Birth of the first ICSI foal in the Benelux

De geboorte van het eerste ICSI-veulen in de Benelux

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ABSTRACT

This paper describes the birth of the first intracytoplasmic sperm injection (ICSI) foal in the Benelux. Oocytes were aspirated from ovaries from slaughtered mares. After *in vitro* maturation, the oocytes were fertilized by ICSI and cultured *in vitro* for 9 days. Two embryos reached the blastocyst stage and they were transferred to the uterus of a synchronized mare. Six days later a single embryonic vesicle was diagnosed by ultrasound. After a normal pregnancy a healthy foal was born on the 27th of October 2009. Parentage testing via microsatellite genotyping confirmed that the foal originated from the transferred embryo.

SAMENVATTING

In dit artikel wordt de geboorte van het eerste intracytoplasmatische sperma-injectie (ICSI)-veulen in de Benelux beschreven. Eicellen werden geaspireerd uit eierstokken van geslachte merries. Na *in vitro* maturatie werden de eicellen bevrucht door middel van ICSI en *in vitro* gekweekt gedurende 9 dagen. Twee embryo's bereikten het blastocyststadium en werden overgeplant in de baarmoeder van een gesynchroniseerde merrie. Zes dagen later werd een vruchtblaasje gediagnosticeerd met behulp van echografie. Na een normale dracht werd op 27 oktober 2009 een gezond veulen geboren. Ouderschapscontrole via microsatellietgenotypering bevestigde dat het veulen zich ontwikkeld had uit het overgeplante embryo.

INTRODUCTION

Conventional *in vitro* fertilization (IVF), which implies culture of matured oocytes with capacitated sperm, is not efficient in horses. Only two IVF foals, produced from *in vivo* matured oocytes, have been born up till now (Palmer *et al.*, 1991). It is not completely clear yet why the *in vitro* fertilization of horse oocytes is so difficult. One theory states that it may be due to a defective capacitation of stallion sperm, which impairs the normal hyperactivation process of the sperm (McPartlin *et al.*, 2009). Hyperactivation is a typical motility pattern which is exhibited by capacitated sperm and it is generally characterized by an increased lateral head displacement and beat asymmetry. It is believed to be necessary for the sperm cell to penetrate the equine zona pellucida (McPartlin *et al.*, 2009). To circumvent this problem of defective hyperactivation and fertilization *in vitro*, ICSI has been used for the *in vitro* production (IVP) of equine embryos. This technique involves the injection of a single sperm cell into the cytoplasm of a mature oocyte using a fine glass needle. It was initially developed for the treatment of male factor infertility in humans and the first ICSI baby was born in Belgium in 1992 (Palermo *et al.*, 1992).

The first ICSI foals resulted from *in vitro* matured equine oocytes which were surgically transferred to the oviduct after ICSI (Squires *et al.*, 1996; Cochran *et al.*, 1998; McKinnon *et al.*, 2000). Subsequently, also embryos which were cultured *in vitro* up to the blastocyst stage and transferred to the uterus, resulted in the birth of healthy foals (Li *et al.*, 2001; Hinrichs, 2005; Galli *et al.*, 2007). Although ICSI is now commercially available for the infertility treatment of both mares and stallions, only a few laboratories perform this practice. In this case report the birth of the first ICSI foal in the Benelux is described.

MATERIALS AND METHODS

Ovaries were collected from slaughtered mares and all follicles larger than 5 mm were aspirated with a vacuum pump (-100 mm Hg), scraped with the aspirating needle and flushed with heparin in phosphate buffered saline (PBS) (25IU/ml). The oocytes were matured during 26 hours in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) based medium in an atmosphere containing 5% CO_2 (Galli *et al.*, 2007). After the removal of the surrounding cumulus cells by means of gentle pipetting, the oocytes with an extruded polar

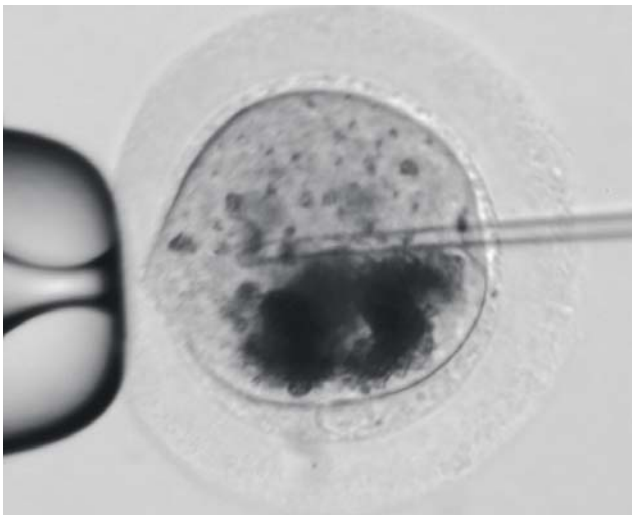

Figure 1. Intracytoplasmic sperm injection (ICSI).

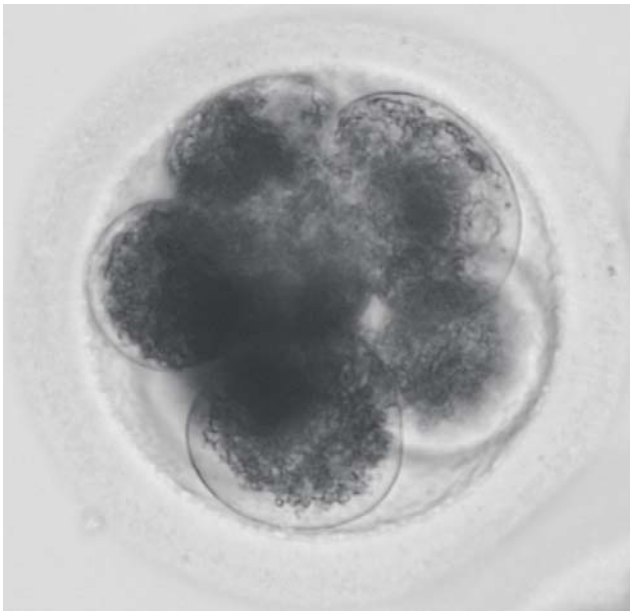

Figure 2. Cleaved equine embryo (day 2.5).

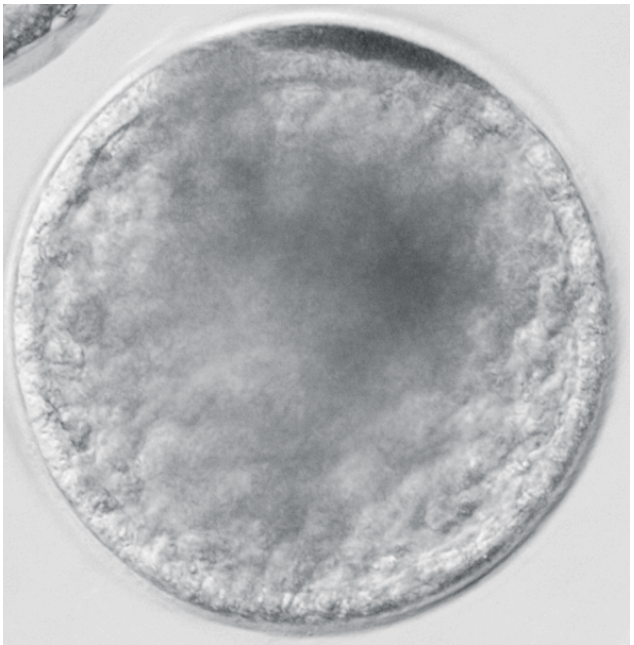

Figure 3. Equine IVP blastocyst (day 9).

body were fertilized by conventional ICSI. Frozen sperm from a stallion of proven fertility was thawed, centrifuged at 750 x g during 40 minutes over a 90%/45% Percoll® gradient, washed with calcium free Tyrode's Albumin-Lactate-Pyruvate (TALP) solution and centrifuged again at 400 x g for 10 minutes. The sperm pellet was resuspended in Synthetic Oviductal Fluid (SOF) medium and stored at 38.5 °C in 5% CO_2. During ICSI the oocytes were kept in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered SOF medium and the sperm in 9% polyvinylpyrrolidone in PBS. All manipulations were performed on a heated plate (38.5 °C) of an inverted microscope. A progressively motile sperm cell was immobilized by crushing the tail on the bottom of the scale and was injected into the cytoplasm of a mature oocyte. The injected oocytes were cultured in groups of 10-20 embryos in 20µl droplets of DMEM-F12 with 10% fetal calf serum at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2. On day 2.5 after fertilization, the embryos which were not cleaved, were removed and half of the medium was changed. On day 6, again half of the medium was changed and on day 9 the embryonic development was evaluated and blastocysts were selected. Two blastocysts were washed in Emcare Holding Medium® and put in a 2 ml tube filled with Emcare Holding Medium®. During the 3 hour transport to the embryo transfer centre, this tube was kept in 50 ml preheated saline and surrounded by 15 l infusion bags at 38.5 °C in an isothermal box. Upon arrival the embryos were washed again in Emcare Washing Medium® and they were both transferred to the uterus of a recipient mare at day 6 post ovulation.

RESULTS

Of the 52 collected oocytes, 27 (52%) extruded the first polar body at 26 hours of maturation and were subsequently injected with a spermatozoon (Figure 1). This resulted in 23 cleaved embryos (Figure 2) of which 2 (8.7%) reached the blastocyst stage at day 9 (Figure 3). On December 18th, 2008, the embryos were transferred to the uterus of a mare that had ovulated 6 days before. Six days after transcervical transfer one single embryonic vesicle of 0.7 cm was diagnosed by rectal ultrasound. On January 12th, 2009, one embryonic heart beat was detected and regular ultrasound examinations revealed a normal development of a singleton conceptus. The recipient mare was transported at 10 months of pregnancy to the Veterinary Faculty at Merelbeke, where she gave birth on October 27th, 2009. A chestnut mare foal of 44 kg was born smoothly and was called SMICSI (Figures 4 and 5). A DNA profile, based on 12 microsatellite markers, was determined for the foal, the sperm donor and the recepient mare. DNA profile comparison assigned the sperm donor as the father and excluded the recipient mare as the biological mother of the foal. The filly is 5 months old now and is developing normally.

DISCUSSION

The IVP of equine embryos has lagged behind that of other large domestic animals. This can be explained by the scarce availability of equine oocytes, the inefficiency of conventional IVF and the suboptimal culture conditions and low blastocyst rates. However, after the introduction of ICSI, the IVP of equine embryos developed into a reasonably efficient and repeatable technique (Hinrichs, 2005). Especially the use of the piezo drill, a device which causes minute vibrations of the injection pipette during ICSI, seemed to be less traumatic for the oocyte and resulted in a rapid evolution in recent years (Choi *et al.*, 2002; *Galli et al.*, 2007).

Nowadays, the combination of ovum pick up, *in vitro* maturation, ICSI, *in vitro* culture and embryo transfer is not only used for research purposes, but is also used clinically. Since only a few normal sperm cells are required, it can be applied for subfertile stallions, for semen which has been frozen-thawed, diluted and frozen again and for sexed semen (Lazzari *et al.*, 2002; Choi *et al.*, 2006; Squires *et al.*, 2008). It has also turned out to be successful in case of problems on the female side, such as advanced age, degenerative endometriosis and cervical laceration (Colleoni *et al.*, 2007). Moreover, ICSI can be beneficial in creating offspring of valuable animals post mortem. These prosperous results are only achieved in a few laboratories in the world. In general, the technique is labor intensive, expensive and not yet optimally efficient. Problems concerning superovulation in horses and a very tight connection between the equine oocyte and the follicle wall imply only limited oocyte collection. Moreover, the IVP process is still suboptimal in horses when compared to other species. The application of IVP of equine embryos on a larger scale, as it happens in cattle, has not been achieved yet in the horse (Blanco *et al.*, 2009).

In this study a cleavage rate of 85% was obtained, which is comparable to the results of other recent publications and which is rather good when it is considered that the ICSI procedure was performed in the conventional way without the use of the piezo drill. However, only 2 out of the 23 injected oocytes (8.7%) reached the blastocyst stage, a fairly low number when compared to other reports of 15.2% (Colleoni *et al.*, 2007) and 23% (Hinrichs *et al.*, 2007). Possible explanations might include differences in oocyte source and culture conditions as well as in the ICSI technique and experience. Even though spectacular blastocyst rates up to 38% have been obtained during *in vitro* culture of equine embryos in DMEM/F12 (Hinrichs *et al.*, 2005), there are still quantitative and qualitative differences when (temporary) *in vivo* culture is performed. Recent studies illustrated a higher percentage of equine ICSI embryos developing to the compact morula or blastocyst stage in sheep oviducts (56%) when compared to culture *in vitro* (20%) (Lazzari *et al.*, 2009). Another intriguing finding was that aberrant expression of a pluripotency gene in IVP blastocysts, when compared to *in vivo* derived embryos, could be normalized by 2-3 days culture in an equine uterus (Choi *et al.*, 2009). Studying gene expression can reveal fundamental differences between equine *in vivo* and *in vitro* embryos (Smits et al., 2009). When compared to *in vivo* derived equine blastocysts, IVP horse embryos have shown to be retarded in the kinetics of development, with more apoptosis and higher levels of chromosomal abnormalities (Tremoleda *et al.*, 2003; Pomar *et al.*, 2005; Rambags *et al.*, 2005).

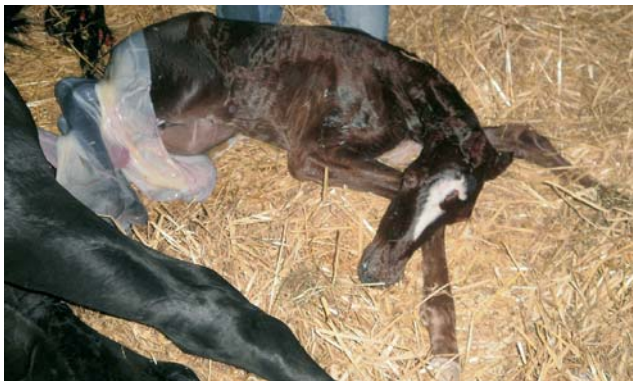

Figure 4. Birth of SMICSI.

Figure 5. SMICSI 3 days old.

Despite these differences, pregnancy rates after an intra-uterine transfer of IVP equine blastocysts are comparable to those after a transfer of their *in vivo* counterparts. The possibility of freezing IVP embryos at rather early stages provides the opportunity of a successful cryopreservation (Galli *et al.*, 2007).

CONCLUSION

Although the IVP of equine embryos has evolved only recently and is not yet optimally efficient when compared to other species, it provides important possibilities for research and clinical application. This first ICSI foal of the Benelux is an important step in the improvement of the IVP processes, which opens possibilities for more research in the domain of equine embryonic development and valuable applications of the technique.

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Uit het verleden

NIEUWE VITRINEKASTEN IN HET DECANAAT TONEN HET DIERGENEESKUNDIG VERLEDEN

Sinds kort is ons 'Veterinair Verleden in de Vitrine', de museale opstelling in de hal van de grote kliniekauditoria, zowaar verdubbeld in omvang. Twee splinternieuwe vitrinekasten prijken nu in de inkomhal van het decanaat. Ze kwamen er in het kader van de viering '75 jaar diergeneeskundig onderwijs in Gent', dank zij de goede zorgen van verscheidene mensen en instanties. We zijn hen daar bijzonder erkentelijk voor.

Net als in de oudere, zal in de nieuwe opstelling thematisch gewerkt worden. Ongeveer halfjaarlijks wordt een nieuwe voorstelling gemaakt van een of ander aspect van ons diergeneeskundig verleden, geïllustreerd aan de hand van instrumenten, producten, brochures, archiefdocumenten, enz. Ere aan wie (of wat) ere toekomt: de eerste presentatie in het decanaatsgebouw toont enkele aspecten van die 75 jaar, samen met een reportage over vorige vieringen van 25 en 50 jaar.

Als illustratie hierbij een detail uit de actuele themapresentatie in de nieuwe kasten. De foto uit *Gids voor de student,* RUG - 1969, genomen in het begin van de zestiger jaren van de vorige eeuw, toont Dr. Antoine De Moor (links), voorganger van prof. Gasthuys en in die tijd assistent van prof. Jean Bouckaert. Hier geeft hij (ongetwijfeld) zeer deskundig uitleg aan enkele studenten, waaronder een Egyptische post graduate. M.B. Morcos en A.H. Said waren de allereerste buitenlandse studenten aan de toenmalige Gentse 'veeartsenijschool'.

Luc Devriese