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In vitro production of horse embryos predisposes to micronucleus formation, whereas time to blastocyst formation affects likelihood of pregnancy

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Abstract. *In vitro* embryo production is an increasingly popular means of breeding horses. However, success is limited by a high incidence of early embryo loss. Although there are various possible causes of pregnancy failure, chromosomal abnormalities, including aneuploidy, are important potential contributors. This study evaluated the frequency of micronucleus formation as a proxy for aneuploidy in *in vitro*-produced (IVP) and *in vivo*-derived horse blastocysts. Associations between IVP embryo morphology, frequency of nuclear abnormalities and the likelihood of pregnancy were investigated. IVP blastocysts exhibited a higher frequency of cells with micronuclei than *in vivo*-derived embryos (10% vs 1% respectively; $P = 0.05$). This indication of chromosomal instability may explain the higher incidence of pregnancy failure after transfer of IVP embryos. However, the frequency of micronuclei was not correlated with brightfield microscopic morphological characteristics. Nevertheless, IVP embryos reaching the blastocyst stage after Day 9 of *in vitro* culture were less likely to yield a pregnancy than embryos that developed to blastocysts before Day 9 (27% vs 69%), and embryos that had expanded before transfer were more likely to undergo embryonic death than those that had not expanded (44% vs 10%). These findings indicate that current embryo culture conditions are suboptimal and that the speed of embryo development is correlated with pregnancy survival.

Additional keywords: aneuploidy, chromosomal instability, intracytoplasmic sperm injection, ovum pick-up.

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Introduction

Over the past decade *in vitro* embryo production (IVEP) has become an increasingly common clinical procedure in equine reproduction. IVEP is used to overcome both female and male subfertility, and allows optimal use of limited stocks of semen. Moreover, IVEP combined with embryo cryopreservation facilitates embryo production outside the breeding season, allows optimal use and easy scheduling of recipient mares and can be used to produce embryos from competing mares without risk of interfering with their competition schedule. IVEP also makes it possible to salvage the genetic material from mares that die suddenly. In order to produce embryos *in vitro*, immature oocytes are harvested either by transvaginal aspiration (ovum pick-up (OPU)) or by follicle scraping (postmortem) and then matured *in vitro* (Hinrichs 2012). Because conventional IVF with equine gametes is not reliably successful, oocytes that reach the MII stage are fertilised by intracytoplasmic sperm injection (ICSI; Hinrichs *et al.* 2002) and subsequently cultured *in vitro* until they reach the blastocyst stage, at which point they are transferred into a suitable recipient mare or are cryopreserved. Although the success of equine OPU-ICSI has improved progressively over the past 10–15 years, equine IVEP results are still variable between laboratories and mares. Recent reports suggest that between 7.2% and 44% of injected oocytes develop into a blastocyst (Hinrichs 2005; Lewis *et al.* 2016) and, although the likelihood of pregnancy after transfer ranges between 40% and 80%, there is a relatively high incidence of pregnancy loss (16–20%) before Day 60 of gestation (Hinrichs

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et al. 2014; Claes *et al.* 2018). Despite the potential contribution of various factors to IVEP failure, in human IVF programs embryo aneuploidy is recognised as the primary contributor to developmental arrest, early embryo death and miscarriage (King 1990; Munné *et al.* 1995; Margalioth *et al.* 2006; Hodes-Wertz *et al.* 2012; Sugiura-Ogasawara *et al.* 2012; Maxwell *et al.* 2016). Although it is known that IVEP increases the number of apoptotic cells in horse embryos (Tremoleda *et al.* 2003; Pomar *et al.* 2005) and cellular aneuploidy is detectable in equine embryos (Rambags *et al.* 2005), the incidence and severity of aneuploidy in equine *in vitro*-produced (IVP) embryos is unknown, and it is unclear whether IVEP increases the risk of aneuploidies arising during the early mitotic divisions in horse embryos.

A recent study on human IVP embryos examined the correlation between the presence of nuclear abnormalities detected by confocal microscopy and aneuploidy detected by cytogenetic analysis and demonstrated that micronuclei (MN; i.e. a small 'nucleus' separate from the main nucleus) are reliable indicators of chromosomal aneuploidy (Kort *et al.* 2016). This was confirmed by Vázquez-Diez *et al.* (2016), who demonstrated in a time-lapse study of mouse embryo development that MN were the result of severely lagging chromosomes. In particular, Kort *et al.* (2016) found that, in blastocysts, the incidence of MN was higher in embryos showing developmental arrest, and suggested that a threshold percentage of cells undergoing normal mitosis is required during the early cell divisions if embryo development is to proceed. If the fidelity of chromosome segregation exceeds this threshold, normal embryo development can continue.

Current techniques for selecting IVP embryos in both animal (Crosier *et al.* 2000; Merton 2002) and human (Gardner *et al.* 2000; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology 2011) assisted reproductive technology (ART) rely on morphological assessment. In addition to morphology, the timing of blastocyst formation *in vitro* is a good indicator of quality for human (Shapiro *et al.* 2008; Chen *et al.* 2014), bovine (van Soom *et al.* 1997) and porcine (Lin *et al.* 2017) embryos. Blastocyst formation at an earlier age has been associated with a higher cell number, a higher ratio of inner cell mass (ICM) to trophectoderm (TE) cells and fewer apoptotic cells. Recent time-lapse studies in human 1- to 8-cell embryos have revealed that chromosome missegregation induces a delay in the completion of the first cleavage divisions (Chavez *et al.* 2012; Vera-Rodriguez *et al.* 2015); therefore, it is possible that slowly developing embryos have a higher incidence of aneuploid cells.

Although several attempts have been made to develop a scoring system for equine IVP embryos (Tremoleda *et al.* 2003; Lewis *et al.* 2016), a morphological classification that takes account of the timing of embryo development and is associated with embryo survival after transfer is lacking. Because the morphology of IVP equine embryos differs substantially from both their *in vivo*-derived equivalents (McKinnon and Squires 1988) and from IVP embryos from other species, the extrapolation of scoring systems is challenging. In fact, *in vivo*-derived equine embryos are, at any given time after fertilisation, developmentally more advanced than IVP embryos (Tremoleda *et al.* 2003; Pomar *et al.* 2005; Rambags *et al.* 2005): they are larger, contain more cells and develop a visible blastocoel earlier than IVP embryos. IVP embryos from other species are more transparent, which makes their morphological quality easier to assess than that of equine IVP embryos.

Therefore, the aims of the present study were to: (1) indirectly quantify chromosomal aneuploidy in IVP and *in vivo*-derived equine blastocysts by evaluating their nuclear morphology; (2) investigate whether a correlation exists between brightfield microscopic morphological characteristics of IVP embryos, their speed of development and the proportion of normal nuclei; and (3) evaluate whether brightfield microscopic morphological characteristics and speed of development could be used to predict pregnancy establishment and survival following embryo transfer.

Material and methods

Experimental design

Experiment 1: indirect quantification of chromosomal aneuploidy in in vivo- and in vitro-derived equine blastocysts by morphological evaluation of individual nuclei

This experiment was designed to investigate the effect of IVEP on post-zygotic embryo aneuploidy. Fourteen *in vivo*-derived Day 6–7 blastocysts and 34 IVP Day 6–13 blastocysts were fixed and stained to image the DNA and actin cytoskeleton. The total number of nuclei was recorded, as was the incidence of MN, cells showing nuclear fragmentation (NF) and mitotic cells (MC), with proportions of MN, NF and MC calculated as a percentage of total cell number (MN%, NF% and MC% respectively; Fig. 1). The incidence of MN, NF and MC was compared between *in vivo*-derived and IVP embryos.

Experiment 2: correlations between speed of blastocyst formation, brightfield microscopic appearance and incidence of MN in IVP embryos

This experiment investigated the possibility of a correlation between the speed of development, morphological parameters and the proportion of abnormal nuclei in IVP embryos. Embryos were divided into two groups based on the speed at which they reached the blastocyst stage (6–9 days after ICSI or beyond Day 9 after ICSI). Each embryo was evaluated using a brightfield microscope at a magnification of ×200 before fixation and immunostaining. The morphological parameters evaluated were selected on the basis of previous reports (Tremoleda *et al.* 2003; Lewis *et al.* 2016), namely the: (1) appearance of the TE (organisation of a presumptive TE layer); (2) presence of extruded cells (presence of dark cells extruded from the blastocyst); and (3) obvious expansion (increase in blastocyst diameter with concomitant thinning of the zona pellucida; Fig. 2). The morphological parameters were subsequently analysed with respect to the number of nuclei, MN%, NF% and MC%.

Experiment 3: correlation between brightfield morphology and pregnancy survival after embryo transfer

To determine whether specific morphological phenotypes were associated with a higher chance of developing into a viable pregnancy, IVP embryos that reached the blastocyst stage at

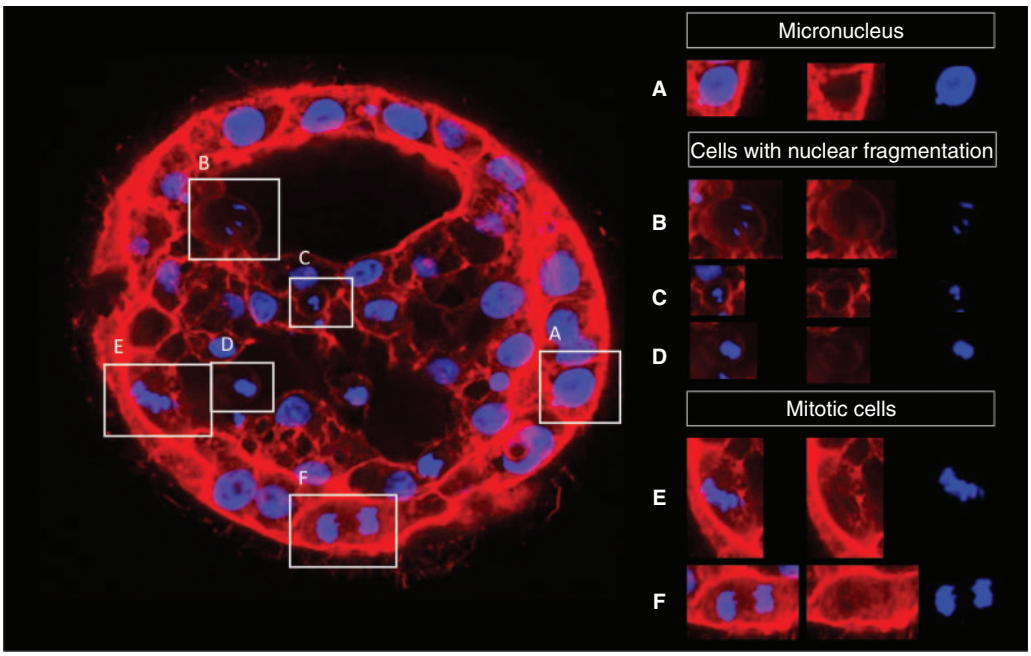

Fig. 1. Confocal micrographs of an equine *in vitro*-produced blastocyst (DNA, blue; F-actin, red) illustrating a nucleus with a micronucleus in the 7 o'clock position (A), cells showing different degrees of nuclear fragmentation (B–D) and mitotic cells in metaphase (E) and telophase (F).

Day 6–9 or beyond Day 9 after ICSI were assessed for the aforementioned morphological parameters under the same conditions as in Experiment 2 and subsequently transferred into a suitable recipient mare (Day 3–5 after ovulation). The likelihood of establishing (Day 7 after transfer) or maintaining (Day 35 after transfer) pregnancy was then correlated with embryo phenotype and speed of development. Retrospective data on the establishment and maintenance of pregnancy after transfer of *in vivo*-derived (i.e. flushed) embryos were used for comparative purposes.

Animals

All animal procedures were approved by Utrecht University's Animal Experimentation Committee (Permission no. 2012. III.03.020).

Experiments 1 and 2

In all, 14 *in vivo*-derived and 34 IVP embryos were used for the immunofluorescence study. A group of nine donor mares, aged between 3 and 11 years, was used to provide the *in vivo*-derived embryos. Fourteen of the IVP embryos were obtained from six donor mares, aged between 4 and 16 years (mean (± s.d.) age 10.2 ± 4.0 years), subjected to OPU; the remaining 20 IVP embryos were produced from oocytes collected post-mortem from ovaries recovered from two different abattoirs on 15 different collection days. Five embryos originated from ovaries recovered from young mares (mean (± s.d.) age 10 ± 2 years; range 9–12 years), eight were from old mares (mean (± s.d.) age 21.4 ± 4.8 years; range 16–33 years) and seven were derived from mares of unknown-age.

Experiment 3

Thirty-seven donor mares were used to produce 66 IVP embryos: 19 IVP embryos were derived from 11 old mares aged between 16 and 26 years (mean (± s.d.) age 18 ± 3 years) and 47 were derived from 26 young mares aged between 2 and 13 years (mean (± s.d.) age 8 ± 3 years). Finally, 54 mares aged between 3 and 15 years (mean (± s.d.) age 9 ± 3 years) and on Days 3–5 after ovulation were used as recipients for ICSI embryo transfer. Retrospective data on pregnancy establishment and maintenance within Utrecht University's commercial embryo transfer program (from 211 transfers of Day 8 *in vivo*-derived embryos performed by the same practitioners) were used for comparative purposes.

In vivo *embryo production*

The oestrous cycle of donor mares was monitored by transrectal palpation and ultrasonography using a scanner equipped with a 7.5-MHz linear transducer (Mylab Gamma; Esaote). During early oestrus, mares were examined every other day and, once the diameter of the dominant follicle exceeded 30 mm, examinations were performed daily. When the follicle exceeded 35 mm in diameter and the mare showed clear uterine oedema indicating oestrus, 1500 IU human chorionic gonadotrophin (Chorulon; Intervet Nederland) was administered to induce ovulation and the mare was inseminated with fresh semen from a single stallion of known good fertility (immunofluorescent staining study) or with chilled–transported or frozen semen from a stallion selected by the mare owner (commercial program). Embryos were recovered on either Day 6.5 or Day 7 after ovulation for the immunofluorescent staining study and on Day 8 for

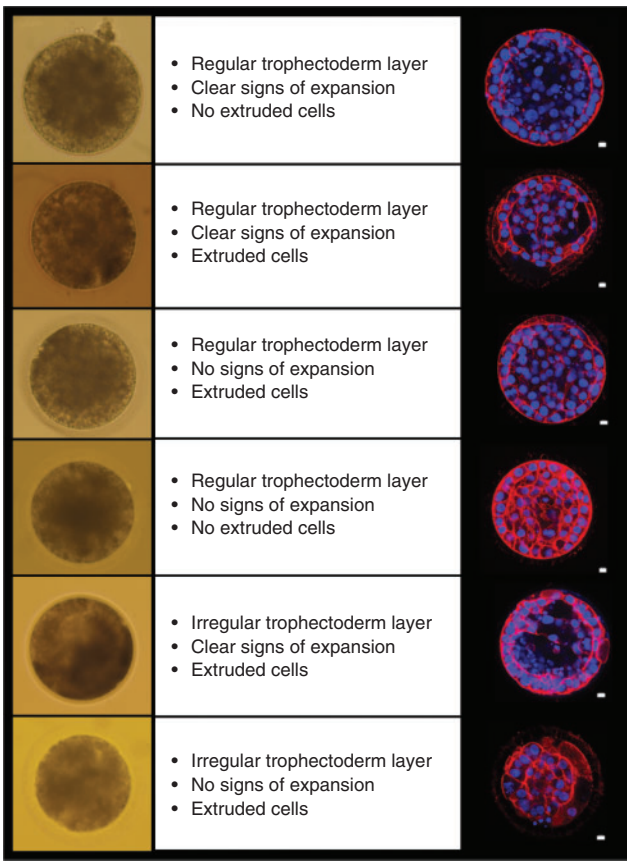

Fig. 2. Examples of brightfield morphological classification of equine *in vitro*-produced (IVP) embryos. Photomicrographs show embryos before fixation using brightfield microscopy (left) and after immunofluorescent staining under confocal laser scanning microscopy (right; blue, chromatin; red, F-actin) with their respective classification (centre column). All IVP blastocysts were scored on the basis of three morphological parameters: (1) the presence of a regular trophectoderm layer; (2) signs of expansion (increase in blastocyst diameter with concomitant thinning of the zona pellucida); and (3) The presence of extruded cells. Scale bars = 10 µm.

embryo transfer by standard uterine flushing with lactated Ringer's solution (Ringer's Lactate Solution for irrigation; Baxter) supplemented with 0.5% v/v fetal calf serum (FCS; GIBCO BRL Life Technologies), as described previously (Stout 2006). The embryos were then washed 10 times with holding medium (SYNGRO; Vetoquinol).

In vitro *embryo production*

Oocyte collection

Equine cumulus–oocyte complexes (COCs) were recovered either by transvaginal ultrasound-guided aspiration (OPU) or by scraping the follicles of ovaries from slaughtered mares.

Recovery of COCs by OPU

The OPU procedure was performed as described previously (Claes *et al.* 2018). Briefly, all follicles >5 mm in diameter were punctured via a transvaginal ultrasound-guided approach using a 12-G double-lumen needle attached to a vacuum pump (Cook Medical). After aspiration of the follicular fluid, the follicle wall was scraped by rotating the needle, and the follicle was flushed 8–10 times with embryo flushing medium (Euroflush; IMV Technologies) supplemented with 0.4% heparin (20 IU mL^{-1}; Leo Pharma) at 38.5°C. The aspirated fluid was recovered in 50-mL tubes (Greiner; Sigma-Aldrich) held in a heated block at 38.5°C (Gewiss; Cenate Sotto Bergamo) and subsequently poured through a 70-µm filter (70-µm Falcon Cell Strainer; Thermo Fisher Scientific). The material on the filter was rinsed into a Petri dish (Greiner Bio-One) and COCs were identified under a dissecting stereomicroscope (Olympus SZ-60) equipped with a warming stage (K-System; Origio) set at 37°C. The COCs were washed four times in HEPES-buffered synthetic oviductal fluid (H-SOF; Avantea) and held in H-SOF overnight in 2-mL tubes (Nunc; Thermo Fisher Scientific) at 22°C.

Recovery of COCs from abattoir-derived ovaries

Ovaries were collected at the abattoir within 15 min after a mare's death and transported at 21–25°C to the laboratory within 5 h, as previously described (Hinrichs *et al.* 2012). At the laboratory, COCs were harvested by scraping the follicle wall with a bone curette and then flushing the lumen with phosphate-buffered saline (PBS; B.Braun) at 37°C supplemented with 0.5% (v/v) penicillin/streptomycin (GIBCO BRL Life Technologies), as described previously (Hinrichs *et al.* 1993). The COCs were identified and held overnight as described above for COCs collected by OPU.

In vitro *maturation*

After overnight holding in H-SOF, COCs were matured *in vitro* in a 50 : 50 mixture of Dulbecco's minimal essential medium (DMEM; Gibco) and Hams F-12 (Gibco) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich), 0.125 µg mL^{-1} epidermal growth factor (Peprotech), 0.1 IU mL^{-1} FSH (Sigma-Aldrich), 0.6 mM cysteine (Sigma-Aldrich), 0.1 mM cysteamine (Sigma-Aldrich) and 0.1% insulin–transferrin–sodium selenite (ITS; Corning Life Science) under mineral oil for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After maturation, cumulus cells and corona radiata were removed by exposing COCs to 0.01% hyaluronidase (Sigma-Aldrich) in H-SOF and gently pipetting through a 155- and then 131-µm capillary plastic pipette (EZ-strip; Origio). Cumulus-denuded oocytes were then washed twice in H-SOF medium supplemented with 10% FCS and were maintained in H-SOF at 38°C until ICSI.

ICSI and embryo culture

Frozen–thawed spermatozoa from stallions with known good fertility via ICSI were selected by a swim-up procedure using a commercial oocyte handling medium (G-MOPS; Vitrolife) supplemented with 10% FCS, as described previously (Choi *et al.* 2015). Oocytes were placed in a 5-µL microdrop of H-SOF under mineral oil at 38°C on a heated stage (Tokai-Hit; Olympus) mounted on an inverted microscope (Olympus IX71) equipped with a micromanipulator (Transfer Man NK2; Eppendorf). Oocytes with an intact oolemma and an extruded first polar body were injected as described by Tremoleda *et al.* (2003). Just before injection, 2 µL motile sperm suspension was mixed with 5 µL clinical-grade polyvinyl pyrrolidone

(PVP; 10%) in HEPES-buffered salt solution (Lucron Bioproducts) to slow sperm cell movement and aid capture. A motile spermatozoon was then immobilised by swiping the injection needle (MIC-50-Angled 30°; Origio) across its tail and against the bottom of the dish. The spermatozoon was then aspirated tail-first into the needle and injected into the ooplasm with a minimum volume of accompanying medium. For sperm injection, the oocyte was held stationary by suction via the holding pipette (MPH-LG-Angled 30°; Origio) with the polar body positioned at 12 or 6 o'clock and the injection needle advanced through the zona pellucida and plasma membrane at the 3 o'clock position. Presumptive zygotes were cultured in 15-μL microdroplets of a commercial human embryo culture medium (Global Medium; LifeGlobal) with 10% FCS under mineral oil (SAGE oil for tissue culture; Origio) in a humidified atmosphere containing 5% CO_2 and 5% O_2 at 38.5°C. Five days after ICSI, cleavage was assessed and cleaved embryos were transferred to DMEM/F-12 with 10% FBS and cultured in a humidified atmosphere containing 5% CO_2 and 5% O_2 at 38.5°C. Embryo development was evaluated daily using an inverted microscope equipped with a ×20 objective (Olympus IMT-2) from Day 6 to Day 13 to identify the day of blastocyst formation.

Morphological assessment of IVP blastocysts

All IVP embryos were evaluated using an inverted microscope equipped with a ×20 objective (Olympus IMT-2) and photomicrographs (Olympus E-330) were taken; various parameters were subsequently evaluated (Fig. 2). First, the developmental rate (time to reach the blastocyst stage) was recorded. Then, TE organisation (clearly visible presumptive TE layer or not), the presence of extruded cells and obvious expansion of the blastocyst were evaluated.

Immunofluorescent study

Fixation and fluorescent labelling of embryos for nuclear evaluation

Blastocysts were fixed in 2% paraformaldehyde for 30 min at room temperature and then stored at 4°C until immunostaining. Fixed embryos were washed three times in PBS containing 3 mg mL^{-1} PVP (Sigma-Aldrich) for 5 min and permeabilised in 0.1% saponin (Sigma-Aldrich) in PBS for 30 min at room temperature. Next, the embryos were incubated in 0.165 μM Alexa Fluor 568 phalloidin solution (Molecular Probes Europe) in PBS-PVP for 30 min at room temperature to label F-actin. After three 5-min washing steps in PBS-PVP, embryos were incubated for 30 min at room temperature in a 1 : 500 dilution of Hoechst 33342 (Sigma-Aldrich) in PBS-PVP to label the DNA. Embryos were mounted in 10-μL droplets of antifade mounting medium (Vectashield; Vector Laboratories) on glass microscope slides (Superfrost Plus; Menzel) using 0.12-mm spacer wells (Secure-Seal Spacer; Thermofisher Scientific) and sealed under a coverslip using nail polish.

Confocal imaging

Immunostained embryos were analysed using a Leica SPE-II DMI4000 confocal laser scanning microscope equipped with a ×65 objective. The images produced by the lasers were merged using Leica Application Suite X (LAS) software. Alexa Fluor 568 phalloidin and Hoechst 33342 were excited using 561- and 405-nm lasers respectively. The whole embryo was imaged by acquiring optical slices at 1-μm intervals, and *Z*-stacks were oversampled at 0.467 μm to ensure that all MN were captured.

Analysis of confocal images

The number of nuclei, MN, NF and MC were counted manually using ImageJ (https://imagej.net/Fiji/Downloads, accessed May 2017; Schneider *et al.* 2012) and MN%, NF% and MC% calculated as described above. The grid tool was used to place a grid over the image and the cell counter plugin was used to count the number of nuclei, MN, NF and MC. The counting tool allowed tracking of the nuclei, MN, NF and MC throughout the *Z*-stack, thereby avoiding double counting. A MN was defined as a small (one-sixth to one-third of nuclear diameter) extranuclear DNA body (i.e. Hoechst 33342 positive) within the same cell as an obvious nucleus (Bhatia and Kumar 2013), a MC was defined as a cell with a mitotic figure (metaphase, anaphase or telophase) and a NF was defined as a cell with a fragmented nucleus (Fig. 1).

Embryo transfer and diagnosis of pregnancy

In vivo-derived ($n = 211$) and IVP ($n = 66$) embryos were transferred into recipient mares by four experienced practitioners (JC, AC, TS, MR, using the technique described previously by Wilsher and Allen (2004) and Cuervo-Arango *et al.* (2018). Embryo recipient synchrony ranged from +1 to −5 days (i.e. the recipient ovulated between 1 day before and 5 days after the donor mare) for *in vivo*-derived embryos. IVP embryos were transferred into recipient mares that had ovulated 3–5 days previously, as described by Claes *et al.* (2018). The pregnancy status of recipient mares was examined by ultrasonography of the genital tract 1 and 5 weeks after transfer.

Statistical analysis

Data were analysed using SPSS Statistics for Mac version 24.0 (IBM Corp.). Independent-samples *t*-tests were conducted to compare the number of nuclei, MN, MC, NF, MN%, MC% and NF% between *in vivo*-derived and IVP blastocysts. A general linear model was used to investigate interactions between development rate, TE organisation, presence of extruded cells and embryo expansion and the number of nuclei, MN, MC, NF, MN%, MC% and NF%. Because no interactions were found, independent-samples *t*-tests were used to explore the effects of delayed development, TE organisation, presence of extruded cells and obvious expansion on the number of nuclei, MN, MC, NF, MN%, MC% and NF% of IVP embryos. Initial and ongoing pregnancy results for IVP and *in vivo*-derived embryos were compared using a Chi-squared test. Chi-squared tests (or independent-samples *t*-tests if expected cell values were <5) were conducted to explore the effects of delayed development, TE organisation, extruded cells and embryo expansion on initial and ongoing pregnancy results for IVP embryos. Significance was set at two-tailed $P < 0.05$.

Results

In total, 100 IVP embryos were produced for this study; 34 were immunostained and 66 were transferred into recipient mares. Among the 34 stained presumptive blastocysts, six of the 20 IVP embryos derived from slaughtered mares were found to be degenerate (two in each of the young, old and unknown age groups) and one of the 14 IVP embryos derived from the OPU program was composed of an unusually low number of cells (60 nuclei); all seven were excluded from further analysis. The pregnancy results for 211 *in vivo*-derived embryos in Utrecht University's commercial embryo transfer program were used as a comparison for the IVP embryo transfer pregnancy results.

Incidence of MN in IVP blastocysts versus in vivo*-derived embryos*

In Experiment 1, the total number of nuclei and MN%, NF% and MC% were evaluated in 14 *in vivo*-derived (six early blastocysts, eight expanded blastocysts) and 27 IVP (Table 1) blastocysts.

IVP embryos showed a 19- and 16-fold higher incidence of cells with MN and NF respectively than *in vivo*-derived embryos (Table 1). Expanded *in vivo*-derived blastocysts were composed of more cells and contained a lower proportion of mitotic cells (MC%) than early *in vivo*-derived blastocysts and IVP embryos. IVP embryos were composed of a similar number of cells, with MC% similar to the *in vivo*-derived early blastocysts.

Correlation between blastocyst morphological characteristics and abnormal nuclei in IVP embryos

In Experiment 2, the possibility of a correlation between blastocyst morphological characteristics (developmental rate, TE organisation, presence of extruded cells and presence of expansion) and abnormal nuclear morphology was explored; results are summarised in Table 2.

Embryos that developed into a blastocyst later than 9 days after ICSI ($n = 15$) had more nuclei when they eventually reached the blastocyst stage than embryos that developed into a blastocyst within 9 days after ICSI ($n = 12$; mean ($\pm$ s.d.) 333.0 ± 29.9 vs 203.5 ± 21.1 nuclei respectively; $P = 0.002$). However, there were no significant differences in MN%, MC% and NF% between these two groups. Moreover, there were no significant differences in the number of nuclei, MN%, NF% or MC% between IVP embryos showing a readily discernible TE layer ($n = 15$) and embryos in which this feature was absent ($n = 12$). Similarly, there were no significant differences for any of the parameters investigated between embryos with ($n = 10$) and without ($n = 17$) extruded cells. However, when nuclear morphology data were analysed in relation to the presence or

Table 1. Total number of nuclei and relative percentage of micronuclei (MN%), cells showing nuclear fragmentation (NF%) and mitotic cells (MC%) in equine blastocysts produced *in vivo* and *in vitro*

Unless indicated otherwise, data are given as the mean $\pm$ s.e.m. Within columns, values with different superscript letters differ significantly ($P < 0.05$)

Embryo production method	Developmental stage	No. embryos	No. nuclei per embryo	MN%	NF%	MC%
In vivo	Early blastocyst	6	480.2 ± 103.4^{a}	0.7 ± 0.6^{a}	1.6 ± 0.8^{a}	3.4 ± 0.4^{a}
	Expanded blastocyst	8	1490.0 ± 193.1^{b}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	2.0 ± 0.4^{a}
In vitro	Blastocyst	27	261.0 ± 21.4^{a}	9.6 ± 1.2^{b}	15.9 ± 2.1^{b}	3.4 ± 0.3^{a}

Table 2. Total number of nuclei and relative percentage of micronuclei (MN%), cells showing nuclear fragmentation (NF%) and mitotic cells (MC%) in equine *in vitro*-produced blastocysts subdivided by developmental rate, presence of a visible trophectoderm (TE) layer, extruded cells and expansion

Unless indicated otherwise, data are given as the mean $\pm$ s.e.m. Within columns, different superscript letters after values for developmental rate (i.e. time to blastocyst formation), TE layer, extruded blastomeres and expansion separately indicate significant differences ($P < 0.05$)

	No. embryos	No. nuclei per embryo	MN%	NF%	MC%
Developmental rate (days)					
6–9	15	203.5 ± 21.1^{a}	10.9 ± 1.9	4.0 ± 0.5	14.6 ± 2.3
10–13	12	333.0 ± 29.9^{b}	8.1 ± 1.3	2.7 ± 0.3	17.6 ± 3.9
TE layer					
Clearly visible	15	276.6 ± 27.9	10.1 ± 1.2	3.1 ± 0.2	12.5 ± 2.0
Not visible	12	241.6 ± 33.9	9.0 ± 2.3	3.7 ± 0.7	20.2 ± 3.8
Extruded cells					
No	17	279.9 ± 26.0	10.4 ± 1.7	3.3 ± 0.5	14.4 ± 1.8
Yes	10	228.9 ± 36.9	8.4 ± 1.5	3.5 ± 0.4	18.5 ± 4.8
Expansion					
No	9	164.2 ± 24.9^{a}	10.9 ± 3.2	4.5 ± 0.9^{a}	24.5 ± 4.6^{a}
Yes	18	309.4 ± 22.3^{b}	8.9 ± 0.9	2.9 ± 0.2^{b}	11.6 ± 1.4^{b}

absence of expansion, compared with embryos that did had not started to expand ($n = 9$), those IVP embryos classified as showing expansion ($n = 18$) had more nuclei (mean (± s.d.) 309.4 ± 22.3 vs 164.2 ± 24.9; $P < 0.001$), a lower MC% (mean (± s.d.) 2.9 ± 0.2% vs 4.5 ± 0.9%; $P = 0.02$) and a lower NF% (mean (± s.d.) 11.6 ± 1.4% vs 24.5 ± 4.6%; $P = 0.002$).

The speed of IVP embryo development and the likelihood of ongoing pregnancy after transfer

IVP embryos ($n = 66$) had a lower likelihood of yielding a pregnancy (41/66; 62%) 7 days after transfer than *in vivo*-derived embryos (187/211; 89%; $P < 0.001$). Within the IVP embryo group, embryos that reached the blastocyst stage later than day 9 after ICSI yielded a lower initial likelihood of pregnancy than embryos that reached the blastocyst stage earlier (3/11 (27%) vs 38/55 (69%) respectively; $P = 0.02$). None of the other morphological parameters examined was significantly correlated with differences in the likelihood of pregnancy. Interestingly, IVP embryos showed a similar incidence of pregnancy loss between Days 14 and 40 of gestation as *in vivo*-derived embryos (7/41 (17%) vs 21/187 (11%) respectively; $P = 0.3$). Moreover, the speed of *in vitro* development to a blastocyst did not significantly affect the likelihood of pregnancy loss, although the numbers were too small for meaningful analysis because only three of the 11 IVP embryos that reached the blastocyst stage later than Day 9 resulted in pregnancy at the first examination. Nevertheless, all three of these pregnancies were ongoing at Day 40. Of the IVP embryos that reached the blastocyst stage at Days 7–9, blastocysts that had visibly expanded were more likely to succumb to pregnancy loss between Days 14 and 40 than blastocysts that had not visibly started to expand (4/9 (44%) vs 3/29 (10%) respectively; $P = 0.04$); the other morphological parameters did not affect pregnancy loss between Days 14 and 40.

Discussion

The results of this study indicate that the *in vitro* culture conditions used negatively affect the developmental competence and, probably, the integrity of chromosome segregation in equine embryos. Indeed, approximately 10% of cells in IVP embryos exhibited one or more MN, whereas less than 1% of the cells in *in vivo*-derived embryos showed similar evidence of aberrant chromosome segregation (Table 1). The higher prevalence of MN in IVP compared with *in vivo*-derived embryos suggests that the *in vitro* culture conditions used compromise the ability of some cells to undergo normal chromosome segregation during mitosis. This is in contrast with mouse embryos, in which similar numbers of MN were reported in *in vivo*-derived and IVP morulae (Vázquez-Diez *et al.* 2016). However, the fact that the *in vivo*-derived embryos in the present study were more advanced in their development than the IVP embryos could explain, in part, the reduction in the prevalence of nuclear abnormalities. Indeed, in human embryos the frequency of nuclear abnormalities has been reported to decrease as embryos develop, with 16% of nuclei abnormal in cleavage stage embryos compared with 5% in blastocyst stage embryos (Kort *et al.* 2016). The highest percentage (65%) of chromosomal abnormalities was seen in arrested human embryos (Kort *et al.* 2016).

MN are a hallmark of mitotic infidelity in cancer cells and are formed when a chromosome 'lags' behind during anaphase, and becomes far enough detached from the rest of the chromosomes to stimulate the formation of a distinct nuclear envelope rather than joining the rest in the nucleus. Although enclosed within a nuclear envelope, the chromosomes in MN may undergo inadequate DNA replication and are more prone to DNA damage during the following S phase (Zhang *et al.* 2015). When the cell next divides, isolation of the chromosome in the MN leads to a copy number asymmetry during the following mitosis, inducing a new missegregation event (Zhang *et al.* 2015). Moreover, due to the compromised DNA repair mechanisms, the chromosomes in the MN accumulate DNA damage and can undergo chromosome rearrangements, *de novo* mutations or chromothripsis (Zhang *et al.* 2015; Liu *et al.* 2018). A recent time-lapse study of mouse embryos revealed that the outcome of MN formation is different for murine morulae than for cancer cells (Vázquez-Diez *et al.* 2016). In that study, the MN in embryos were found to lack kinetochores and were thus unable to correctly segregate during mitosis, resulting in random inheritance of the MN by only one of the daughter cells (Vázquez-Diez *et al.* 2016). The authors suggested that this form of inheritance, together with the possible absence of chromothripsis, may be a mechanism by which the incorporation of damaged DNA into the genome is prevented. In the present study, MN were identified solely on the basis of positive DNA staining combined with their size and position relative to the main nucleus. One limitation of this methodology is that the identity of the additional pieces of DNA as MN is not corroborated, and nor does it allow the study of DNA damage occurring as a consequence of MN formation. In the future, more robust studies could analyse a larger number of IVP embryos and combine the immunofluorescent staining of DNA with markers for the nuclear envelope (e.g. lamin B) to verify their identification as micronuclei. Additional staining for DNA double-strand breaks (e.g. with phosphorylated γH2AX or p53 binding protein 1 (53BP1)) would also help characterise the consequences of MN formation to DNA integrity in horse embryos.

Nevertheless, the apparently higher incidence of MN in IVP than *in vivo*-derived equine embryos is an indication that the former suffer from a higher level of chromosomal instability. Because aneuploidy has been proposed to be the major factor responsible for human embryo developmental arrest and pregnancy failure (King 1990; Munné *et al.* 1995; Margalioth *et al.* 2006; Hodes-Wertz *et al.* 2012; Maxwell *et al.* 2016), this may explain the lower likelihood of pregnancy for IVP compared with *in vivo*-derived embryos after transfer into recipient mares. In human IVF, aneuploidy appears to be more frequent in slowly developing embryos (Magli *et al.* 1998, 2007). However, we did not see a significant difference in the incidence of MN between IVP blastocysts developing before or after Day 9 after ICSI. This may be because we did not examine the incidence of MN at the same time point in these two groups of embryos. Instead, we waited until the delayed embryos had developed into a blastocyst. During this 'time gap', a process of cell selection could have induced apoptosis in some of the aneuploid cells, thus reducing the apparent incidence of MN.

Unexpectedly, IVP embryos that developed into a blastocyst beyond Day 9 after ICSI showed a higher cell number at blastocyst formation and similar MN%, NF% and MC% to embryos developing more rapidly. That these slowly developing embryos showed a reduced likelihood of pregnancy after transfer (27% vs 69%) could be a consequence of perturbation of the process of cell lineage segregation. In this respect, mouse embryos showing delayed development have been reported to show a reduced percentage of ICM cells (Kelley and Gardner 2016). The present study estimated cell proliferation by counting cells in the M phase of mitosis; this method underestimates the real number of mitotic cells because it does not take account of cells in the G_1, S and G_2 phases. Future studies should include the use of proliferation markers, such as Ki67, to more specifically evaluate the relationship between rate of cell proliferation and pregnancy survival in equine IVP embryos.

Similar to Lewis *et al.* (2016), we found it challenging to correlate brightfield microscope morphological characteristics of IVP blastocysts with the incidence of nuclear abnormalities. An example of obvious discrepancies between microscopic morphology and incidence of nuclear abnormalities can be seen in Fig. 3. Overall this indicates that although morphology can help in the selection of blastocysts for transfer, it cannot be used to reliably assess IVP embryo nuclear or chromosomal integrity. Interestingly, although the number was low, embryos that had visibly expanded at the time they were first recognised as a blastocyst showed an increased likelihood of pregnancy loss (44% vs 10%) than embryos that had not yet started to expand. This could be because the expanded embryos had progressed further with development, and that later developmental processes, such as cell lineage allocation, may be more profoundly disturbed by continued *in vitro* culture. Overall, this suggests that the prompt identification of IVP blastocysts before significant expansion is crucial for satisfactory results after transfer, even though it may be tempting to wait for expansion as an obvious sign of blastocyst formation.

In conclusion, the results of this study indicate that *in vitro* culture leads to an increased incidence of nuclear fragmentation and MN formation in equine embryos, where the latter is a proxy for aneuploidy arising during mitotic divisions. However, these abnormalities are not reliably correlated with brightfield microscope morphological characteristics, and morphological assessment of equine IVP blastocyst quality remains challenging. Furthermore, the results of the present study indicate that IVP embryos showing delayed development (i.e. do not reach the blastocyst stage until Day 10 or later) have a significantly lower likelihood of resulting in a pregnancy after transfer, and that it is important to identify and transfer equine blastocysts before expansion has started to avoid an increased risk of early pregnancy loss in recipient mares.

Clearly, further studies are needed to investigate the mechanisms that predispose to MN formation and the fate of the cells carrying MN during early development of equine IVP embryos. Based on our observations it is evident that current *in vitro* culture conditions need optimisation to reduce chromosomal instability and improve the developmental competence of resulting embryos.

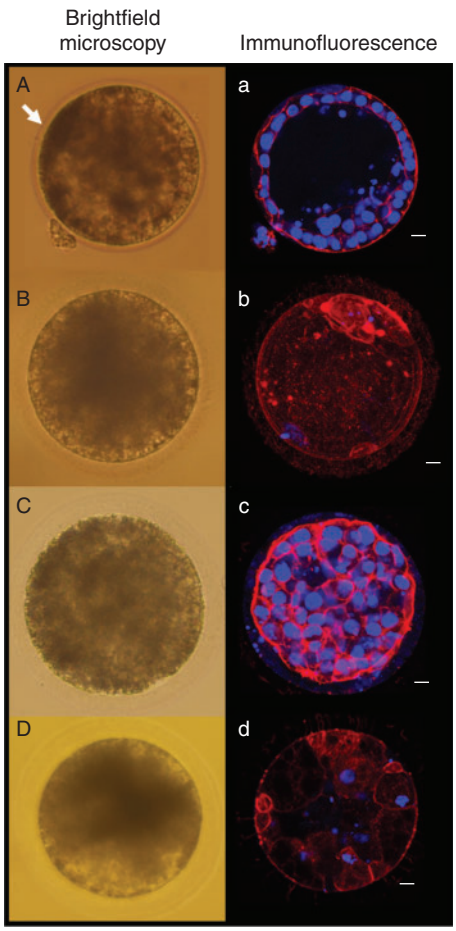

Fig. 3. Photomicrographs of *in vitro*-produced (IVP) equine blastocysts before fixation under brightfield microscopy (*A–D*) and after immunofluorescent staining under confocal laser scanning microscopy (*a–d*; blue, chromatin; red, F-actin) illustrating the challenge of scoring IVP equine embryos for quality. (*A*) An embryo scored as exhibiting expansion, a continuous trophectoderm (TE) layer and extruded cells (arrow; presence of a dark area). Confocal microscopy (*a*) confirmed the brightfield classification. (*B*) Embryo scored as a blastocyst without expansion, with a continuous and regular TE layer and without extruded cells. Confocal imaging (*b*) revealed that it was a degenerated embryo, composed only of cells showing nuclear fragmentation. (*C*) Embryo scored as degenerated, without expansion, with an irregular TE layer and no extruded cells. Confocal microscopy (*c*) showed that the embryo was an early blastocyst that had just started forming a blastocoel. (*D*) Embryo classified as degenerated without expansion, an irregular TE layer and no extruded cells. The confocal image (*d*) demonstrated that it contained only cells showing nuclear fragmentation. Scale bars = 10 μm.

Conflicts of interest

The authors declare no conflicts of interest.

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