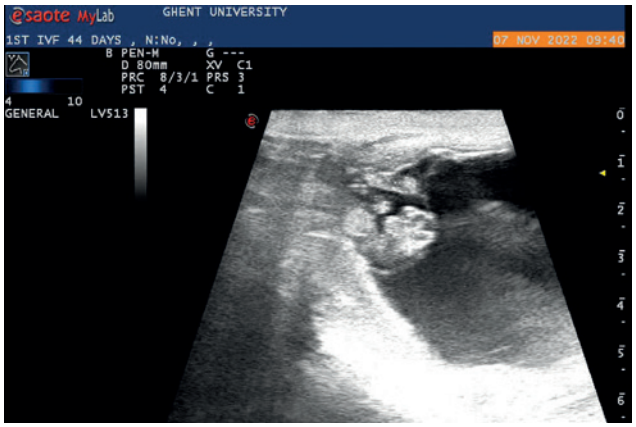

Figure 2. IVF fetus at 44 days.

On Day 4, after ovulation of the recipient mare, the embryo was thawed and transferred. Five days after the transcervical transfer, one embryonic vesicle of 2.9 mm diameter was visualized by transrectal ultrasonography. Regular ultrasonographic examinations throughout the gestation revealed a normal development of a single conceptus (Figure 2). The recipient mare gave birth without any complications after 323 days of gestation. The newborn colt of 38 kg was called PIVFI DRACARYS Di08 (Figure 3). Blood samples of the sperm donor and the foal were sent to an external laboratory (Zoolyx, Belgium) for genotyping of short tandem repeats for parentage testing. The results qualified the sperm donor as the sire and excluded the recipient mare as the biological dam of the foal. At the time of writing, the foal was nine months old and developing normally.

Figure 3. Birth of the IVF foal.

DISCUSSION

Since the development of ICSI, IVP of equine embryos has developed into a reliable and reproducible method, unlike the conventional IVF technique, which has continued to face reproducibility problems in horses. In Palmer and Bézard's protocol, mature oocytes were collected from stimulated dominant follicles and early embryos (24 to 60 hours) were transferred into the oviduct of the recipient mare by flank laparotomy (Bézard, 1992; Palmer et al., 1991). Ever since, methods for in vitro maturation of equine oocytes have been developed and the use of in vitro-matured oocytes has proven to be more efficient (Lazzari et al., 2020), given the challenge of superovulation in mares (Squires and McCue, 2007). Moreover, the culture of embryos to the blastocyst stage allows transcervical transfer (Smits et al., 2012) instead of oviductal transfer through flank laparotomy, making the procedure much less invasive. In their innovative approach, Felix et al. (2022) developed a complete protocol for conventional IVF, which includes in vitro maturation of equine oocyte, in vitro sperm capacitation and in vitro embryo culture to blastocyst stage (Felix et al., 2022). A noteworthy success was achieved, reporting an outstanding fertilization rate of 90% and a blastocyst rate of 74%. These results are promising and mark a significant progress in advanced reproductive techniques, since blastocyst rate achieved using ICSI in an experimental setting range from 27% up to 54% (Brom-de-Luna et al., 2019). Therefore, conventional IVF seems to offer the possibility to produce embryos in a more cost-efficient manner.

During conventional IVF, it is challenging to assess oocyte maturation before the initial co-incubation of oocytes and sperm, since oocytes are still surrounded by cumulus cells, making it difficult to observe the presence of a polar body. Assessing the presence of a polar body on the uncleaved presumed zygotes after denuding offers the possibility of retrospective evaluation of the rate of maturation. Due to constraints linked to the timing of the experiment, this evaluation could not be carried out in the present study. As a result, it was not possible to evaluate crucial aspects such as fertilization, cleavage and blastocyst rate. Nevertheless, the main objective of this experiment was to determine whether another laboratory could reproduce the complex protocol described by Felix et al. (2022).

The achievement here marks a crucial milestone, demonstrating the reliability and feasibility of this technique. Further research is needed to determine whether this procedure can be performed consistently and whether a high rate of fertilization and blastocysts can be obtained. In addition, the impact of individual stallion variability is another aspect worth exploring since it is conceivable that the ability of spermatozoa to capacitate successfully during incubation varies between stallions.

While ICSI might be more effective with limited sperm doses and in case of severe male infertility where sperm has difficulty penetrating the oocyte, conventional IVF offers distinct advantages. From a practical point of view, this technique can be performed by persons with less training and with less costly equipment than that required to operate the micromanipulator used with ICSI. Certainly, the injection process during ICSI represents a complex series of manipulations requiring a long experience. It is worth noting that the skill and meticulous effort needed for oocyte maturation and embryo culture remains an essential aspect, requiring sustained work and specialized equipment (Scoggin et al., 2024), and is independent of the ICSI or IVF procedure. Another major advantage of the conventional IVF procedure is that fertilization occurs through the self-selection of the sperm that will penetrate the oocyte on its own, while the ICSI procedure involves immobilizing subjectively selected sperm. The technique of IVF therefore appears to be less invasive, and better mimics the natural course of fertilization that occurs in the oviduct.

CONCLUSION

The use of conventional IVF in the horse remains a relatively recent development and is currently under investigation. This holds significant promise for fundamental research and clinical application in reproductive strategies and assisted reproductive tech-

niques. The replication of the results of Felix et al. (2022) and the development of the first IVF in vitro-produced blastocyst in Europe mark a crucial stage in the refinement of in vitro production processes and opens up new possibilities.

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