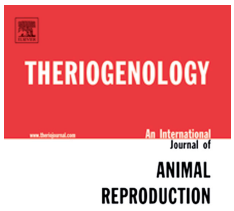

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Andrés Gambini, Adrián De Stéfano, Javier Jarazo, Carla Buemo, Florencia Karlanian, Daniel Felipe Salamone

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EMBRYO AGGREGATION DOES NOT IMPROVE THE DEVELOPMENT OF INTER-SPECIES SOMATIC CELL NUCLEAR TRANSFER EMBRYOS IN THE HORSE

Authors: Andrés Gambini[1, 2], Adrián De Stéfano[1], Javier Jarazo[1], Carla Buemo[1, 2], Florencia Karlanian[1], Daniel Felipe Salamone[1, 2, C]

[1]. Laboratory of Animal Biotechnology, Faculty of Agriculture, University of Buenos Aires, Buenos Aires, Argentina.

[2]. National Institute of Scientific and Technological Research, Buenos Aires, Argentina.

Authors emails:

gambini@agro.uba.ar; adrian_de_stefano@hotmail.com; javierjarazo@hotmail.com

carlabuemo@hotmail.com; florkarlanian@hotmail.com

[C] **Corresponding Author:** Salamone, Daniel Felipe. Tel.: +54 11 4524 8000; fax: +54 11 4514 8737. Email: salamone@agro.uba.ar. Postal Address: Av. San Martin 4453 (C1417DSE), Buenos Aires, Argentina.

Abstract

The low efficiency of Inter-Species Somatic Cell Nuclear Transfer (iSCNT) makes it necessary to investigate new strategies to improve embryonic developmental competence. Embryo aggregation has been successfully applied to improve cloning efficiency in mammals, but it remains unclear whether it could also be beneficial for iSCNT. In this study we first compared the effect of embryo aggregation over *in vitro* development and blastocyst quality of porcine, bovine and feline zona-free parthenogenetic (PA) embryos to test the effects of embryo aggregation on species that were later used as enucleated oocytes

donors in our iSCNT study. We then assessed whether embryo aggregation could improve the *in vitro* development of zona-free equine iSCNT embryos after re-construction with porcine, bovine, and feline ooplasm. Bovine and porcine aggregated PA blastocysts had significantly larger diameters compared to non-aggregated embryos. On the other hand, feline and bovine aggregated PA embryos had higher blastocyst cell number. Embryo aggregation of Equine-Equine SCNT was found to be beneficial for embryo development as we have previously reported but the aggregation of three Zona-Free Reconstructed Embryos (ZFREs) did not improved embryo developmental rates on iSCNT. *In vitro* embryo development of non-aggregated iSCNT was predominantly arrested around the stage when transcriptional activation of the embryonic genome is reported to start on the embryo of the donor species. Nevertheless, independent of embryo aggregation, equine blastocyst-like structures could be obtained in our study using domestic feline enucleated oocytes. Taken together, these results showed that embryo aggregation enhance *in vitro* parthenogenetic embryo development and embryo quality but effects vary depending on the species. Embryo aggregation also improves, as expected, the *in vitro* embryo development of Equine-Equine SCNT embryos, however we did not observe positive effects on equine iSCNT embryo development. Among oocytes from domestic animals tested in our study, the feline ooplasm might be the most appropriate recipient to partially allow preimplantation embryo development of iSCNT equine embryos.

Keywords

Embryo, Aggregation, SCNT, Equine, Parthenogenetic, Inter-species

1. Introduction

Differentiated somatic cells can be reprogrammed to the totipotent state when transferred into enucleated oocytes by means of somatic cell nuclear transfer (SCNT) [1]. Inter-species Somatic Cell Nuclear Transfer (iSCNT) is achieved by transferring a donor cell into the cytoplasm of an enucleated

oocyte from another different species/family/order/class. iSCNT can be utilized to investigate the interactions between the somatic cell and the ooplasm. Furthermore, iSCNT can be used to produce viable embryos, pregnancies and deliveries [2-8]. In recent years, iSCNT has been used to understand the mechanisms involved following the fusion of a somatic cell with an enucleated oocyte (reviewed by Long et al. [9]). Nevertheless, producing a viable embryo following activation of reconstructed cloned embryo remains a complex phenomenon.

Current stem cell and cell reprogramming research could benefit from employing iSCNT and using oocytes from species that are more accessible and abundant. In particular, iSCNT may facilitate obtaining pluripotent stem cells by producing inter-species cloned embryos [10, 11], as well as the study of mitochondrial/genomic DNA compatibility [12]. A few studies using iSCNT with mouse [15], cattle [16, 17] and sheep [5] enucleated oocytes fused with equine cells have reported low blastocysts rates. However, iSCNT remains an exciting tool for species with limited availability of oocytes, such as the horse, and for endangered species where assisted reproduction is needed. Moreover, the genus Equus has the unusual characteristic of being able to produce viable offspring when crossing individuals of different species, even with different phenotypic and karyotypic characteristics [13, 14]. This characteristic makes the equine an interesting model for iSCNT studies.

To overcome the low efficiency of iSCNT, it is necessary to investigate new strategies to improve its embryo developmental competence. Embryo aggregation has been successfully applied to improve cloning efficiency in several mammals [18-24], but it remains unclear whether it could also be beneficial for iSCNT. Therefore, we sought to evaluate the effects of embryo aggregation on the *in vitro* embryo development efficiency of iSCNT in the equine. We first investigated the effects of embryo aggregation on *in vitro* development and blastocyst quality of porcine, bovine and feline zona-free parthenogenetic (PA) embryos. We then tested if embryo aggregation improved *in vitro* development of equine cloned embryos generated by iSCNT with enucleated oocytes from porcine, bovine and domestic feline.

2. Materials and methods

2.1. Chemicals

Except otherwise indicated, all chemicals were obtained from Sigma Chemicals Company (St. Louis, MO, USA).

2.2. Experimental design

Two experiments were performed in our study. In experiment one, we produced non aggregated and aggregated porcine, bovine and feline zona-free parthenogenetic embryos to test the effects of embryo aggregation on the *in vitro* embryo development and blastocyst quality. In experiment two, tested species in experiment one were used to produce interspecific equine zona-free cloned embryos, and to evaluate the effects of embryo aggregation on the *in vitro* development. Additional, non-aggregated and aggregated homospecific zona free equine cloned embryos were produced as control.

In experiment one, *in vitro* matured zona-free oocytes from porcine, bovine and feline species were parthenogenetically activated. For each species, immediately after activation, Zona-Free Parthenogenetic Embryos (ZFPE) were culture in a microwell system, placing one (1x-non-aggregated) or three (3x-aggregated) ZFPE per microwell. Therefore, experimental groups for experiment one were: porcine: 1x (non-aggregated) and porcine 3x (aggregated); bovine: 1x (non-aggregated) and bovine 3x (aggregated), and feline: 1 x (non-aggregated) and feline 3 x (aggregated). *In vitro* culture was performed for eight days, and we measured blastocyst size and cell number (Fig.1).

In experiment two, *in vitro* matured zona-free oocytes from porcine, bovine, feline and equine species were enucleated by micromanipulation and then fused in all cases with equine skin fibroblasts to produce Zona-Free Reconstructed Embryos (ZFREs). For each species, immediately after activation, ZFREs were culture in a microwell system, placing one (1x-non-aggregated) or three (3x-aggregated) ZFREs per microwell. Therefore, experimental groups for experiment two were: Equine-Porcine: 1x (non-aggregated) and Equine-Porcine 3x (aggregated); Equine-Bovine: 1x (non-aggregated) and Equine-Bovine 3x (aggregated); Equine-Feline: 1x (non-aggregated) and Equine-Feline 3x (aggregated), and Equine-Equine: 1x (non-aggregated) and Equine-Equine 3x (aggregated). *In vitro* culture was performed for eight days, and some obtained blastocysts were used for *in vitro* embryo culture after Day 8 (Fig. 2).

2.3. Oocyte collection and *in vitro* maturation

2.3.1. Porcine

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory at around 25 to 30 ºC within 3 h of collection. Cumulus-oocyte complexes (COCs) from 3 to 6 mm follicle diameter were aspirated using an 18 gauge needle attached to a 10mL disposable syringe. Compact COCs were selected and matured in 100 µL droplets of tissue culture medium bicarbonate-buffered TCM-199 (31100-035; Gibco, Grand Island, NY, USA) under mineral oil (M8410), supplemented with 0.3 mM sodium pyruvate (P2256), 100 mM cysteamine (M9768), 5µg/mL *myo*-Inositol (I5125), 1 µg/mL insulin-transferrin-selenium (ITS; 51300-044, Gibco) 1% antibiotic-antimycotic (ATB; 15240-096, Gibco), 10% porcine follicular fluid (follicular fluid was obtained from follicles of 3 to 6 mm of diameter, centrifuged at 1900 X g for 30 min at 5 °C, filtered and then aliquoted and stored at -20 °C), 5 ng/mL basic Fibroblast Growth Factor (F3685) and 10 µg/mL of FSH (NIH-FSH-P1, Folltropin, Bioniche, Caufield

Junction Caufield North, Victoria, Australia). Maturation was performed at 38.5 ºC in a humidified atmosphere of 6.5% CO_2 in 90% air for 42 to 44 h.

2.3.2.Bovine

Cow ovaries were transported from a local slaughterhouse to the laboratory in a thermo container at 24 to 27°C. COCs were aspirated from follicles with a diameter of two to eight millimeters. Oocytes covered with at least three layers of granulosa cells were selected for maturation. The maturation medium was TCM-199, containing 2 mM glutamine (G8540), 10% fetal bovine serum (FBS; 10499-044, Gibco), 10 µg/mL FSH, 0.3 mM sodium pyruvate, 100 mM cysteamine, and 1% ATB. Groups of 25 COCs were *in vitro* matured in 100 µL droplets of maturation medium covered with mineral oil. Maturation conditions were 6.5% CO_2 in humidified air at 38.5 °C during 22 to 24 h.

2.3.3.Feline

Ovaries were recovered from queens subjected to ovariectomy and transported to the laboratory within 2 h. The COCs were released from follicles by repeatedly puncturing and scraping the ovaries. The maturation medium was TCM-199 containing 1 IU/mL hCG (Ovusyn, Syntex SA, Buenos Aires, Argentina), 10 ng/mL Equine chorionic gonadotropin (eCG, Novormon 5000, Syntex SA), 2.2 mM calcium lactate (L2000), 0.3 mM sodium pyruvate, 3 mg/mL BSA (A6003) and 1% ATB. *In vitro* maturation conditions were 6.5% CO_2 in humidified air at 38.5 °C for 24 hours.

2.3.4.*Equine*

Slaughterhouse ovaries were collected and transported to the laboratory within four to seven hours, at 26 to 28 °C. Equine oocyte recovery was performed by a combination of scraping and

washing of all visible follicles using a syringe filled with DMEM/Nutrient Mixture F-12 medium (DMEM/F12; D8062), supplemented with 20 IU mL-1 heparin (H3149). Oocytes were matured for 24 to 26 h in 100 µL microdrops of TCM-199 supplemented with 10% FBS, 2.5 µL/mL ITS, 1 mM sodium pyruvate, 100 mM cysteamine, 100 µg/mL of FSH and 1% ATB, under mineral oil. Maturation conditions were 6.5% CO_2 in humidified air at 38.5 °C.

2.4. Cumulus and zona pellucida removal

In all the species, cumulus cells were removed by vortexing them for 2 min in hyaluronidase solution [H4272; 1 mg/mL in Hepes-buffered Tyrodes medium containing albumin, lactate and pyruvate (TALP-H)]. Oocytes were observed under stereoscopic microscopy to confirm nuclear maturation by the presence of the first polar body. Zona pellucida of matured oocytes was removed by incubating oocytes for 3 to 6 min in 1.5 mg/mL pronase (P8811) in TALP-H on a warm plate. Zona-free matured oocytes (ZF- matured oocytes) were washed in TALP-H and placed in microdrops of Synthetic Oviductal Fluid (SOF) until activation for zona-free parthenogenetic embryos (ZFPEs) for experiment one, or until enucleation for SCNT or iSCNT embryos in experiment two.

2.5. Cell culture (experiment two only)

Only equine fibroblasts were used in this study, and were obtained from a skin biopsy of an Argentinean Criollo horse. Fibroblasts were cultured in Dulbecco´s modified Eagle´s Medium (DMEM; 11885, Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 1% ATB, and 1µL/mL ITS in 6.5% CO_2 in humidified air at 38.5 ºC. After the primary culture was established, fibroblasts were expanded, frozen in DMEM with 20% FBS and 10% DMSO, and stored in liquid nitrogen. Donor cells were induced into quiescence by being grown to 100% confluence for three to four days prior SCNT. Cells were trypsinized (25300, Gibco) before use and resuspended in DMEM with 10% FBS.

2.6. Oocyte enucleation (experiment two only)

Enucleation of all oocytes used for experiment two was performed as described by Gambini et al. [23]. Briefly, aspiration of the metaphase plate was performed in TALP-H containing 0.3 µg/mL of cytochalasin B (C6762) with a blunt pipette under UV light. Prior to enucleation, oocytes were incubated in a microdrop of SOF containing 1 µg/mL Hoechst bisbenzimide 33342 (H33342) for 5 to 10 min. Zona-free enucleated oocytes were kept in a SOF microdrop until nuclear transfer.

2.7. Cell fusion and embryo reconstruction (experiment two only)

Zona-free enucleated oocytes were individually washed for a few seconds in 50 µL drops of 1 mg/mL phytohemagglutinin (L8754) dissolved in TCM-Hepes, and then dropped over an equine donor cell resting on the bottom of a 100 µL TALP-H drop; consequently these two structures were attached. Formed cell couplets were washed in fusion medium [0.3 M mannitol (M9546), 0.1 mM $MgSO_4$ (M7506), 0.05 mM $CaCl_2$ (C7902), 1 mg/mL polyvinyl alcohol (P8136)], and then fused in a fusion chamber containing 2 mL of warm fusion medium. A double direct current pulse of 1.2 kV/cm V, each pulse for 30 µs, 0.1 s apart was utilized for fusion. Couplets were individually placed in a 10 µL drop of SOF medium supplemented with 2.5 % FBS and incubated under mineral oil, at 38.5 ºC in 5% CO_2 in air. Twenty minutes after the first round of fusion, non-fused couplets were re-fused. Fusion rates were recorded**.** Two hours after the first round of fusion, Zona-free Reconstructed Embryos (ZFREs) were subjected to activation.

2.8. Activation

ZF-matured oocytes or ZFREs derived from porcine, bovine, feline and equine was performed according to tested protocols for each species.

2.8.1.*Bovine and feline*

ZF-matured oocytes and Equine-Bovine and Equine-Feline ZFREs were activated in TALP-H containing 5 mM ionomycin (I24222; Invitrogen) for 4 min. After this initial activation, ZFREs were subsequently treated with 1.9 mM 6-dimethylaminopurine (6-DMAP, D2629) in SOF for 3 h.

2.8.2.*Porcine*

ZF-mature oocytes and Equine-Porcine ZFREs were electrically activated by a single direct current pulse of 1.2 kV/cm for 80 µs, followed by incubation for 3 h of 1.9 mM 6-DMAP in a 100µL drop of SOF medium. For Equine-Porcine ZFREs this was the second or third electrical pulse (one or two for cell-ooplasm fusion and one for activation).

2.8.3.*Equine*

For Equine-Equine ZFREs, chemical activation was achieved by a 4 min treatment in TALP-H containing 8.7 mM ionomycin followed by a 4 h individual culture in SOF supplemented with 1 mM 6-DMAP and 5 µg/mL cycloheximide (C7698). None PA embryos were produced with equine oocytes.

2.9. In vitro embryo culture until day 8 and embryo aggregation

In vitro culture of ZFPEs and ZFREs was carried out in microwells containing 50 µL microdrops of SOF medium under mineral oil. These microwells were produced using a heated glass capillary lightly pressed to the bottom of a 35 x 10 mm Petri dish [23]. Experimental groups were designed for each species according to the number of ZFPEs or ZFREs placed per each microwell immediately after activation (Fig. 1 and 2): Group 1x: one ZFPE or ZFRE per microwell (non-aggregated embryos), Group 3x: three ZFPEs or ZFREs per microwell. Culture conditions were 5% O_2, 5% CO_2 and 90% N_2 in a humidified atmosphere at 38.5 ˚C. Day 0 was considered the day of activation. A similar ratio of

embryo/culture medium was maintained for all experimental groups. Cleavage was assessed 72 h after activation, and rates of blastocyst formation and their diameter were recorded by a millimeter eyepiece at Day 8.

Additionally, some parthenogenetic blastocysts of each experimental group from experiment one were fixed at Day 8 in 4% formaldehyde (F1635) in Dulbecco phosphate buffered saline (DPBS; 14287-072; Gibco, Grand Island, NY) for 20 min, rinsed in DPBS with 0.4% BSA (A7906) for 20 min and then permeabilized in 0.1% Triton-X (T9284) in DPBS for 15 min. Then PA blastocysts were stained with 0.5% propidium iodide for 30 min at room temperature and mounted on a glass slide in 70% glycerol under a coverslip. Embryos were analyzed on a Nikon Confocal C.1 scanning laser microscope. Some iSCNT blastocysts from experiment two were stained with 1 µg/mL Hoechst for cell counting under the microscope.

2.10. In vitro embryo culture after day 8 (experiment two only)

Some Equine-Equine SCNT blastocysts from each experimental group were kept in *in vitro* culture from Day 8 until Day 16 to 17 unless they collapsed earlier. On Day 12, blastocysts were placed in 100 µl microdrop of DMEM/F12 medium containing 15% FBS and 1% ATB. Blastocyst diameter was measured every two days using a millimeter eyepiece. Additionally, cloned blastocysts from Equine-Feline experimental group were kept on *in vitro* culture to study embryo growth beyond Day 8. No blastocysts from Equine-Porcine or Equine-Bovine were obtained to perform this experiment.

2.11. Statistical analysis

Differences among treatments in each experiment were determined using GraphPad Prism software version five. Blastocyst rates were analyzed by Chi-square or Fisher's exact test. Mann-Whitney test was performed to analyze differences in embryo size and cell number among experimental groups. Significant differences were considered with a p value <0.05.

3. Results

3.1. Experiment one: In vitro embryo development of aggregated PA bovine, feline, and porcine embryos.

We assessed the *in vitro* embryo development to blastocyst stage of non-aggregated (1x experimental group) and aggregated (3x experimental group) zona-free bovine, feline, and porcine PA embryos to test the effects of embryo aggregation on the species that were later used as ooplasm donors for the iSCNT study. Furthermore, we analyzed the quality of obtained blastocysts through recording embryo size and cell number. Embryo aggregation at one cell stage improved the cleavage rates in porcine but not in bovine and feline. Although no differences were found in the number of blastocysts obtained per ZFPEs among groups, blastocyst rates per microwell were higher for aggregated groups in all the species (Table 1). We noticed that Bovine and porcine aggregated PA embryos were significantly larger compared to the non-aggregated embryos. Mann-Whitney test was performed to analyze differences in embryo size and cell number among experimental groups. Blastocyst diameter in microns: Experimental Group porcine 1x, 181.3 ± 51.21 (n = 12) vs. 3x, 256.7 ± 102.5 (n = 23); bovine 1x, 152 ± 38.67 (n = 20) vs. 3x, 201.3 ± 48.97 (n = 19); feline 1x, 228.8 ± 89.54 (n = 30) vs. 255.0 ± 117.7 (n = 31). In contrast, bovine and feline aggregated PA embryos had higher blastocyst cell number. Cell number: Experimental Group porcine 1x, 28.14 ± 9.04 (n = 7) vs. 3x, 49.57 ± 23.94 (n = 7); bovine 1x, 83.00 ± 41.83 (n = 13) vs. 3x, 129.2 ± 49.56 (n = 20); feline 1x, 115.2 ± 72.70 (n = 13) vs. 3x, 259.8 ± 130.6 (n = 19). Embryo aggregation improves *in vitro* embryo development and embryo quality but effects vary depending on the species (Fig. 3 and Fig. 4).

3.2. Experiment two: in vitro embryo development of aggregated SCNT and iSCNT cloned equine embryos.

To assess whether embryo aggregation could improve the efficiency of iSCNT in equine, a total of 250 Equine-Equine SCNT and 383 iSCNT ZFREs were produced and cultured *in vitro* for 8 days. The number of cells and the diameter of the iSCNT blastocyst and the *in vitro* embryo growth after Day 8 were recorded. We observed that equine fibroblasts fused better with porcine enucleated oocytes (Table 2). Additionally, cleavage, two to four cell, four to eight cell, eight to 16 cell, >16 cell and blastocyst rates per embryo (microwell) and per ZFRE were recorded for all experimental groups (Table 3). A significant improvement of blastocyst rates per embryo was observed in aggregated homospecific Equine-Equine SCNT embryos. Furthermore, aggregation did not involve the use of additional oocytes to obtain blastocysts, since no significant differences in blastocyst rate per ZFRE were observed in Equine-Equine Experimental Group. The aggregation of three ZFREs did not improve embryo developmental rates on iSCNT, and embryo developmental arrest varied depending on the species: 70% of Equine-Porcine iSCNT embryos arrested at two to four cell stage; 53% of Equine-Bovine iSCNT embryos and 58% of Equine-Feline iSCNT embryos arrested at four to eight cell stage. Intriguingly, three Equine-Feline iSCNT embryos reached blastocyst-like stage at Day 8 (Fig.5), and sizes in microns were: 100, 88 and 82. Equine-Equine SCNT blastocysts were able to grow *in vitro* when they were kept in culture media after Day 8. The numbers of embryos analyzed per group were: 1x, n = 5 and 3x, n = 4. Mean embryo sizes in microns per Day ± SD were: Day 8, 125.29 ± 22.68; Day 10, 358.97 ± 122.47; Day 12, 589.87 ± 214.36; Day 14, 1479.68 ± 477.57; and Day 16, 2143.33 ± 665.29. In contrast, *in vitro* embryo growth after Day 8 was not observed in iSCNT Equine-Feline blastocyst. Only two Equine-Feline iSCNT blastocyst-like structures were successfully processed for cell counting at Day 10, and cell number were: 33 and 27. These results showed that embryo aggregation improved embryo development in Equine-Equine SCNT embryos, but did not improve survival in iSCNT equine embryos.

4. Discussion

Embryo aggregation showed to be beneficial for PA zona-free preimplantation embryo development in porcine, bovine, and domestic feline. However, effects on embryo quality varied among these species. While embryo aggregation increased the PA blastocyst size in bovine and porcine but not in feline, blastocyst cell number was improved by aggregation in bovine and feline but not in porcine. Moreover, consistent with our previous reports [23, 24] we also observed positive effects of embryo aggregation in homospecific cloned embryos (SCNT Equine-Equine embryos). Conversely, the mentioned benefits of embryo aggregation did not improve the developmental competence of equine iSCNT embryos.

Parthenogenetic mammalian embryos have been used as a model to understand some of the mechanism involved in embryo development. In our study, parthenogenetic embryos were produced to study the effect of the aggregation in embryos with nuclear-cytoplasmic compatibility. Firstly, embryo aggregation was evaluated within each species and an improvement of the developmental competence to blastocyst stage was detected. A threefold increase was observed in blastocyst rates in 3x aggregated groups in all the analyzed species. Interestingly, embryo aggregation significantly improved blastocyst size in bovine and porcine blastocyst but not in the feline. In contrast, embryo aggregation improved PA blastocyst cell number in bovine and feline but not in the porcine. Cell number and blastocyst size were considered as two independent indicators of embryo quality. Blastocyst size is mostly correlated with the grade of blastocyst expansion. We observed that an increased in the blastocyst cell number not necessarily correlates to an increased ability of the blastocyst to expand or *vice versa.* Comparisons with previous reports are challenging when the timing of aggregation is different. Aggregation of 8 cells embryos improves inner cell mass cell number in PA bovine embryos [25]. However, a report in pigs showed that SCNT embryo aggregation also improves blastocyst cell number [26], but aggregation was performed at the four cell stage in this study. Taken together, these differences may reflect different mechanisms underlying embryo development according to the species and suggest that timing of aggregation may

affect the potential benefits of this technique. Studies focusing on establishing the best timing of aggregation are needed.

In vitro embryo development of non-aggregated equine iSCNT were predominantly arrested around the initiation of transcription activation of the embryonic genome (Zygote Genome Activation, ZGA) reported for the donor species. Indeed, this phenomenon is critical for a normal embryonic development, and the initiation of ZGA varies depending on the species: pigs in four cells [27], cattle in eight cells [28], feline four to eight cells [29] and equine four to eight cells stage [30]. The maternal to zygotic transition is concomitant with a dramatic reprogramming of gene expression and molecular events that depends on the recruitment and the degradation of maternal factors [31]. The incompatibility between donor cell and recipient oocyte during iSCNT could cause failures during these processes and affect embryo developmental competence. Therefore, failure to develop to blastocyst stage in some of our experimental groups could be due to nucleus-cytoplasm incompatibility. This idea is supported by previous studies where failures in reprogramming and ZGA appears to be one of the major problems associated with the low developmental rates observed in iSCNT embryos [32-37].

The results of embryo aggregation of Equine-Equine SCNT obtained in this study are consistent with our previous reports [23, 24] which suggest that embryo aggregation immediately after embryo activation is a repeatable and reliable technique. However, embryo aggregation showed no positive effect on *in vitro* embryo development of iSCNT embryos. The benefits observed for PA and Equine-Equine SCNT embryo aggregation in our study, in addition to the advantages reported for SCNT embryo aggregation in others mammals [18-24, 26, 38-40], suggest that embryo aggregation effects may be influenced by the nuclear-cytoplasmic compatibility within the embryo. Some of the reported effects of embryo aggregation are the increased number of available cells to achieve an embryo and a potential epigenetic complementation [18]. Up to date, only one study reported achieving development to blastocyst stage by producing Equine-Bovine iSCNT embryos. However, aggregation of two embryos did not improve embryo development in this study [15]. Recently, our laboratory reported that embryo

aggregation could improve the embryo development in Cheetah-Cat iSCNT embryos but there were no effects on pluripotent gene expression [40]. These observations suggest that embryo aggregation effects on iSCNT embryos could also depend on the species and the phylogenetic relationship between them.

Surprisingly, equine blastocyst-like structures (embryos compacted at Day 5 with a certain degree of expansion at Day 8) could be obtained in our study using domestic feline enucleated oocytes independent of embryo aggregation. It has been previously reported that the success of iSCNT embryo development depends on phylogenetic proximity [41]. Interestingly, horses are more phylogenetically related to cat than to cow or pigs. Horses (orders Perissodactyla) and cats (orders Carnivora) share a single evolutionary origin named “Pegasoferae” [42], and coincidentally they have a similar timing of ZGA. Hence, those might be some of the reasons why domestic feline enucleated oocytes showed to be a better recipient for an equine genome compared to bovine and porcine. Nevertheless, phylogenetically distant species can also sustain embryonic development during iSCNT [43, 44]. Equine iSCNT was previously reported by some authors who used bovine oocytes [5, 15-17] and mouse [15]. Up to date, only one study reported obtaining equine blastocyst stages by using bovine enucleated oocytes [15]. In our study we observed that enucleated domestic cat oocytes can partially support *in vitro* development of equine cloned embryos until compaction or early blastocyst-like stages. However, the failure to grow *in vitro* after Day 8 in addition to the low number of cells and the smaller size compared Equine-Equine SCNT blastocysts, are indicators of the low embryo quality of Equine-Feline iSCNT obtained embryos.

5. Conclusion

In summary, our results showed that embryo aggregation improved the *in vitro* embryo development and embryo quality of porcine, bovine and feline parthenogenetic embryos but effects differed according the species. Aggregated Equine-Equine SCNT embryos showed higher blastocyst rates compared to non-aggregated embryos; however, no positive effects of embryo aggregation were observed on iSCNT embryo development. We detected a developmental arrest of iSCNT embryos around the

estimated time of major ZGA. Finally, among enucleated oocytes tested in our study, the feline might be the most appropriate to partially allow *in vitro* preimplantation development of equine iSCNT embryos.

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8. Figure Legends

Figure 1: Schematic illustration of our experimental design for zona-free aggregated parthenogenetic embryo production (experiment one). IVM, *In vitro* Maturation; ZP, Zona Pellucida; ZF, Zona-Free; ZFPA, Zona-Free Parthenogenetic Embryo; 1x, non-aggregated experimental group; 3x, aggregated experimental group.

Figure 2: Schematic illustration of our experimental design for zona-Free SCNT and iSCNT equine aggregated embryo production (experiment two). SCNT, Somatic Cell Nuclear Transfer; iSCNT, Interspecific Somatic Cell Nuclear Transfer; IVM, *In vitro* Maturation; ZP, Zona Pellucida; ZF, zona-free; ZFRE, Zona-Free Reconstructed embryo; 1x, non-aggregated experimental group; 3x, aggregated experimental group.

Figure 3: Effects of parthenogenetic embryo aggregation on porcine, bovine, and feline blastocyst quality. Graphs plot Mean with SEM. **(A)** Non-aggregated and aggregated blastocyst diameter at Day 8 from all experimental groups. **(B)** Non-aggregated and aggregated blastocyst cell number at Day 8 from all experimental groups. * Statistically different (Mann-Whitney test, $p < 0.05$).

Figure 4: Images of porcine, bovine and feline Day 8 aggregated and non-aggregated blastocyst stained with propidium iodide. (A) Non-aggregated (1x) zona-free parthenogenetic bovine blastocyst. **(B)** Aggregated (3x) zona-free parthenogenetic bovine blastocyst. **(C)** Non-Aggregated (1x) zona-free parthenogenetic porcine blastocyst. **(D)** Aggregated (3x) zona-free parthenogenetic porcine blastocyst. **(E)** Non-Aggregated (1x) zona-free parthenogenetic feline blastocyst. **(F)** Aggregated (3x) zona-free parthenogenetic feline blastocyst. Scale bars = 50 μm.

Figure 5: Zona-Free SCNT and iSCNT equine embryos. (A) Day 8 aggregated SCNT equine blastocyst (165μm diameter). **(B)** Day 8 aggregated Equine-Feline iSCNT blastocyst-like structure (100 μm). **(C)** Day 5 non-aggregated Equine-Bovine iSCNT from 1x experimental group. Note the inability to compact of Equine-Bovine cloned embryos at Day 5.

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Table 1: *In vitro* embryo development of aggregated zona-free PA bovine, feline and porcine embryos until Day 8.

Experimental Groups		No. of ZFPEs	No. of embryos (microwells)	No. of cleaved ZFPEs (%)	No. of Blastocyst	% of blastocyst per microwell	% of blastocyst per ZFPEs
Porcine	1x	178	178	117 (65.73)[a]	23	12.92[a]	12.92
	3x	291	97	250 (85.91)[b]	31	31.96[b]	10.66
Bovine	1x	113	113	108 (95.57)[a]	21	18.58[a]	18.58
	3x	105	35	96 (91.42)[a]	20	57.14[b]	19.04
Feline	1x	104	104	61 (58.65)[a]	15	14.42[a]	14.42
	3x	111	37	85 (76.57)[a]	23	62.16[b]	20.72

[a, b] Values within species in same column with different superscript indicate significant differences at $P<0.05$(Fisher's exact test).

Abbreviation: PA, Parthenogenetic; ZFPEs, Zona-free parthenogenetic embryos.

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Table 2: Fusion rates of the first round of fusion between equine skin fibroblasts and enucleated oocytes from porcine, bovine, feline, and equine species.

Experimental Group (Cell-Ooplasm)	No. of couplets	No. of fused couplets (%)
Equine-Porcine	177	147 (83)[a]
Equine-Bovine	118	71 (60)[b]
Equine-Feline	87	59 (67.8)[b]
Equine-Equine	200	152 (76)[ab]
Total	**582**	**429 (73.7)**

[a,b] Values with different superscripts in a column indicate significant differences at P<0.05 (Chi square test).

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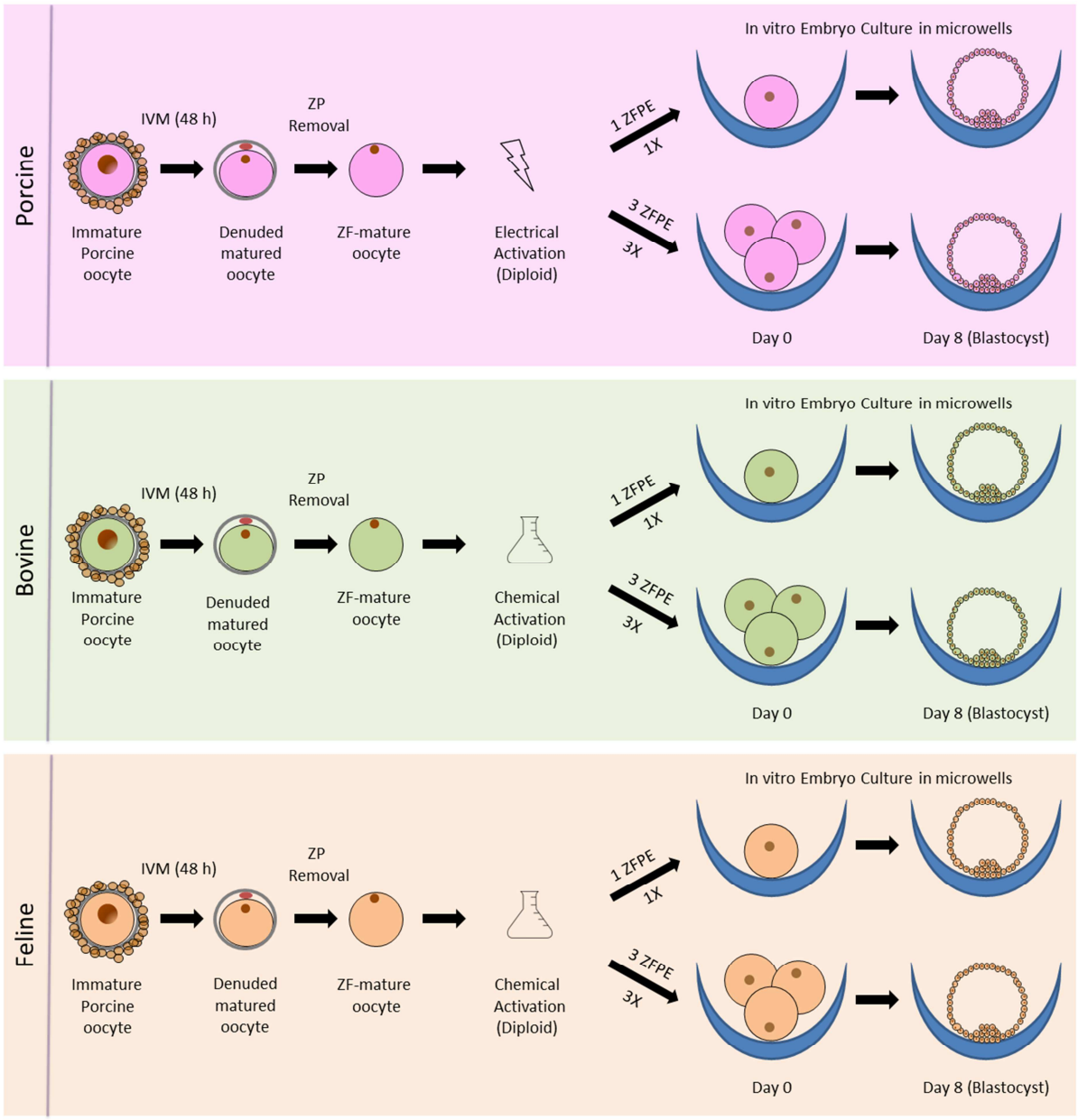
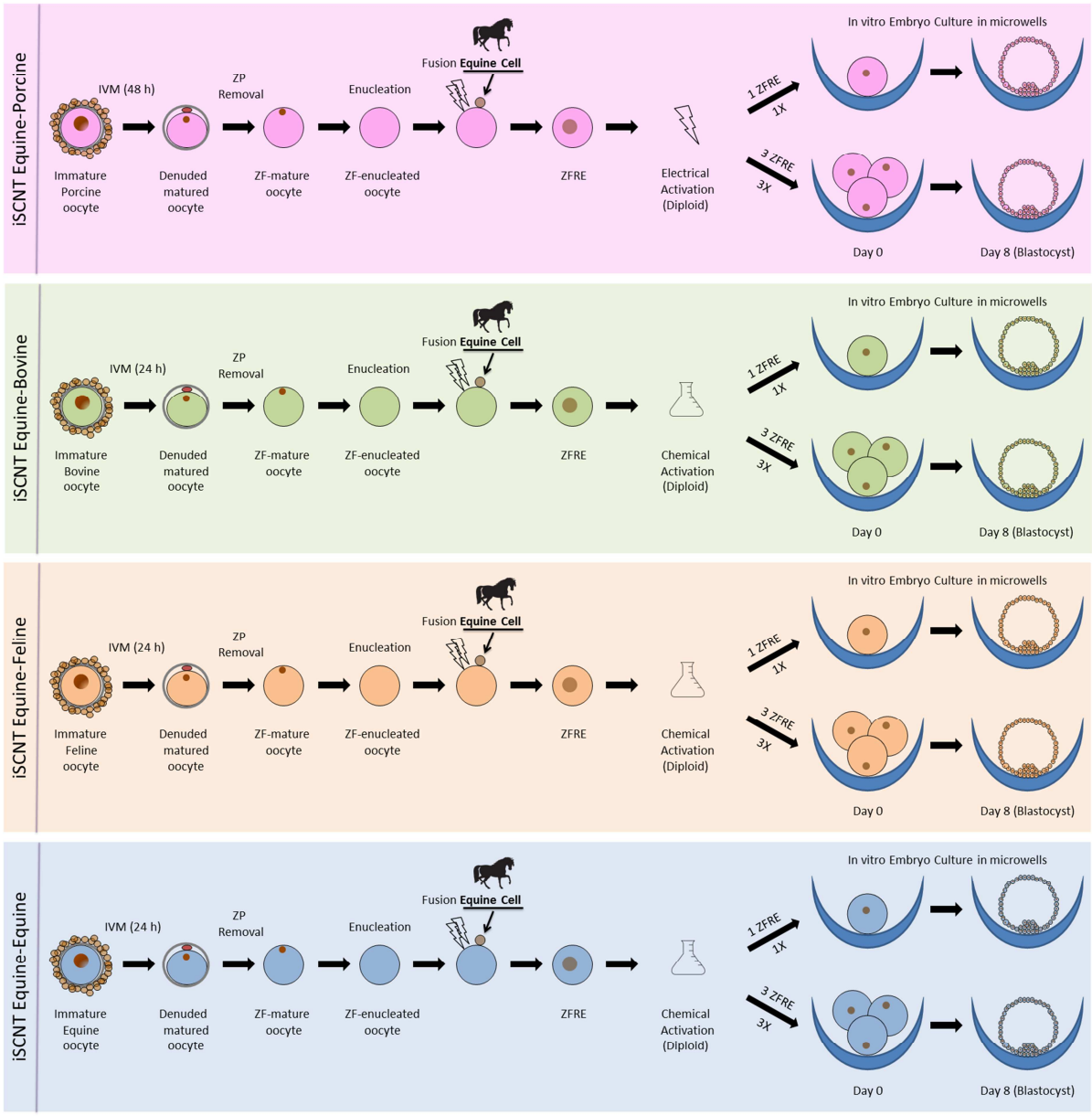
Table 3: *In vitro* embryo development of aggregated equine SCNT and iSCNT embryos using porcine, bovine and feline enucleated oocytes until Day 8.

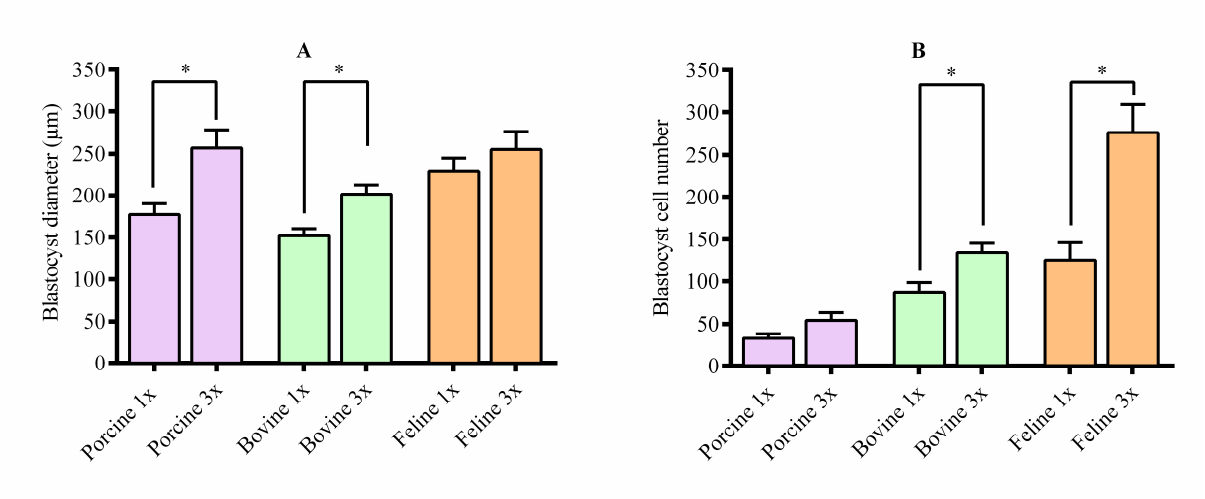
Experimental Groups (Cell-Ooplasm)		No. of ZFREs	No. of embryos (microwell)	No. of cleaved embryos (%)	Embryo development stages						
					2 to 4 cells (%)	4 to 8 cells (%)	8 to 16 cells (%)	≥ 16 cells (%)	No. of blastocysts	% Blastocyst per ZFREs	% Blastocyst per cleaved microwell
Equine-Porcine	1x	64	64	43 (67)^{bd}	30 (69.76)^{a}	15 (34.88)^{a}	2 (4.65)^{a}	1(2.32)^{a}	0	0^{a}	0^{a}
	3x	87	29	27 (93.10)^{ae}	0^{b}	7 (25.92)^{a}	10 (37.03)^{b}	10 (37.03)^{a}	0	0^{a}	0^{a}
Equine-Bovine	1x	49	49	38 (77.55)^{be}	8 (21.05)^{c}	20 (52.63)^{c}	10 (26.31)^{b}	0^{a}	0	0^{a}	0^{a}
	3x	54	18	17 (94.44)^{ac}	0^{bc}	2 (11.76)^{a}	6 (35.29)^{b}	9(52.94)^{a}	0	0^{a}	0^{a}
Equine-Feline	1x	57	57	48 (84.21)^{acd}	7 (14.58)^{cd}	28 (58.33)^{b}	8 (16.66)^{b}	3 (6.25)^{a}	2	3.51	4.16^{a}
	3x	72	24	24 (100)^{a}	0^{bd}	7 (29.16)^{a}	10 (41.66)^{b}	6 (25.00)^{a}	1	1.38	4.16^{a}
Equine-Equine	1x	145	145	115 (79.31)^{bcde}	N/R	N/R	N/R	N/R	8	5.52	6.95^{a}
	3x	105	35	34 (97)^{a}	N/R	N/R	N/R	N/R	12	11.42^{b}	35.29^{b}

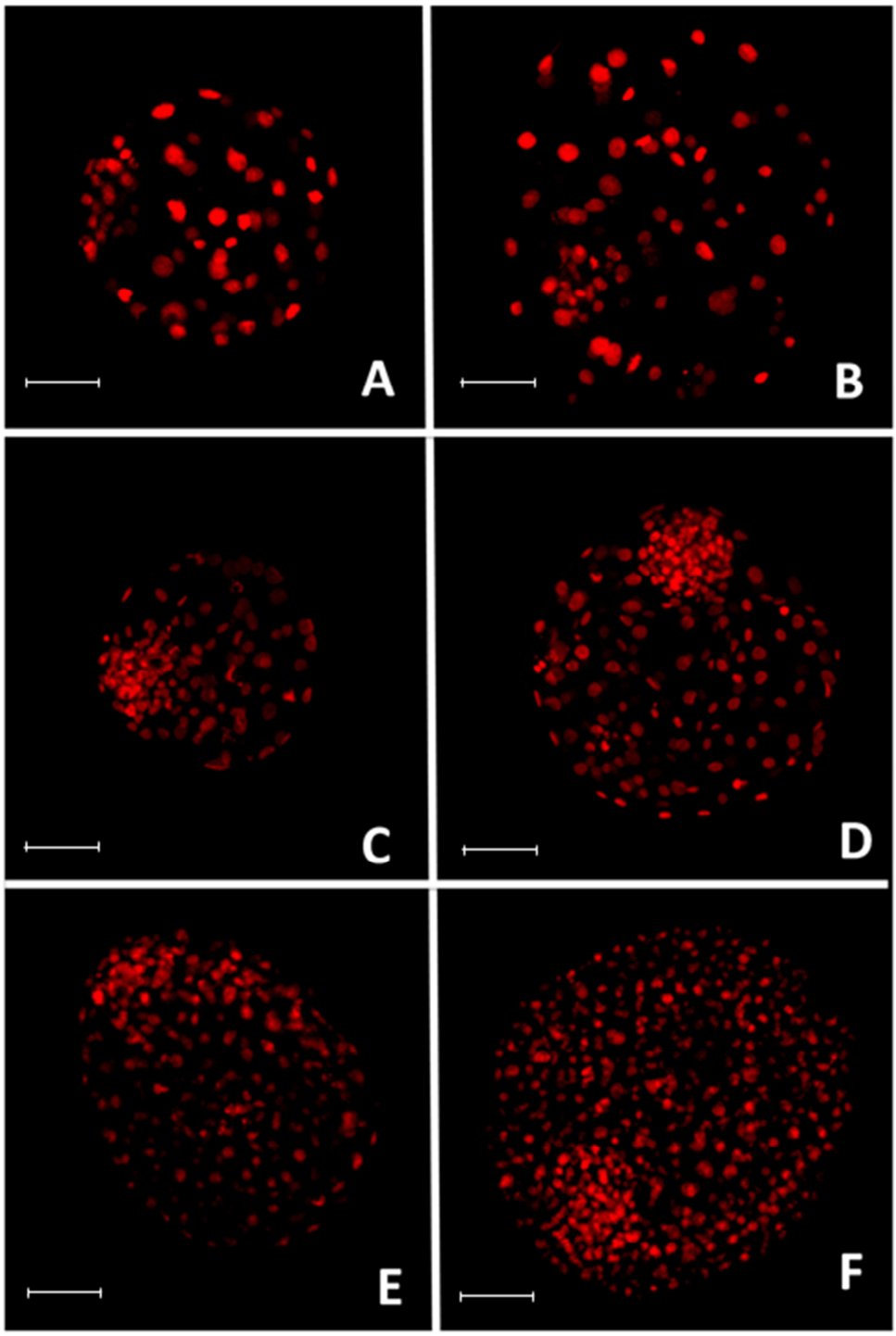
$^{a, b, c, d, e}$ Values with different superscripts in a column indicate significant differences at P<0.05 (Fisher's exact test).

Abbreviation: SCNT, Somatic Cell Nuclear Transfer; iSCNT, Inter-Species Somatic Cell Nuclear Transfer; ZFREs, Zona-Free Reconstructed Embryos; N/R, Not recorder.


Porcine
IVM (48 h)
ZP Removal
Immature Porcine oocyte
Denuded matured oocyte
ZF-mature oocyte
Electrical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFPE
1X
3 ZFPE
3X
Day 0
Day 8 (Blastocyst)
Bovine
IVM (48 h)
ZP Removal
Immature Porcine oocyte
Denuded matured oocyte
ZF-mature oocyte
Chemical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFPE
1X
3 ZFPE
3X
Day 0
Day 8 (Blastocyst)
Feline
IVM (48 h)
ZP Removal
Immature Porcine oocyte
Denuded matured oocyte
ZF-mature oocyte
Chemical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFPE
1X
3 ZFPE
3X
Day 0
Day 8 (Blastocyst)


iSCNT Equine-Porcine
IVM (48 h)
ZP Removal
Enucleation
Fusion Equine Cell
Immature Porcine oocyte
Denuded matured oocyte
ZF-mature oocyte
ZF-enucleated oocyte
ZFRE
Electrical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFRE
1X
3 ZFRE
3X
Day 0
Day 8 (Blastocyst)
iSCNT Equine-Bovine
IVM (24 h)
ZP Removal
Enucleation
Fusion Equine Cell
Immature Bovine oocyte
Denuded matured oocyte
ZF-mature oocyte
ZF-enucleated oocyte
ZFRE
Chemical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFRE
1X
3 ZFRE
3X
Day 0
Day 8 (Blastocyst)
iSCNT Equine-Feline
IVM (24 h)
ZP Removal
Enucleation
Fusion Equine Cell
Immature Feline oocyte
Denuded matured oocyte
ZF-mature oocyte
ZF-enucleated oocyte
ZFRE
Chemical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFRE
1X
3 ZFRE
3X
Day 0
Day 8 (Blastocyst)
iSCNT Equine-Equine
IVM (24 h)
ZP Removal
Enucleation
Fusion Equine Cell
Immature Equine oocyte
Denuded matured oocyte
ZF-mature oocyte
ZF-enucleated oocyte
ZFRE
Chemical Activation (Diploid)
In vitro Embryo Culture in microwells
1 ZFRE
1X
3 ZFRE
3X
Day 0
Day 8 (Blastocyst)

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