# Effect of oocyte source and transport time on rates of equine oocyte maturation and cleavage after fertilization by ICSI, with a note on the validation of equine embryo morphological classification

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## Abstract

Production of equine embryos using intracytoplasmic sperm injection (ICSI) is rapidly gaining interest in the horse industry. Due to increasing client demand, equine practitioners with limited experience in embryology are attempting to set up clinical ICSI programs, with little success. We report here studies performed with the purpose of establishing an equine in vitro maturation (IVM)/ICSI program. We addressed three objectives: to determine (1) the effect of oocyte source (transvaginal follicle aspiration [TVA] vs. abattoir) on maturation, cleavage and blastocyst rates; (2) the impact of time of oocyte recovery (soon after death vs. delayed [median time 7.5 h]) on these parameters in abattoir-derived ovaries; and (3) the correlation of post-ICSI embryo morphology with histologically-confirmed nuclear status. Maturation rates were greater for TVA-derived than for abattoir-derived oocytes (67% vs. 32%, respectively; $P < 0.01$). Duration of ovary transport did not affect oocyte maturation (34-48%) or cleavage rates (70-73%). Chromatin staining revealed that light-microscopic evaluation was accurate in determining oocyte maturation to metaphase II (14/15, 93%), but was not accurate in classification of either normal embryo cleavage (8/31 cleaved embryos possessed normal nuclei) or blastocyst formation (7/15 embryos classified as blastocysts were verified on staining). Early embryo and blastocyst viability was confirmed by transfer of some embryos into recipient mares. These findings indicate that oocyte source (TVA vs. abattoir) can affect results in an equine IVM/ICSI system, and suggest that new laboratories should use a systematic approach of comparison of nuclear chromatin staining with morphological classification to validate embryo development after ICSI.

**Keywords**: Horse, ICSI, blastocyst, oocyte, embryo

## Introduction

The ability to produce foals using ICSI has the potential to improve our understanding of early embryo development and has clinical applications in the salvage of valuable equine genetics.[1] In mares, this is either following post-mortem oocyte recovery or by collecting oocytes from living sub-fertile mares that cannot provide an embryo for transfer[2,3] and in stallions, by offering a mechanism to produce embryos and foals when only limited reserves of semen are available.

The *in vitro* production of viable blastocysts and subsequent pregnancies, encompasses many complex and interdependent procedures, as well as requiring knowledge of cell culture and embryological techniques, such that the development of a successful equine ICSI program has been described as "practically unattainable in most situations".[4] Interest in the use of equine ICSI in both commercial and research applications has rapidly increased worldwide, and client demand has resulted in an increasing number of equine practitioners and equine laboratories with limited experience in *in vitro* embryo production embarking on the establishment of commercial equine ICSI laboratories. Given the challenges, successes have been limited. Groups that report blastocyst development after equine ICSI typically achieve a < 10% blastocyst rate per injected *in vitro*-matured (IVM) oocyte.[5-8] Although it is generally understood that equine ICSI is feasible, in reality, only a few laboratories have reported high blastocyst rates (>20%) using IVM oocytes[9-12] and only two of these have reported >20% blastocyst rates for this procedure more than once (~25-43%).[3,11-15]

The rate of oocyte maturation is another major determinant of commercial success. If blastocyst rates are low (<10%), production of one blastocyst would take over ten mature (metaphase II [MII]) oocytes. For many laboratories, oocyte recovery and *in vitro* maturation rates currently conspire against this. Rates of *in vitro* maturation are variable (44 to 61%), particularly when collecting oocytes post-mortem, as in genetic salvage cases.[11,16]

Clinically, in the case of genetic salvage, a mare often dies or is euthanized at a location distant from the ICSI facility. Under these circumstances ovaries/oocytes must be optimally retrieved and transported to the ICSI facility. Despite anecdotal reports, currently, only a single report has

detailed the clinical production of foals by ICSI under these conditions. In that report, pregnancies were obtained following ovary removal at the location of death and transport of both whole ovaries or recovered oocytes.[2]

Another aspect affecting development of an ICSI program is the ability of the practitioner to appropriately identify viable embryos. While some laboratories have validated their classification of embryos as blastocysts by chromatin staining for nuclear status or by achieving high pregnancy rates (>50%) after transfer, some laboratories have assessed blastocyst development based only on morphological appearance.[3,6,12,17,18] The morphology of *in vitro* produced (IVP) equine blastocysts differs from both the morphology of IVP blastocysts in other species and from that of equine *in vivo*-derived blastocysts, thus identification of viable embryos may be problematic for the inexperienced eye. However, to the best of our knowledge, other than early reports from the laboratory in Texas which reported both morphological cleavage and cleavage with normal nuclei (41% to 88% of morphologically cleaved embryos had normal cleavage on nuclear evaluation),[19-21] essentially no information is available on the relationship of light-microscopic morphological classification to actual embryo status as shown by evaluation after nuclear staining.

In this study, we performed three evaluations to establish basic parameters for the development of an equine ICSI program. We determined (1) the effect of oocyte source (TVA from live mares vs. abattoir-derived) on maturation, cleavage and blastocyst rates after ICSI; (2) the relative impact of oocyte recovery soon after death as opposed to a delay following the transport of entire ovaries and (3) the correlation of post-ICSI embryo morphology on light microscopy with histologically-confirmed nuclear status.

**Materials and methods**

All procedures were conducted in agreement with the principals of the University of Liverpool Veterinary Ethics Committee and with the approval of the Royal College of Veterinary Surgeons.

Oocyte recovery from abattoir-derived ovaries

Ovaries were obtained from mares, of unknown age and breed, slaughtered at a UK abattoir for purposes unrelated to the study. Slaughter was conducted in accordance with EU legislations EC 852/2004, 853/2004 and 854/2004. Ovaries were obtained within 15 min post-mortem, placed in a polythene bag within a polystyrene foam container and maintained at ambient temperature (18-20°C) until all tissues had been processed. Ovaries were either transported to the ICSI laboratory (transport time 4 hr) or to a local facility as dictated by the study protocols detailed below. On arrival, all visible follicles were aspirated using a 14 G needle and vacuum pump (Rocket Medical PLC., Watford, Herts, UK) set at approximately 200 mmHg. All plastics (MILA international, Erlanger, Kentucky) were sterilized in ethylene oxide with a minimum gas-off time of 4 wk. A syringe and needle were used to flush each follicle one to two times with flush medium (M199 with Hanks salts, 0.4% Fetal Bovine Serum (FBS) (Life Technologies Ltd, Paisley, UK), 25 μg/ml gentamicin, 8 IU/ml heparin (Sigma-Aldrich Ltd, Gillingham, Dorset, UK). Ovaries were then sliced at 5-10 mm thickness and additional visible follicles were aspirated and scraped using the 14 G needle with vacuum. Aspirated fluid was collected into sterile 500-ml bottles and passed through an embryo filter (Emcare ICPbio Reproduction, Auckland, NZ). The filter was rinsed with flush medium and cumulus-oocyte complexes (COCs) were located in the recovered tissue under a dissection microscope at 60-120X. Identified COCs were moved to manipulation medium (M199 with Hanks salts, 10% FBS, 25 μg/ml gentamicin). All manipulations were conducted at ambient temperature (approximately 18-20°C).

Oocyte collection from live mares

Oocytes were recovered by TVA from six sport-horse type mares aged 7-14 years. The TVA procedures were conducted as previously described.[10] All visible follicles > 0.5 cm were aspirated; each follicle was flushed up to six times with flush medium. The aspirated fluid was processed within 1-2 hr of aspiration, as described above for fluid aspirated from follicles in abattoir-derived ovaries.

Oocyte holding

Recovered COCs were divided into groups of eight to 14 and placed in individual 1-ml glass vials (Thermoscientific Inc., Waltham, MA) in EH Medium (40% M199 with Hanks salts, 40% M199 with Earle's salts, 20% FBS and 25 μg/ml gentamicin).[22] Vials were sealed and held overnight (12-18 hr) at room temperature (20°C) protected from light.

Oocyte maturation

Maturation dishes (pre-prepared with medium and oil) were pre-equilibrated for 12 hr before use. Cumulus-oocyte complexes were removed from the EH holding vial, washed in maturation medium (M199 with Earle's salts, 10% FBS, 25 μg/ml gentamicin with 5 mU/ml FSH [Sioux Biochemical Inc., Sioux Center, IA]) and incubated in groups of 9-12 at a ratio of 10 μl medium per COC in a 4-well dish (Thermoscientific), under mineral oil (Vitrolife Ltd., Warwick, UK) for 30 hr at 38.3°C in 5% $CO_2$ in air. Oocytes were then denuded of cumulus cells by repeated pipetting in manipulation medium containing 80 IU/ml hyaluronidase (Vitrolife). Oocytes were evaluated by light microscopy using a dissection microscope at 500X. Those with a visible polar body were classified as metaphase II (MII), oocytes with an intact oolemma without a polar body were classified as intact and those with an irregular oolemma or shrunken cytoplasm were classed as degenerating (DEG). Confirmation of correlation of oocyte morphological classification with chromatin status was performed in experiment 3.

ICSI and Embryo Culture

Frozen-thawed sperm from one fertile stallion was used for ICSI. For each ICSI session, a single 0.5-ml straw of semen was thawed at 37°C for 30 sec. To perform swim-up, 200 μl of thawed semen was placed under 3 ml of frozen-thawed Sp-Talp[23] and incubated at 37°C for 30 min. Sperm suspension from the top of the Sp-Talp layer was used for ICSI.

Conventional ICSI was performed using an Integra micromanipulator (Research Instruments, Falmouth, UK) by an embryologist (KS) with extensive experience in human clinical ICSI. A standard sharpened ICSI needle (5 μm inner diameter; Research Instruments) was used. Denuded MII oocytes were placed in individual 10-μl droplets of manipulation medium and 1-2 μl of sperm suspension was placed in a separate 5-μl droplet of a commercial 9% PVP solution (ICSI™; Vitrolife). Motile spermatozoa were immobilized by crushing of the flagellum with the ICSI needle until kinking was observed and aspirated into the ICSI needle flagellum first. The oocyte was positioned with the polar body at 6 o'clock and the ICSI needle was advanced through the zona pellucida and into the oocyte cytoplasm, essentially to the opposite side of the oocyte. Puncture of the oolema was confirmed by visualization of cytoplasmic contents in the ICSI needle after suction and the spermatozoon was then deposited in the cytoplasm with a minimum of medium. Micromanipulator platform temperature was controlled in relation to ambient temperature in an attempt to perform all ICSI manipulations at 37°C.

Injected oocytes were washed twice in pre-equilibrated culture medium (DMEM-F12 [Sigma Aldrich] with 10% FBS and 25 μg/ml gentamicin)[12] to remove manipulation medium and were placed in 20-μl droplets of culture medium under oil (four to ten injected oocytes/droplet, i.e. 2-5μl per injected oocyte) in a 4-well dish, (Thermoscientific) at 38.3°C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$. Except when noted below, embryos were assessed for cleavage on Day 3 (Day 0 = Day of ICSI), at which point uncleaved oocytes were moved to a different droplet within the same dish. Medium in the original droplet was refreshed by adding 20 μl of fresh culture medium and then removing 20 μl. From Day 7 to Day 11, embryos were assessed for blastocyst development daily by visualization under an inverted microscope at 200X. Embryos were classified as blastocysts morphologically based on an increase in diameter from the previous day and a multicellular appearance with presence of an apparent uniform outer cell layer.

Transcervical embryo transfer

Embryos were loaded into 0.25 ml straws using pre-equilibrated culture medium, warmed to 38.3°C. A stainless steel gun with a disposable sheath (both IMV Technologies, France) was used for the transfer. Altrenogest (Regumate, MSD Animal health, Buckinghamshire, UK; 0.044 mg/kg bwt PO q 24 hr) was administered to each recipient mare beginning on the day of transfer. Pregnancy

diagnosis was first performed via transrectal ultrasonography on Day 14 (Day 0= Day of ICSI). If an embryonic vesicle was not present, examinations were repeated every 2-3 days until Day 20 at which point the mare was declared non- pregnant and altrenogest treatment was discontinued. If an embryonic vesicle was visualized on one of these examinations, examination was repeated on days 21, 28, 45, 60 and 90. Altrenogest treatment was continued until Day 120.

Oviductal embryo transfer

Transfer of embryos to the oviduct was performed by flank laparotomy under standing sedation as described previously,[24] with the following major modifications: 1) a 15-cm oblique incision was made in the paralumbar fossa contralateral to the site of ovulation, beginning just ventral to the cranioventral aspect of the tuber coxae and extending cranially and ventrally perpendicular to the last rib; 2) injected oocytes/embryos were loaded into a modified 6 F dog urinary catheter attached to a 1 ml all-plastic syringe with < 0.5 ml of culture medium for transfer.

Evaluation of oocytes and embryos for chromatin status

Oocytes or presumptive embryos were briefly fixed in buffered formal saline and mounted on a slide with 7 µl mounting medium (9:1 glycerol:PBS) containing 5 µg/ml Hoechst 33258 and examined by fluorescent microscopy (range 350-461nm) at 400X. Oocytes were classified as MII, MI, GV (having an intact nucleus) or degenerating (no chromatin or abnormal chromatin). Zygotes were evaluated for presence of two pronuclei and two polar bodies. Cleaved embryos were evaluated to determine the number and status of nuclei. Only nuclei that appeared to be normal were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation or fragmentation) were disregarded. Embryos were confirmed to be blastocysts if they contained more than 64 normal nuclei and showed arrangement of an outer rim of nuclei in a presumptive trophoblast layer.

*Preliminary study.* To establish oocyte collection and maturation procedures, ovaries were obtained from the abattoir and transported to the main laboratory for processing. After EH holding, oocytes were placed in culture in maturation medium as described above, then evaluated morphologically for presence of a polar body after 30 hr culture.

*Experiment one: the effect of oocyte source (abattoir vs. in vivo) on maturation, cleavage and blastocyst rates after ICSI.* Oocyte maturation rates recorded in the preliminary study were low. This study was conducted to determine whether the oocyte source affected maturation rates. Oocytes were obtained from abattoir-derived ovaries as described above, or from live mares via TVA. After IVM, the proportion of oocytes having polar bodies was recorded for each treatment, and these oocytes were then subjected to ICSI. Injected oocytes were cultured, and evaluated for blastocyst development from Day 7 to Day 11. Embryos classified morphologically as having developed to blastocyst were either transferred transcervically to the uterus of a recipient mare, or were stained for evaluation of chromatin status.

*Experiment two: effect of ovary transport time on maturation, cleavage and blastocyst rate after ICSI.* The maturation rate was higher with *in vivo*-derived oocytes in Experiment 1. It was hypothesized that the low maturation rates found in abattoir-derived oocytes in Experiments 1 and 2 might be related to the delay during transport of the ovaries to the laboratory. To test this hypothesis, abattoir-derived ovaries were divided into two treatments. Group 1: local facility (LOC) ovaries collected from mares slaughtered from 7 am to 1 pm were processed at a facility near the abattoir. Recovered oocytes were placed into EH medium as described for overnight holding above, then transported to the laboratory. Group 2: LAB, ovaries from mares slaughtered from 1-5 pm were collected and transported to the main laboratory for processing. Recovered oocytes were placed into EH medium for overnight holding.

After IVM and ICSI, injected oocytes were examined from Day 7 to 11 of culture for development to blastocyst, at which time all presumptive embryos were stained for evaluation of nuclear status. In one replicate, for the purpose of evaluating embryo viability, at 66 hr after ICSI, the embryos showing the greatest degree of development (uniform morphological cleavage and > six

cells) as assessed under light microscopy were transferred to the oviduct of a recipient mare on Day 1 after ovulation.

*Experiment three: correlation of embryo morphology with nuclear status.* Staining of embryos at Days 7 to 11 in Experiments 2 and 3 suggested that morphological classification was not accurately reflecting the nuclear status of embryos. To improve our understanding of the association between equine embryo morphology and nuclear status, a systematic, sequential embryo staining study was performed.

Oocytes recovered from abattoir-derived ovaries and processed at the main laboratory were matured *in vitro*, then subjected to ICSI and embryo culture as described above, with the modifications that 0.27mM pyruvate was added to the oocyte injection medium and the sperm preparation method was changed to direct swim up.[10]

Staining and assessment of chromatin status was performed at the following times: A) immediately after IVM in oocytes classified as MII on the basis of presence of a polar body; B) at 20 hours post-ICSI to evaluate pronuclear status; C) at Day 2, 3, 4, or 7 after ICSI to determine nucleus number and correlation of morphological cleavage classification with nucleus number; and D) after seven to 11 days of culture. In group D, embryos classified morphologically as blastocysts were stained and evaluated on the day they were identified, then all remaining presumptive embryos were stained and evaluated on Day 11 of culture. Two Day-9 presumptive blastocysts from this group were not stained but were transferred to the uterus of a recipient mare five days after ovulation to evaluate viability.

**Statistical analyses**

Differences in rates of oocyte maturation, cleavage, and blastocyst development were compared among groups by Chi-square analysis, with Fisher's Exact Test used between treatments when a value of less than ten was anticipated for any parameter.

**Results**

Preliminary study

Five replicates were performed, and 49 oocytes were recovered and subjected to IVM. The rate of maturation to MII was 39%.

Experiment one: effect of oocyte source (abattoir vs. in vivo) on maturation, cleavage and blastocyst rates after ICSI

Fifteen TVA procedures were performed, and 99 oocytes were recovered (Table 1). Three replicates were performed with abattoir-derived ovaries. Overall maturation rates were greater for oocytes obtained by TVA than for oocytes from abattoir-derived ovaries (67 % vs. 32%, respectively; $P < 0.01$). There was no significant difference between groups in morphological cleavage rate after ICSI (50 -53 %).

On Day 8 after ICSI, on visual appraisal, based on increased diameter and the apparent presence of a uniform outer cell layer, four embryos (two TVA and two abattoir), were considered to have developed to the blastocyst stage. Three of these were transferred transcervically (one single [abattoir]; one double [TVA]) to the uteri of two recipient mares six days after ovulation. No pregnancies resulted. The fourth presumed blastocyst was stained with Hoechst 33258; on evaluation, this proved to be a degenerated embryo.

Experiment two: effect of ovary transport time on maturation, cleavage and blastocyst rates after ICSI

Three replicates were performed (264 oocytes). The time from mare death to placement of oocytes in EH media for the LOC treatment ranged from 0.5- 4 hr (median 2.25 hr); in the LAB treatment this ranged from 5-10 hr (median 7.5 hr). Three metaphase II oocytes were lost during manipulation and not injected. There was no significant difference in oocyte maturation or cleavage rates between LOC and LAB treatments (Table 2).

Transfer of the four most advanced embryos at 66 hr culture in one replicate resulted in two embryonic vesicles (4 mm and 5 mm in diameter) detected by transrectal ultrasonography of the recipient mare on Day 17 after ICSI. These vesicles were lost by Day 24. None of the 61 cleaved

embryos cultured to Day 11 were classified as blastocysts morphologically. However, on evaluation after staining, one blastocyst was identified in this group based on the presence of > 64 cells and an organized outer layer of nuclei.

Experiment three: correlation of embryo morphology and nuclear status

Staining and evaluation of chromatin status was performed at sequential steps to validate morphological classification of oocyte and embryo status. In one replicate, 18 oocytes presumed to be in MII based on presence of a polar body were stained to confirm meiotic status; three were lost during manipulation and 14/15 (93%) were confirmed in MII (Fig. 1A). A total of 109 oocytes were subjected to ICSI. Over four replicates, the rate of pronuclear (PN) formation at 20 hr after ICSI was 43% (10/23 oocytes; Fig. 1B); a further eight injected oocytes were lost during processing. Over six replicates, 46 presumptive embryos were stained on Day 2, 3, 4, or 7 after ICSI. Of these, 31 (64%) demonstrated apparent morphological cleavage, but only eight of these (17%) had two or more normal nuclei on staining. In 3/46 embryos (6.5%), the number of nuclei matched the number of visible blastomeres and were appropriate for age (Fig. 2A); five embryos possessed two to six normal nuclei, but nucleus number was lower than expected for day.[16] The other 23 stained embryos that appeared cleaved morphologically possessed only degenerated nuclei or were completely anuclear (Fig. 2B).

In five replicates, presumptive embryos were cultured to determine blastocyst development (Table 3). Seven metaphase II oocytes were used in other studies and not injected. After culture for 7 to 11 days, 17 presumptive embryos were classified as having developed to the blastocyst stage based on morphological criteria. Of these, 15 were stained for nuclear evaluation and two were transferred to the uterus of a recipient mare (results below). Evaluation of the 15 presumptive blastocysts after staining revealed seven confirmed blastocysts (e.g. Fig. 3A1-3, Fig, 4) and eight degenerating embryos (e.g. Fig, 3C1-2). In these replicates, at Day 11 all remaining structures (not classified as blastocysts) were stained, and one of these was also revealed to be a blastocyst (Fig, 3D1-2). Notably, as uncleaved oocytes were placed in a separate droplet at Day 4 but were kept in culture, we could evaluate changes in these oocytes over time. Several known uncleaved oocytes increased in diameter on Day 9 (e.g. Fig. 3B1), which, on simple morphological evaluation, could have led to mistaken classification of these structures as blastocysts (Fig. 3B2-3).

Transcervical transfer of two Day-9 presumptive blastocysts in the final replicate, to the uterus of a recipient mare five days after ovulation resulted in two embryonic vesicles (3 mm and 10 mm in diameter) detected by transrectal ultrasonography on Day 14 after ICSI. The smaller vesicle was manually reduced at Day 16 and the remaining vesicle developed normally. A healthy colt foal was born at 345 days gestation (Fig. 5). Overall, in these five replicates, including the two transferred embryos, the rate of "blastocyst" development based on morphological assessment was 17/138 (12.3%); however, the confirmed blastocyst development per injected oocyte was 10/138 (7.2%).

**Discussion**

This study highlights factors that may affect the development of an effective equine ICSI program. We found an increased rate of meiotic maturation in oocytes derived from live mares by TVA compared to oocytes recovered post-mortem. The maturation rate of post-mortem derived oocytes was not affected by ovary transport time (up to 10 hr). Notably, when assessing embryo development in the first two experiments, we found discrepancies between our morphological classification of the embryos on light microscopy and the findings on staining for nuclear evaluation. This led us to evaluate the accuracy of our morphological classifications via step-wise oocyte and embryo staining in Exp. 3.

The rate of nuclear maturation in our preliminary study with abattoir-derived oocytes was low (39%). To determine if this was related to oocyte source, or to our untested IVM system, we compared maturation rates of TVA vs. abattoir-derived oocytes. This showed that with TVA oocytes the IVM system produced acceptable rates of maturation to MII (67%) whereas maturation remained low for oocytes from abattoir-derived oocytes, serving to validate the IVM system. We then evaluated whether the long transport time was associated with the low maturation rates in abattoir-derived oocytes; however, maturation remained low for oocytes collected after a median time of 2.25 hr or 7.5 hr. It is possible that changes in oocyte meiotic competence had already occurred within our

shortest handling duration (2.25 hr in the LOC group), thus lowering the maturation rates. A study by Guignot et al. also found no difference in maturation rates across transport times (1.5- 4 hr vs. 6-8 hr);[26] while Hinrichs et al. did find a higher maturation rate in oocytes collected < 1 hr of slaughter than in oocytes collected 5-9 hr after slaughter. Due to abattoir location and available processing facilities, the LOC group represented the most rapid processing time possible in this study. Of course, transport time is only one factor affecting rates of meiotic maturation and developmental competence in abattoir-derived ovaries, others including temperature of transport, mare age and health, and exposure of the ovaries to toxic compounds at the abattoir. Several studies have investigated optimal transport temperatures for equine ovaries and it was suggested that for periods < 1 hr, ovaries should be kept at 30-37°C but for longer periods, room temperature (22-24°C; as in this study) was more appropriate and resulted in the highest developmental competence.[27-29]

The final blastocyst rate (number of confirmed blastocysts per injected oocyte) achieved was 7.2%, which is similar to that reported by some established ICSI laboratories[6,8] but lower than optimal (20-40%).[3,10,30] All embryos in this study were produced by conventional ICSI. Historically, the first equine ICSI pregnancy was produced using conventional injection and surgical transfer of early embryos.[31] Some laboratories applied chemical oocyte activation due to low cleavage rates after ICSI,[1] although soon after, workers employing Piezo drill-assisted ICSI indicated that chemical activation was unnecessary.[19] On the basis of successes with the Piezo drill,[12,21,33] it has been hypothesized that this technique is an important aid to equine ICSI. In the only study in which Piezo-assisted and conventional (laser-assisted) ICSI were directly compared, no significant difference was recorded, however, blastocyst rates per injected oocyte were low for both methods (4.8% and. 5.1%, respectively).[6] The same authors have reported a live foal produced following conventional ICSI.[34] That study, and ours report blastocyst development at a much lower rate (7.4 % and 7.2% respectively) than do those that use Piezo-assisted ICSI (> 25%),[10,11,14] but clearly there are a myriad of other potential differences among these laboratories. There is a need for a systematic, prospective study within the same laboratory to establish whether Piezo-assisted ICSI offers a significant advantage over conventional ICSI. This is important, as the inclusion of the Piezo drill represents a significant additional investment for new laboratories and introduces an additional requirement for technical expertise.

Difficulties were experienced in correctly interpreting the morphology of cleaved embryos. Initially, we used a human grading system, which was shown not to be appropriate for application to equine embryos. The systematic staining of cleaved embryos revealed that only three of 31 embryos, empirically classified as morphologically cleaved, actually possessed the number of nuclei appropriate for age, and we found that many embryos contained anuclear blastomeres.[25] The significance of this latter finding is unclear. A recent study in humans reported that presence of anuclear blastomeres had no significant effect on birth rate.[35]

Blastocyst identification was also problematic. Even when interpretation was based on images and information from laboratories working in equine ICSI,[10,36] we still encountered discrepancies between morphological blastocyst appearance and nuclear status (Figure 3). It was noted that several known uncleaved oocytes increased in diameter between Days 7 and 9 after ICSI, and these were shown to have no nuclear chromatin upon staining (Figure 3B1-3). This phenomenon has not to the best of our knowledge been presented previously. Certain time-dependent cell-cycle and development stages can occur independently of nuclear status,[37] therefore it could be hypothesized that there are cytoplasmic developmental clock mechanisms driving oocyte expansion in the absence of cleavage. Our findings reinforced the importance of removing uncleaved oocytes to limit uncertainty in later assessments of blastocyst development, and of staining embryos for DNA to definitively establish blastocyst development. In practical terms, chromatin integrity cannot be evaluated in presumed blastocysts designated for transfer. Here, the benefits gained from coupling morphological assessments with definitive evaluations of nuclear status were invaluable. Morphology can be used to reliably select true blastocysts, as shown by the high initial pregnancy rate of IVP blastocysts after transfer reported by experienced laboratories (82/101, 82%;[30]; 9/13, 69%;[3]). In our case, morphological selection of two blastocysts in the final replicate of the study resulted in two embryonic vesicles.

To the best of our knowledge, this is the first report to document the disparity between morphological and histological assessment in equine IVP embryos. When setting up an ICSI

laboratory, it is tempting, as we did in Experiments 1 and 2, to transfer structures presumed to be embryos to recipient mares in an attempt to establish pregnancies and thus validate the program. However, in retrospect, this was counter-productive as evaluating morphology alone proved to be inaccurate and misleading. Our data and the related figures from Exp. 3 highlight the need for objective verification of embryo status. We hope that by sharing our experience of identifying discrepancies between morphological classification and actual embryo developmental stage, we have highlighted that morphology must be confirmed by a method such as nuclear staining when laboratories are initially evaluating their rate of blastocyst production.

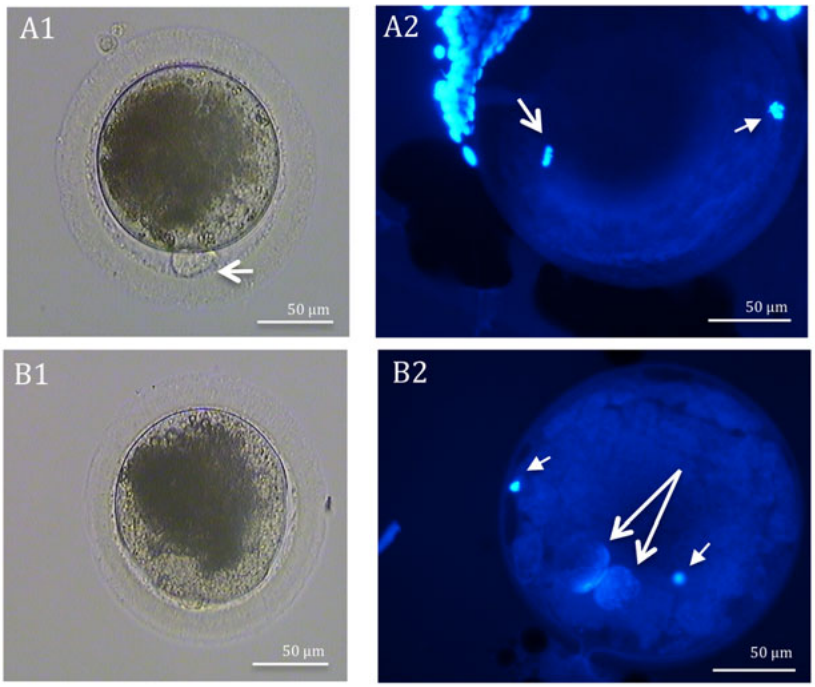


**Figure 1**: Photomicrographs of oocytes evaluated after IVM to validate MII status (A) and after ICSI to validate fertilization status (B). A1, B1: bright field microscopy 300X; A2, B2: after staining with Hoechst 33258, under fluorescence 400X. A1: MII oocyte with polar body (arrow); A2: metaphase plate (arrow) and polar body (closed arrow); B1: Injected oocyte 22 hours after ICSI B2: Fertilization confirmed by visualization of two pronuclei (staining incomplete; arrows) and two polar bodies (closed arrows).

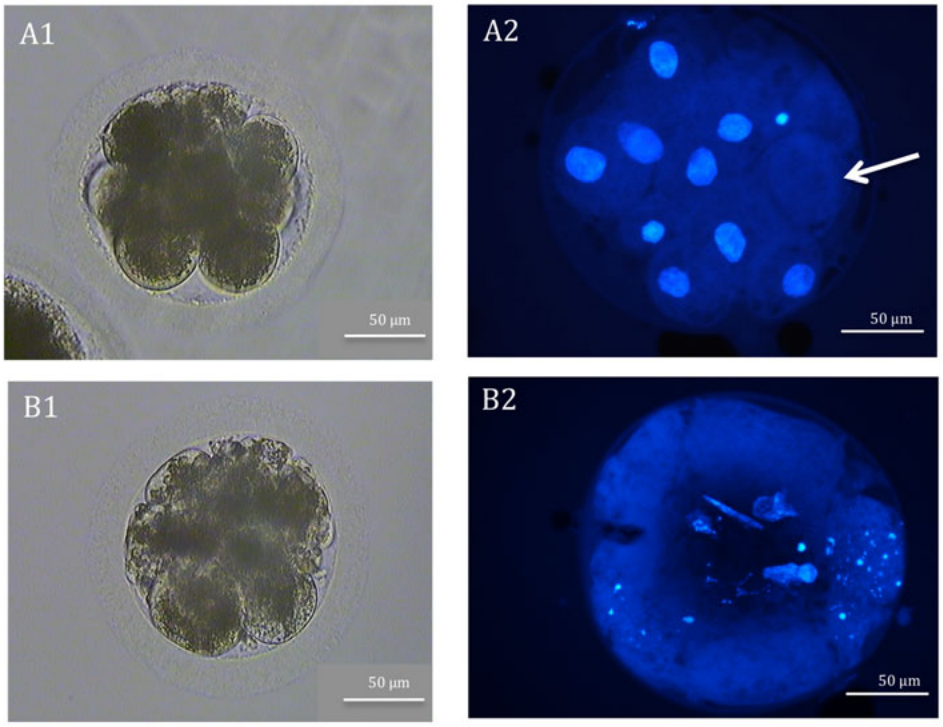


**Figure 2**: Photomicrographs of two embryos 96 hours after ICSI. A1, B1: bright field 300X; A2, B2 after staining with Hoechst 33258 400X. A1: apparent 10-cell embryo; A2: 9 normal nuclei and one anuclear blastomere (arrow); B1: apparent 8-cell embryo; B2: no normal nuclei.

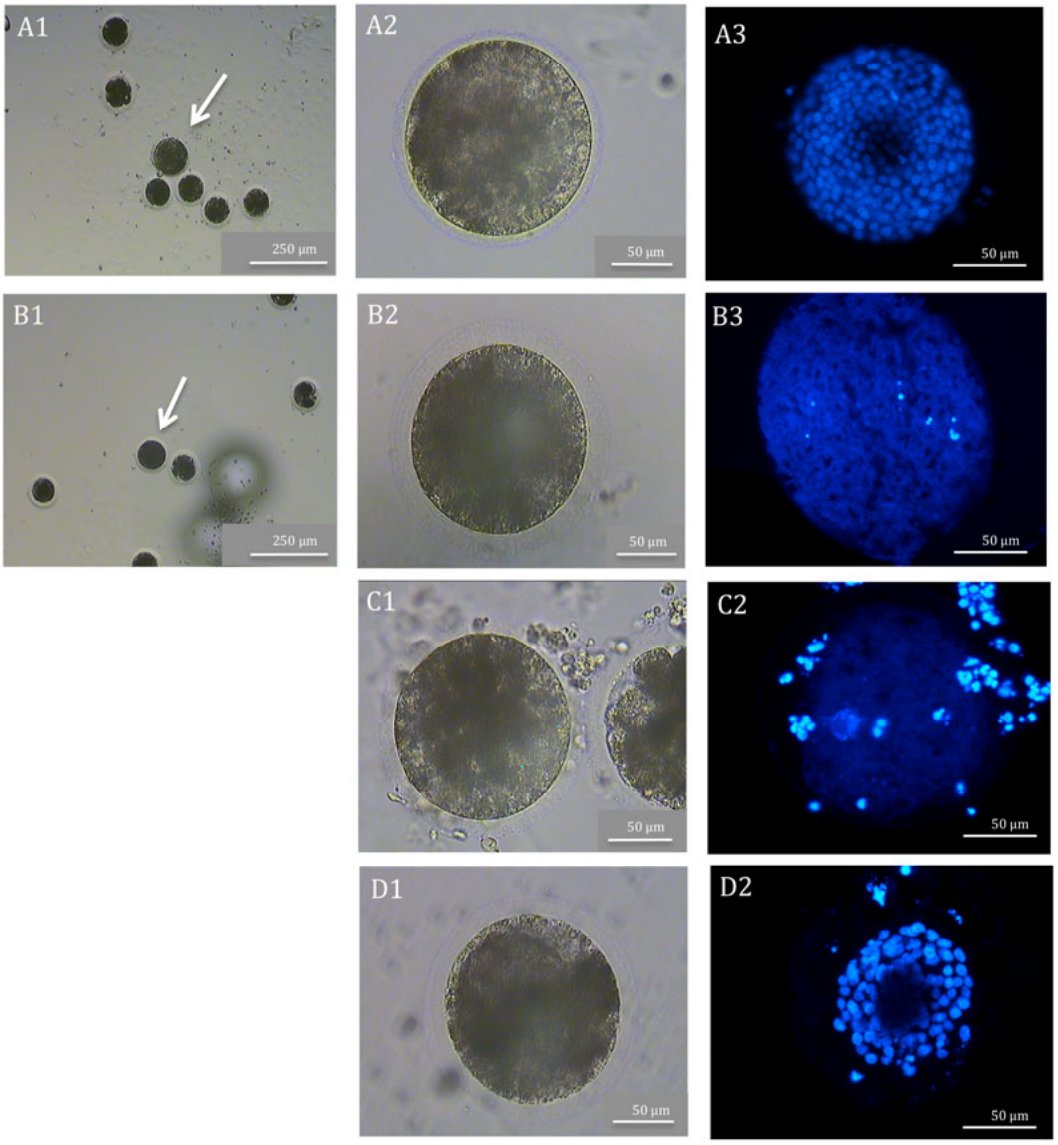


**Figure 3**: Photomicrographs of presumptive blastocysts at Day 9 after ICSI, showing discrepancies between morphological classification and nuclear status. A1 and B1: bright field images (60X) of presumptive embryos shown in panels A2-A3 and B2-B3, respectively, showing relative increase in diameter (arrows). B1-3; structure arising from uncleaved oocyte. Left: bright field (300X); right, after Hoechst 33258 staining (400X). A2: Presumed blastocyst shown in A1, with presumptive trophoblast layer (outer layer of organized cells) and thinning zona pellucida (ZP). A3: Status as blastocyst (presence of >64 cells with organized outer layer of nuclei) confirmed after staining. B2: structure pictured in B1, having similar morphology to blastocyst; B3: structure confirmed as a degenerating uncleaved oocyte, with no visible chromatin. C1: presumed blastocyst (from cleaved embryo) due to increase in size and thinning of ZP. C2: staining revealed structure to be degenerating. D1: presumed degenerating embryo (from cleaved embryo) due to irregular cytoplasmic outline and lack of size increase. D2: staining revealed structure to be blastocyst (penetration of the stain is incomplete).

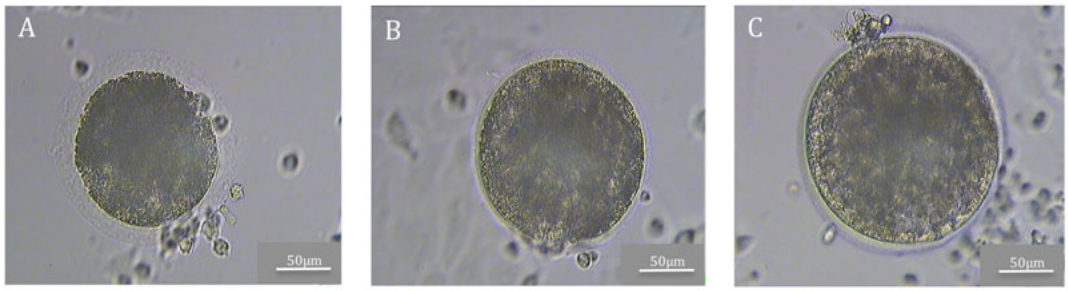


**Figure 4**: Photomicrographs (Bright field 300X) showing sequential development of a blastocyst on Days 7 through 9 (A-C) after ICSI (blastocyst status was subsequently confirmed by staining). An increase in diameter and in organization of the presumptive trophoblast layer can be seen.

**Figure 5:** Photograph of ICSI foal at 5 days old.

Editor's note: The photographs in this manuscript are available in color in the online edition of Clinical Theriogenology.

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Table 1: The effect of oocyte source (abattoir derived vs. TVA) on maturation, cleavage and blastocyst rates after ICSI. TVA= Transvaginal aspiration from live mares. * = Presumed status based on morphology. Day 0= day of ICSI. Mares were examined for pregnancy from Days 14-20.

| | Replicates *n* | Ovaries *n* | Oocytes *n* | MII *n* | Injected *n* | Cleaved* *n* | Blastocysts* *n* | Embryos transferred (n, time post ICSI) | Pregnancies *n* |
|---|---|---|---|---|---|---|---|---|---|
| Abattoir derived | 3 | 24 | 56 | 18 (32%)[a] | 18 | 9 (50%) | 2 | 1, Day 8 | 0 |
| TVA | 15 | 52 (26 mares) | 99 | 66 (67%)[b] | 66 | 35 (53%) | 2 | 2, Day 8 | 0 |

Table 2: Effect of ovary transport time on maturation, cleavage and blastocyst rate after ICSI. * = Presumed status based on morphology. Blastocyst rates based on confirmation by DNA staining with Hoechst 33258 or pregnancy.

| | Median time death- EH media (hr) | Ovaries *n* | Oocytes *n* | MII *n* | Injected *n* | Cleaved* *n* | Blastocysts *n* | Embryos transferred (n, time post ICSI) | Pregnancies *n* |
|---|---|---|---|---|---|---|---|---|---|
| Local facility (LOC) | 2.25 | 114 | 166 | 57(34%) | 57 | 40 (70%) | 0 | 4 (66hr) | 2 |
| Laboratory (LAB) | 7.5 | 86 | 98 | 41 (48%) | 38 | 25 (66%) | 1 | 0 | 0 |

Table 3: Results of Day 11 staining in experiment 3. Blastocyst rates based on confirmation by DNA staining with Hoechst 33258 or pregnancy.

| Ovaries *n* | Oocytes *n* | MII *n* | Injected *n* | Cleavage *n* | Morulae *n* | Presumed Blastocysts *n* | Confirmed Blastocysts *n* | Embryos transferred *n* | Day 14 Pregnancies *n* |
|---|---|---|---|---|---|---|---|---|---|
| 214 | 326 | 145 (44%) | 138 | 82 (59%) | 3 | 17 | 10 (7.2%) | 2 | 2 |