REPRODUCTION
RESEARCH

Aggregation of *Leopardus geoffroyi* hybrid embryos with domestic cat tetraploid blastomeres

Matteo Duque Rodriguez[1,2], Andrés Gambini[1,3], Laura D Ratner[1,2], Adrian J Sestelo[4], Olinda Briski[1,2], Cynthia Gutnisky[2,6], Susana B Rulli[5], Rafael Fernández Martin[1,2], Pablo Cetica[2,6] and Daniel F Salamone[1,2]

[1]*Facultad de Agronomía, Departamento de Producción Animal, Laboratorio Biotecnología Animal (LabBA), Universidad de Buenos Aires, Buenos Aires, Argentina,* [2]*Instituto de Investigaciones en Producción Animal (INPA), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina,* [3]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz, Ciudad Autónoma de Buenos Aires, Argentina,* [4]*Ecoparque Interactivo, Secretaría de Ambiente de la Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina,* [5]*Instituto de Biología y Medicina Experimental- Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina and* [6]*Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal (INITRA), Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina*

Correspondence should be addressed to D F Salamone; Email: salamone@agro.uba.ar

Abstract

Heterospecific embryo transfer of an endangered species has been carried out using recipients from related domestic females. Aggregation of an embryo from an endangered species with a tetraploid embryo from the species to be transferred could improve the development of pregnancy to term. The main objective of the present study was to analyze embryo aggregation in domestic cat model using hybrid embryos. For this purpose, we compared *in vitro* development of synchronic (Sync) or asynchronic (Async) and asynchronic with a tetraploid (Async4n) aggregation of domestic cat IVF embryos. Furthermore, aggregated blastocyst quality was analyzed by evaluation of the total cell number, cell allocation by mitotrackers staining of embryonic cells, expression of *Oct4*, *Nanog*, *Sox2*, *Cdx2* genes, number of OCT4+ nuclei, and presence of DNA fragmentation. Additionally, the developmental rates of Async4n aggregation of domestic cat with *Leopardus geoffroyi* hybrid (*hLg*) embryos were evaluated. Async aggregation increased blastocyst cell number and the number of OCT4+ nuclei as compared to non-aggregated diploid (2n) and tetraploid (4n) embryos. Moreover, blastocysts produced by Async4n aggregation showed reduced rates of fragmented DNA. No differences were found in the expression of the pluripotent genes, with exception of the *Cdx2* expression, which was higher in 4n and aggregated embryos as compared to the control group. Interestingly, hybrids embryos derived by Async4n aggregation with domestic cat embryos had similar rates of blastocysts development as the control. Altogether, the findings support the use of two-cell-fused embryos to generate tetraploid blastomeres and demonstrate that Async4n aggregation generates good quality embryos.

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Introduction

Efforts for the preservation of wild felids are needed for the maintenance of animal population and genetic variability among individuals. In addition to the natural breeding, conservation programs can be enhanced potentially by using assisted reproductive technologies (ART) to achieve optimal genetic management and to overcome infertility issues (Pukazhenthi 2016, Comizzoli & Holt 2019). Among ART, cryopreservation of gametes combined with *in vitro* embryo production, such as *in vitro* fertilization (IVF), are plausibly powerful tools for rescuing endangered animals or preserving genetics of critically endangered species. However, the lack of gametes has been one of the main factors to explain why the knowledge of *in vitro* embryo production in felid species has grown at slower rate over the last decades (Gómez *et al.* 2004, 2009).

To sort out these problems, the domestic cat (*Felis catus, Fc*) has been used as a model to develop reproductive technologies for the preservation of wild endangered felids (O'Brien *et al.* 2002). Moreover, domestic cat oocytes can be fertilized by wide range of species and can be used as a recipient for heterospecific embryo transfer of small wild felids species (Gómez *et al.* 2004). However, maternal–fetal incompatibility has been one of the limitations for successful offspring production, especially if the species is phylogenetically distant (Gómez *et al.* 2009). The aggregation of a feline endangered embryo with tetraploid blastomeres from

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domestic cats to generate chimeric embryos could improve the development to term after transfer of embryos to heterospecific recipients.

Tetraploid (4n-cells) complementation contributes to extra-embryonic tissue and reduces maternal–fetal failures during implantation and placentation by improving placenta compatibility (Lin *et al.* 2011, Curnow *et al.* 2000). Tetraploid complementation has been used to study pluripotency and developmental capacity in mice by injecting stem cells or induced pluripotent stem cells (iPS cells) into a tetraploid blastocyst. As a result, a viable live-born animal derived entirely from those pluripotent cells were obtained (Zhao *et al.* 2009). Moreover, it has been demonstrated that the more developmentally advanced blastomeres contributed disproportionately or entirely to the inner cell mass (ICM) when they are aggregated to embryos at earlier cleavage stages (asynchronic aggregation) (Schramm & Paprocki 2004, Hiriart *et al.* 2013).

Hybrids can represent a powerful tool to generate knowledge about *in vitro* development since it has half of the genetic material of the endangered species (Yin *et al.* 2006). Hybridization between felid species is well known and healthy offspring by interbreeding occurs in several species, including *Prionailurus bengalensis* (Leopard cat) and *Felis silvestris lybica* (African wild cat) (Yin *et al.* 2006, Gómez *et al.* 2009). (Moro *et al.* 2014) demonstrated, using intracytoplasmic sperm injection (ICSI), that cat oocytes can hybridize and develop till the blastocyst stage, even with a distantly related species (leopard, *Panthera pardus*). A South American species listed as near threatened is *Leopardus geoffroyi*, but the lack of availability of homologous embryos limits its study. However, experiments with hybrid embryos of *Leopardus geoffroyi* and domestic cat (hLg) could help to establish the basic knowledge needed to do successful heterospecific tetraploid aggregation. The three objectives of the present study were to (1) evaluate different electrofusion conditions to generate tetraploid IVF embryos, (2) compare *in vitro* development, cell allocation, total cell number, *Oct4,* Nanog, *Sox2*, *Cdx2* gene expression, and presence of OCT4 and DNA fragmentation in blastocysts generated by different aggregation strategies (synchronic, asynchronic and asynchronic with tetraploid), and (3) as a proof of concept, to evaluate *in vitro* development of hLg embryos generated by asynchronic with tetraploid aggregation.

Materials and methods

Experimental design

Experiment 1 Generation of *tetraploid blastomeres* by *electrofusion* of two-cell IVF domestic cat embryos

After 24 h of IVF, two-cell embryos were subjected to electrofusion with different electric pulse voltages. Embryos were fused and exposed to the pulse but non-fused embryos (Exp-*p*/nf) were cultured *in vitro*. Fusion was determined 24 h after the pulse and blastocyst rate was evaluated on day 8. Embryos with the highest fusion rates and developmental competence were selected for karyotype analysis at the 4–8 cell stage. Four biological replicates were performed for this experiment.

Experiment 2 Async4n aggregation of domestic cat embryos

Four-cell embryos were aggregated with a two-cell tetraploid IVF embryos (two-cell fused embryos) (Async4n). This experimental group was compared to four-cell diploid embryo aggregated with a four-cell diploid embryo (Sync) and four-cell diploid embryo aggregated with a two-cell diploid embryo (Async) (Fig. 1). Embryo aggregation was determined after 48 h. Blastocyst rate, total cell number and mitotracker distribution were evaluated at day 8 in all experimental groups. Additionally, for examining presence of OCT4 protein, DNA fragmentation and gene expression, two additional groups were included (non-aggregated 2n and 4n blastocysts) and Sync aggregation were excluded. Blastocysts were either fixed for immunofluorescence and TUNEL or stored later in RNA at −20°C for gene expression. Three biological replicates were performed for this experiment.

Experiment 3 Async4n aggregation of domestic cat embryos with *Leopardus geoffroyi* hybrid embryos

Finally, as a proof of concept for interspecific tetraploid complementation, *in vitro* matured *Fc* oocytes were co-incubated with *Leopardus geoffroyi* (Lg) and *Felis catus* (*Fc* control) spermatozoa to produce hLg and *Fc* embryos, respectively. Then four-cell diploid hLg embryos were Async4n aggregated with two-cell tetraploid *Fc* IVF embryos (hLgAsync4n). This experimental group was compared to four-cell diploid *Fc* embryo Async4n aggregated with two-cell tetraploid *Fc* IVF embryos (Async4n) (Fig. 1). For both the experimental and control group, aggregation was determined after 48 h and blastocyst rate was evaluated on day 8. Three biological replicates were performed for this experiment.

Reagents

Except as otherwise indicated, all chemicals were obtained from Sigma Aldrich Chemical Company. Media was prepared weekly and filtered through 0.22 mm pores (#4192 Acrodisc; Pall Corp., Ann Arbor, MI, USA) into sterile tubes.

Ethics and animal welfare statement

Animal procedures were approved by the 'Comité institucional de cuidado y uso de animales de laboratorio of Universidad de Buenos Aires' (CICUAL, protocol number 2017/6). The castrations were carried out in centers or veterinary clinics by authorized veterinarians that follow the animal welfare standards. Sperm of *Leopardus geoffroyi* were collected during the routine veterinary evaluation of the specimens. Animals were housed in zoological institutions distributed throughout Argentina, working

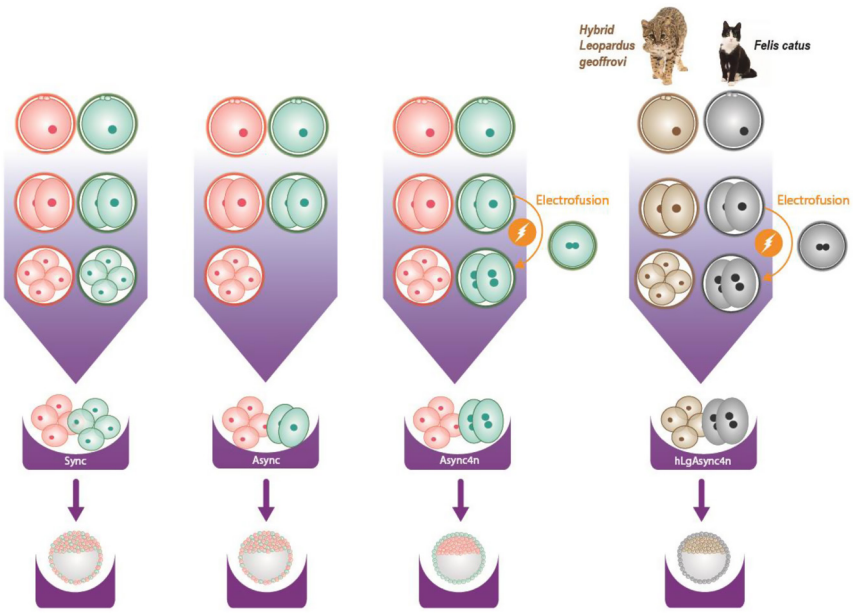

Figure 1 Experimental design illustration. Schematic representation of the experiments 2–3. (A) Four-cell diploid IVF embryos were aggregated with a four-cell diploid embryo (Sync). (B) Four-cell diploid IVF embryos were aggregated with a two-cell diploid embryo (Async). (C) Four-cell diploid IVF embryos were aggregated with a two-cell tetraploid embryo (Async4n). (D) Four-cell hLg diploid IVF embryos were Async4n aggregated with a two-cell *Fc* tetraploid embryo (hLgAsync4n). For A, B and C, before aggregation, some embryos were stained with red and green mitotrackers (see experimental design for detailed descriptions).

under the highest standards of care established by regional associations for wildlife management. All sperm collection were complied with the Code of Ethics of Latin American Zoo and Aquarium Association (ALPZA 2018), World Association of Zoos and Aquariums (WAZA) Code of Ethics and Animal Welfare and WAZA Caring For Wildlife The World Zoo and Aquarium Animal Welfare Strategy. In addition, the sperm study was approved by the CICUAL from Facultad de Ciencias Veterinarias, Universidad de Buenos Aires (protocol number 2018/54).

Oocyte collection and in vitro maturation

Ovaries were recovered from queens subjected to ovariectomy and transported to the laboratory within 2 h. Ovaries were washed in Tyrode's albumin lactate pyruvate medium buffered with HEPES (TALP-H, (Bavister & Yanagimachi 1977)). Cumulus–oocyte complexes (COCs) were recovered from follicles by repeatedly puncturing and scraping the ovaries with a 18 g needle. Only COCs grade 1 were selected based on previously established morphological criteria (Wood & Wildt 1997). Oocytes were matured *in vitro* for 22 h in 100 µL drops covered with mineral oil (M8410) of bicarbonate-buffered Tissue Culture Medium (TCM-199, 11150–059, Thermo Fisher Scientific) containing 1 IU/mL hCG (Ovusyn, Syntex SA, Buenos Aires, Argentina), 10 ng/mL eCG (Novormon 5000, Syntex SA), 25 ng/mL EGF (PHG0314, Thermo Fisher Scientific), 2.2 mM calcium lactate (L2000), 0.3 mM pyruvate (P2256), 0.3% w/v BSA (A6003) and 3% v/v antibiotic-antimycotic (ATB; penicillin, streptomycin and amphotericin B; 15240-096; Thermo Fisher Scientific). *In vitro* maturation was performed in a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

Sperm collection

After orchiectomy of domestic cat males, the testicles and epididymites were transported to the laboratory within 2 h in TALP-H and refrigerated for 24–48 h at 4°C. Testicles were equilibrated at room temperature for 2 h, and then the epididymite and proximal vasa deferential were isolated. Spermatozoa were collected as described by Jiménez *et al.* (2013). Briefly, Spermatozoa were collected by mincing the cauda epididymis in 3 mL TALP-H at 38°C. After 10 min, the washed tissue was filtered to obtain spermatozoa, and 3 mL TALP-H were used to rewash the tissue retained in the filter. Then, samples were evaluated for motility, morphology, acrosome status, viability and functional integrity of the sperm membrane was performed. For *Leopardus geoffroyi* sperm, straws were thawed by exposure to air for 10 s and then immersion in a 37°C water bath for 30 s. The contents of the straws were poured into a sterile 1.5 mL microtube pre-warmed to 37°C. The sperm suspension was diluted (1:3, v/v) by slow (drop by drop) addition of a modified Tyrode's solution. For domestic cat, sperm collection and evaluation were carried out on the same day of IVF.

In vitro fertilization and embryo culture

In vitro matured oocytes were co-incubated with 2 × 10^5/mL motile spermatozoa under 5% CO_2 in air at 38.5ºC for 18–20 h. After gametes co-incubation, presumptive zygotes were cultured *in vitro* in 50 µL droplets of modified Tyrode's medium in 5% CO_2, 5% O_2, and 90% N_2. Cleavage was assessed 48 h post-fertilization, and blastocyst rate was evaluated at day 8. Blastocyst were fixed for immunocytochemistry or kept in 10 µL of RNA later (AM7020, Ambion) at −20°C until gene expression analysis.

Electrofusion

Membrane fusion of two-cell embryos was performed at 24 h after IVF using a double-direct current pulse with different electric pulse voltages (0.8, 2, 4, and 8 kV/cm) for 30 µs and 0.1 s apart in fusion medium (0.3 M mannitol (M9647), 0.1 mM $MgSO_4$ (63138), 0.05 mM $CaCl_2$ (C7902) and 1 mg/

mL polyvinyl alcohol (P8136)) at 38.5°C. Presumptive fused and non-fused embryos were cultured *in vitro* in 50 µL drops of modified Tyrode's medium in 6.5% CO_2 in air at 38.5°C. Fusion was assessed 60 min after pulse and cleavage was determined 24 h later.

Karyotyping

Four to eight cell embryos were incubated in 1.5 mg/mL pronase (P8811) in TALP-H on a warm plate at 38.5°C for 5 min. Zona pellucida-free embryos (ZF embryo) were incubated for 6 h in 1.25 mg/mL colchicine (C3915). Then, they were transferred to 0.8% w/v trisodium citrate hypotonic solution (F71497) in distillated water for 15 min at 37° C. Subsequently, embryos were fixed on a glass slide in a mixture of methanol: acetic acid solution (3:1 v/v). After air drying, fixed embryos were stained with 5% v/v Giemsa solution (Merck 1.09204.1002) in distilled water for 10 min. Chromosomes were examined under a 100× objective lens, and chromosome number for each embryo was determined. Embryos were classified as either diploid ($2n=38$), tetraploid ($4n=76$) or aneuploid.

Embryo aggregation Sync, Async, and Async4n

For embryo aggregation, the zona pellucida of two-cell and four-cell embryos were removed by incubation in 1.5 mg/mL pronase (P8811) in TALP-H on a warm plate at 38.5°C. ZF embryo were washed in TALP-H and Sync, Async and Async4n aggregated in a microwell. Briefly, microwells were produced using a heated glass capillary slightly pressed to the bottom of a 35 × 10 mm petri dish. Microwells were covered with 100 µL drops of modified Tyrode's medium in 6.5% CO_2 in air at 38.5°C. Embryo aggregation was performed by placing randomly two ZF embryos per microwell. After 48 h of culture, wells with one structure were considered as containing an aggregated embryo and blastocyst formation rate and cell number were evaluated on day 8. Embryos presenting more than one structure per microwell at 48 h after were considered as non-aggregated.

Mitotracker staining

To investigate cell distribution after aggregation of ZF embryos, we analyzed the distribution of mitotrackers staining of embryonic cells after Sync, Async and Async4n aggregation. In blastocysts stage, the area with the highest concentration of staining was defined and the intensity of this area was determined and compared with the estimated intensity of the same area if the total intensity of the embryo was homogeneously distributed. Diploid two-cell and tetraploid two-cell embryos were stained with green mitotracker and diploid four-cell embryos were stained with red mitotracker before aggregation. Briefly, embryos were incubated in modified Tyrode's medium with 20 mM of green mitotrackers (M7514, Thermo Fisher Scientific) or 0.5 mM red mitotrackers (M7512, Thermo Fisher Scientific) for 45 min in humidified conditions at 39°C. Then, embryos were washed in TALP-H and the zona pellucida was removed, followed by aggregation and cultured *in vitro* as previously described (section: Embryo aggregation: Sync, Async, and Async4n), by placing one embryo of each color per microwell. Embryo development and mitotrackers fluorescence were evaluated at day 8.

Gene expression analysis

Pools of five blastocysts were stored at −20°C in RNA later until RNA extraction for each biological replicate. The Arcturus PicoPure RNA isolation kit (Applied biosystems) was used for total RNA isolation, according to manufacturer's instructions and eluted in 11 µL. First-strand cDNA was synthesized by using random primers and the SuperScript II reverse transcriptase (Life Technologies) with 10 µL of total RNA in a 20 µL final reaction. qPCR analysis was performed with gene-specific primers (Table 1), using SYBR green detection on a BioRad CFX96 thermocycler. At least three biological replicates of each group were evaluated. Samples were loaded as duplicates (technical replicates) in a 15 µL reaction containing 8 µL Sybr green, 1 µL of each primer (20 mmol/µL), 1 µL cDNA template, and 4 µL Milliq water. Data were analyzed by the ΔΔCT method using the geometric mean of Actin and Gapdh as internal standard.

Immunofluorescence and TUNEL assay

Blastocysts were fixed for 20 min in 4% v/v paraformaldehyde (F1635) in DPBS (14287-072, Thermo Fisher Scientific) and permeabilized for 15 min with 0.2% v/v Triton X-100 (T9284) in DPBS. Non-specific immunoreactions were blocked by 30 min incubation

Table 1 Sequence-specific primers used for quantification of differentially expressed transcripts.

Gene	GeneBank Accession no.	Primer sequences (5′–3′)		Amplicon size (bp)	Temp. (°C)	Effcy (%)
		Forward	Reverse			
POU5F1	EU366914	TGAGAGGCAACCTGGAGAAC	AACCACACTCGGACCACATC	112	61	98
NANOG	EU366913	GGCTCCAGAATTTAACCACAAG	TGGAATAACGAACAGGTCGG	195	61	93
SOX2	AANG01283909	TTACCTCTTCCTCCCACTCC	CCCATTTCCCTCGTTCTTC	250	62	97
CDX2	ACBE01001761	CAGTGAAAACCAGGACGAAAG	CCGGATGGTGATGTAACGAC	104	61	97

Temp., temperature; Effcy, efficiency.

with 3% v/v BSA and 0.1% v/v Tween-20 (Promega, H5152) in DPBS (blocking solution). OCT4 localization was analyzed by immunofluorescence and DNA fragmentation by TUNEL assay following manufacture's protocol (DeadEndTM Fluorometric TUNEL System; Promega G3250). Incubation with primary antibody against OCT4 (goat polyclonal IgG, SC-8628 Santa Cruz Biotechnology, dilution 1:100) was performed overnight at 4°C. Then, embryos were rinsed in a blocking solution for 15 min and incubated with the secondary antibody (donkey anti-goat IgG 647, dilution 1:1000) for 2 h at 39°C. Nuclei were counterstained with 20 mg/mL Hoechst bisbenzimide 33342 (H33342) for 15 min to stain DNA. Stained blastocysts were mounted on glass slides, in 70% v/v glycerol under a coverslip and stored at 4°C for 24 h before microscopic fluorescence evaluation. Embryos were analyzed on an inverted microscope Olympus Disk Spinning Unit-IX83 Spinning Disc Confocal. Analysis of total cell number, OCT4 positive (OCT4+) and TUNEL positive (TUNEL+) nuclei quantification was performed manually with FIJI image processing software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Comparisons of two groups were performed using Mann–Whitney *U*-test. Comparisons of three groups were performed using Kruskal–Wallis test, with Dunn's multiple comparisons test. Embryo *in vitro* developmental rates were compared using two-tailed Fisher's exact test. A *P*-value > 0.05 was considered as non-significant.

Results

Experiment 1 Generation of tetraploid blastomeres by electrofusion of two-cell IVF domestic cat embryos

To establish the optimal electrofusion conditions to produce tetraploid blastomeres, we compared embryonic development rates of two-cell IVF embryos subjected to different electric pulse voltages. Results are shown in Table 2. Although the highest fusion rate was achieved with 12 kV/cm, embryo development was compromised, and no blastocysts were obtained. Conversely, fusion rates of more than 50% were achieved with 8 kV/cm DC pulses without compromising embryo developmental competence. Exp-*p*/nf embryos developed to the blastocyst stage at a similar rate. To confirm that fused two-cell embryos fused with 8 kV/cm were tetraploid, we performed a karyotype analysis at the 4–8 cell embryo stage. The number of tetraploid blastomeres was higher in fused embryos as compared to the control and to Exp-*p*/nf groups (Table 3). In addition, 82% of the Exp-p/nf embryos were aneuploids.

Experiment 2 Async4n aggregation of domestic cat embryos

To determine if aggregation of embryos at different cell stages impaired subsequent *in vitro* development, we compared blastocyst developmental rates after Sync, Async and Async4n aggregation. As tetraploid complementation was reported to restrict the 4n cells to extra-embryonic lineages, we evaluated embryo development and quality of Async4n aggregation. We observed that, independent of the aggregation method and ploidy, embryos in a single microwell formed a unique structure and developed up to the blastocyst stage at similar rates (Table 4). Interestingly, blastocysts from the Async4n aggregation group showed a significantly higher number of cells as compared to blastocysts from the Sync aggregation group (Fig. 2).

Mitotracker staining distribution analysis of embryonic cells after Sync, Async and Async4n aggregation revealed that no differences were found for the red mitotracker (most advanced embryo) among the three groups. However, after Async or Async4n aggregation, less advanced blastomers showed no preference to accumulate in specific areas, which could be associated with the exclusion of ICM (Fig. 3).

To evaluate embryo quality, we analyzed gene expression of pluripotency (*Oct4,* Nanog*, Sox2*), and trophectoderm (TE) markers (*Cdx2*) in 4n, Async, and Async4n aggregated blastocyst. No differences in expression of pluripotency genes were observed among groups (Fig. 4). However, 4n, Async and Async4n aggregated embryos showed an increase in *Cdx2* gene expression as compared to the control group (2n non-aggregated blastocyst). These results indicated that *Cdx2*, a transcription factor required for proper TE specification, is over expressed in 4n and Async aggregated embryos and is suggestive of trophoblastic cell quality.

Table 2 *In vitro* development of domestic cat two-cell IVF embryos presumptive fused with different electric pulse voltages. Values with different letters in a column are significantly different ($P < 0.05$, Fisher's exact test).

Electric pulse voltage (kV/cm)	**No. of Two-cell IVF embryos**, *n* (%)	**Fused embryos**, *n* (%)	**Cleavage embryos from fused**, *n* (%)	**Blastocysts from fused embryos**, *n* (%)
0.8	35	0 (0)[a]	–	–
2	34	7 (26)[b]	3 (43)[a]	2 (29)[a,b]
4	54	16 (30)[b]	12 (75)[b]	5 (31)[a]
8	71	38 (54)[c]	33 (87)[b]	17 (45)[a]
12	15	15 (100)[d]	12 (80)[b]	0 (0)[b]
Control	173	–	–	62 (36)

Table 3 Karyotype analysis of 4–8 cell embryos.

Experimental groups	*n*	Diploid embryos, *n* (%)	Tetraploid embryos, *n* (%)	Aneuploid embryos, *n* (%)
Fused	15	0 (0)[a]	10 (67)[a]	5 (33)[a]
Exp-p/nf	11	2 (18)[a]	0 (0)[b]	9 (82)[b]
Control	23	18 (78)[b]	0 (0)[b]	5 (22)[a]

Fused: two-cell embryos exposed to 8 kv/cm electric pulse voltage. Exp-p/nf: two-cell embryos exposed to 8 kv/cm electric pulse voltage but non-fused. Control: embryos not exposed to electric pulse. Different superscript letters indicate statistical significance (Fisher's exact test, *P*-value < 0.05).

Blastocysts obtained by electrofusion (4n), Async and Async4n aggregation were evaluated to determine blastocyst cell numbers, OCT4+ cell and the presence of fragmented nuclei by TUNEL assay (Table 5 and Fig. 5). 2n blastocysts were evaluated as control. Async and Async4n aggregation had a similar blastocyst cell number between them, although a higher cell number compared to non-aggregated embryos (4n and 2n), suggesting that embryo aggregation increases blastocyst cell number. Moreover, Async4n aggregation showed statistically lower DNA fragmentation than the other groups. These results indicate that the proportion of fragmented nuclei related to the apoptosis process is lower in Async4n aggregation.

Experiment 3 Async4n aggregation of domestic cat embryos with Leopardus geoffroyi hybrid embryos

As a proof of concept, we explored whether hybrid embryos from different species could Async4n aggregate with *Fc* 4n embryos and develop up to the blastocyst stage. Overall, the rate of Async4n aggregation of hLg and *Fc* embryos that formed a single structure and developed up to the blastocyst stage (Fig. 6) was not different from the control group (Table 6).

Discussion

Reproduction of endangered felids using ART remains challenging due to the limited access to gametes and suitable surrogate recipients capable of producing healthy term pregnancies. The use of related domestic cats as surrogate recipients becomes an interesting alternative although the efficiency is limited by maternal–fetal

Table 4 *In vitro* development of Sync, Async and Async4n aggregation of ZP-free IVF embryos.

Groups	*n*	Wells	Aggregate embryos, *n* (%)	Aggregate develop to blastocyst, *n* (%)
Sync	144	72	57(79)	34 (60)
Async	118	59	53 (90)	39 (74)
Async4n	84	42	33(79)	26 (79)

Sync: four-cell diploid IVF embryos were aggregated with a four-cell diploid embryo. Async: four-cell diploid IVF embryos were aggregated with a two-cell diploid embryo. Async4n: four-cell diploid IVF embryos were aggregated with a two-cell tetraploid embryo.

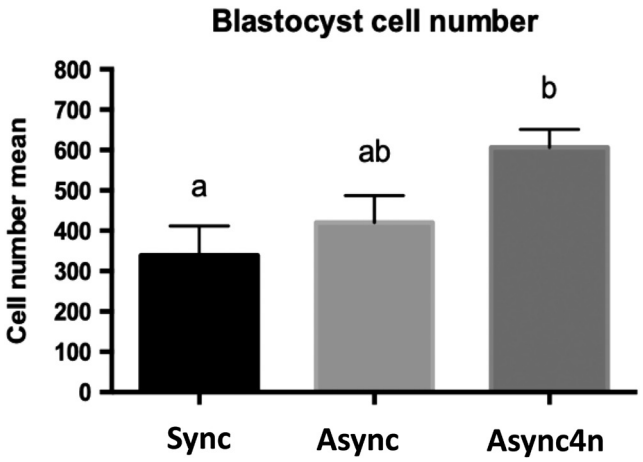

Figure 2 Blastocyst cell numbers mean ± s.e.m. Total blastocyst cell number of aggregated embryos. Sync: four-cell diploid IVF embryos were aggregated with a four-cell diploid embryo. Async: four-cell diploid IVF embryos were aggregated with a two-cell diploid embryo. Async4n: four-cell diploid IVF embryos were aggregated with a two-cell tetraploid embryo. Different superscript letters indicate statistical significance (Fisher's exact test, *P*-value < 0.05).

incompatibilities. To overcome this limitation, tetraploid complementation may improve *in vitro* embryo quality and the compatibility of extra-embryonic tissue with the recipient species. To the best of our knowledge, the data reported here demonstrated for the first time the domestic cat Async4n aggregation with 2n embryos increasing blastocyst cell number and C*dx2* expression.

In our study, we established suitable conditions to successfully generate tetraploid embryos without compromising embryo development. In the pig, similar rates of fused embryos were achieved using a lower electric pulse voltage (Lin *et al.* 2015). We showed that a higher electrical pulse is necessary to achieve two-cell embryo fusion in domestic cats and this may be due to the amount of lipids and/or thicker zona pellucida. We confirmed that 67% of the fused embryos were tetraploid. Aneuploidy was increased in 4- to 8-cell stage embryos subjected to the electrical pulse that did not fuse, even though blastocyst development was not affected. Additionally, in accordance with our results, it has been reported in cattle that aneuploidy is a result of asynchronous cleavage between two-cell embryos and asynchronous nuclear status of blastomeres within the embryo suggesting that blastomeres need to be at a certain stage of the cell cycle for consistent production of tetraploids (Curnow *et al.* 2000). In agreement with our results, previous reports showed that higher electric pulse voltage affects efficiency of electrofusion and embryo survival (Meghji & Burnstock 1995, Suo *et al.* 2009, Razza *et al.* 2016).

It has been shown that embryo aggregation improves *in vitro* and *in vivo* embryo development and early pregnancy rates in mouse, equine and cattle (Jeong *et al.* 2008, Gambini *et al.* 2012, Buemo *et al.* 2016). Our study

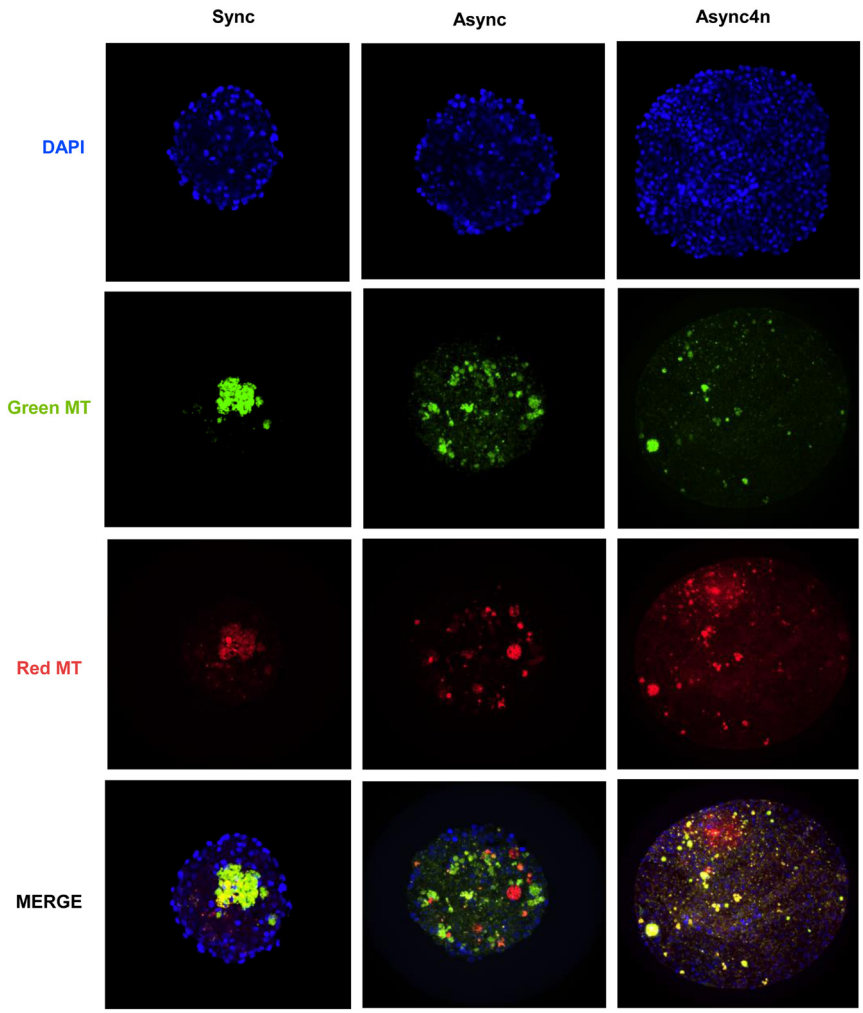

Figure 3 Mitotracker distribution in blastocyst produced by Sync, Async and Async4n aggregation. Fluorescent analysis of zona pellucida free embryos stained with green and red mitotrackers. Each picture represents one section of the total blastocyst. The nuclei are shown in blue (DAPI).

is the first to evaluate Sync, Async and Async4n embryo aggregation in domestic cat embryos. No difference was observed in blastocyst rate among aggregation groups, although blastocysts in the Async4n aggregation group showed higher total cell numbers. Our green mitotracker results suggest that embryonic cells from less advanced blastocysts contributed mainly to the trophoblast; observations that are in agreement with other reports in cattle and pigs (Picard *et al.* 1990, Jeong *et al.* 2019). In cattle, it was shown that when day 8 ICMs (most advanced embryo) were aggregated with day 5 morulae, day 8 ICM cells preferentially participated in the formation of the ICM of resulting composite embryos and the majority of the offspring produced were mostly or entirely from the day 8 ICM phenotype (Picard *et al.* 1990). Additionally, after aggregation of developmentally asynchronous cow embryos, the less advanced blastomeres were excluded at compaction, resulting in ICM blastocyst formation only from the most advanced embryos (Wells & Powell 2000).

By analyzing pluripotency gene expression levels and early differentiation genes of 2n, 4n, Async and Async4n aggregated embryos, we found differences in relative expression of *Cdx2*, an earliest transcription factor essential for formation and maintenance of TE lineage, but there were no differences in the relative expression levels of the three pluripotency genes among groups. Higher *Cdx2* gene expression was found in 4n, Async and Async4n aggregated blastocysts as compared to 2n control blastocysts. Similar to our results, it has been reported that blastocysts from cattle and humans frequently contain polyploid cells, which are more abundant in TE than in ICM (Viuff *et al.* 2002). Having this into consideration, 4n or polyploid blastomeres generally contribute to TE development, explaining the higher expression of *Cdx2* in 4n and Async4n aggregated blastocysts (Viuff *et al.* 2002, MacKay & West 2005). Similarly, embryo aggregation in miniature pigs enhanced the expression of *Cdx2* as compared to that of non-aggregated embryos (Siriboon *et al.* 2014).

We evaluated the distribution and presence of OCT4 protein in 2n, 4n, Async and Async4n aggregated blastocysts. Results of studies in other species have indicated that embryo aggregation is a promising method for improving gene expression and developmental competence in blastocysts (Terashita *et al.* 2011). Bovine, porcine, non-human primate and feline blastocysts stain

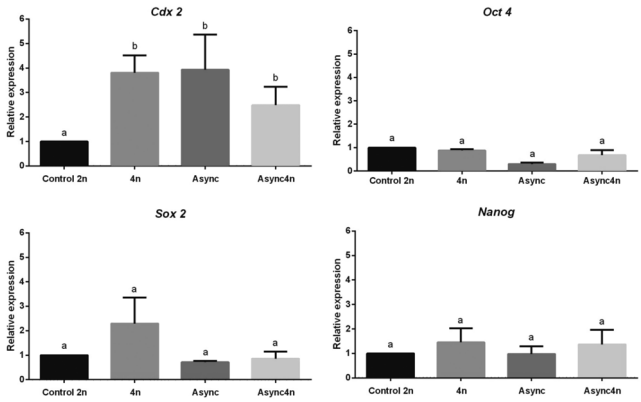

Figure 4 Relative transcript abundance of *Oct4*, *Nanog*, *Sox2* and *Cdx2* genes in blastocysts produced by electrofusion, Async, and Async4n aggregation. Control 2n, diploid blastocyst; 4n, tetraploid blastocyst produced by electrofusion of two-cell embryos; Async, four-cell diploid IVF embryos were aggregated with a two-cell diploid embryo. Async4n, four-cell diploid IVF embryos were aggregated with a two-cell tetraploid embryo. Values with different superscripts within columns are significantly different ($P < 0.05$, ANOVA test).

positive for OCT4 protein in TE, unlike the mouse model where the gene is downregulated in TE and is mainly expressed in the ICM (Gómez *et al.* 2010, Moro *et al.* 2015). Lee and Lee (2007) observed that expression levels of OCT4 were higher in aggregated embryos than in control IVF embryos. Our results suggest that higher cell numbers in Async and Async4n aggregated blastocysts increase the number of OCT4+ cells. Likewise, to our results, aggregation of porcine somatic cell nuclear transfer (SCNT) embryos at the four-cell stage improved the percentage of OCT4+ cells in cloned blastocysts (Terashita *et al.* 2011).

Table 5 OCT4+ cell number by immunocytochemistry and DNA fragmentation by TUNEL assay in blastocyst produced by electrofusion, Async, and Async4n aggregation.

Group	*n*	Cell number, mean	OCT4+ cell, mean (%)	Tunnel+ cell, mean (%)
2n	5	142[a]	110 (77)[a]	49 (35)[a]
4n	5	140[a]	106 (76)[a]	43 (31)[a]
Async	5	443[b]	381 (86)[b]	114 (26)[a]
Async4n	5	457[b]	392 (86)[b]	71 (16)[b]

2n, diploid blastocysts; 4n, tetraploid blastocyst produced by electrofusion of two-cell embryos; Async, four-cell diploid IVF embryos were aggregated with a two-cell diploid embryo; Async4n, four-cell diploid IVF embryos were aggregated with a two-cell tetraploid embryo. Different superscript letters indicate a statistical significance. (Fisher's exact test, *P*-value < 0.05).

In addition to cell number, apoptosis is another important indicator of embryo quality which occurs during the preimplantation development in both *in vivo* and *in vitro* produced embryos, and it may contribute to embryonic loss (Hao *et al.* 2003, Lin *et al.* 2015). We evaluated the proportion of fragmented nuclei, as related to the apoptosis process, to determine the quality of Async and Async4n aggregation. Async4n aggregated embryos showed a lower proportion of fragmented nuclei as compared to the other groups. Similar to our finding, in pigs, aggregated embryos showed fewer apoptotic cells (Misica-Turner *et al.* 2007); however, expression levels of Bax (a pro-apoptotic gene) were higher in tetraploid and octaploid than in diploid mouse embryos (Wu *et al.* 2017). Therefore, future experiments to elucidate the influence of Async4n aggregation in blastocyst should be done.

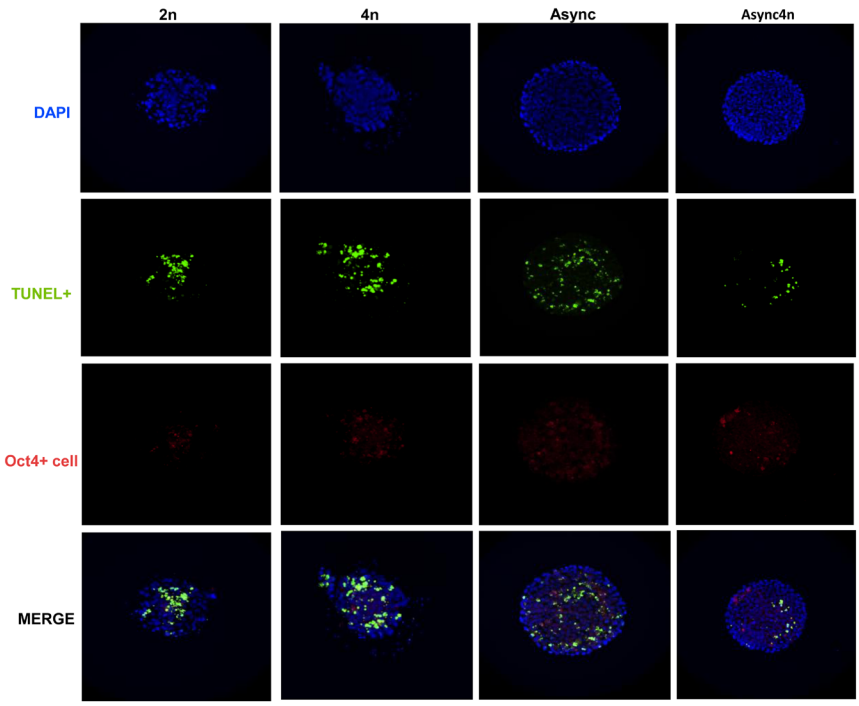

Figure 5 Representative OCT4+ immunofluorescent staining and TUNEL assay of blastocysts produced by electrofusion, Async, and Async4n aggregation. Blastocyst produced by electrofusion, Async, and Async4n aggregation analyzed by DAPI staining, DNA-fragmentation level and OCT4+ cells by immunofluorescence. Values with different superscripts within columns are significant differences ($P < 0.05$, Fisher's exact test).

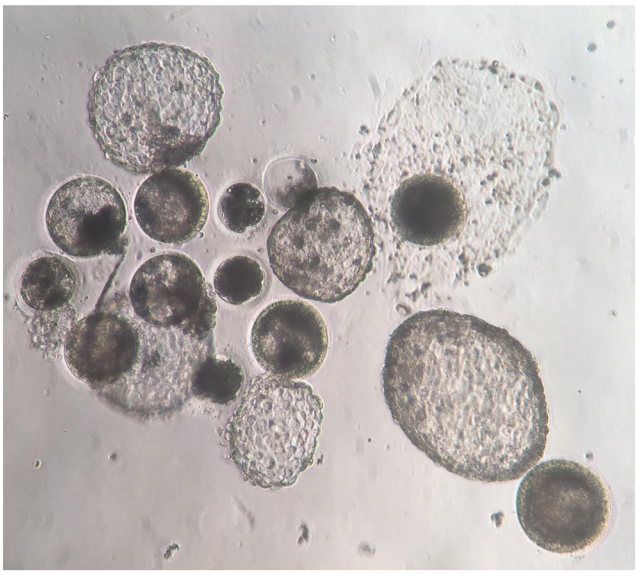

Figure 6 Hybrid blastocysts produced by heterospecific fertilization of domestic cat oocytes with *Leopardus geoffroyi* sperm.

Finally, we Async4n aggregated hLg embryos with *Fc* tetraploid embryos to evaluate the *in vitro* capacity of the resultant interspecies hybrid embryos to aggregate and develop up to blastocyst stage. Our findings showed no differences compared to the homospecific control group. Similar results were obtained in sheep and goat chimeric embryos created by aggregation of a single 4-cell and two 8-cell stage blastomeres (Meinecke-Tillmann & Meinecke 1984).Therefore, further experiments should be performed to evaluate embryo quality and pregnancy rate after Async4n aggregation using IVF or SCNT embryos from endangered feline species with domestic cat 4n embryos.

In conclusion, 4n blastocysts produced by electrofusion and embryo aggregation exhibit significantly higher blastocyst cell numbers. Additionally, Async4n aggregation reduces the incidence of fragmented nuclei and increases *Cdx2* gene expression without altering the expression of pluripotent genes. To date, no data have been generated on production and quality of tetraploid blastocysts and Async4n aggregation in feline species.

Our findings demonstrate that Async4n aggregation with hLg and *Fc* embryos is feasible and possibly a valuable tool for producing preimplantation embryos with improved developmental competence, a critical factor for successful application of assisted reproductive technology for conserving endangered felid species.

Table 6 *In vitro* development after Async4n aggregation with hybrid embryos.

Group	*n*	Wells	Aggregate embryos, *n* (%)	Aggregate developed to blastocyst, *n* (%)
Async4n	38	19	14 (74)	12 (86)
hLgAsync4n	22	11	7 (64)	5 (71)

Async4n, four-cell *Fc* diploid IVF embryos were Async4n aggregated with a two-cell *Fc* tetraploid embryo. hLgAsync4n, four-cell *hLg* diploid IVF embryos were Async4n aggregated with a two-cell *Fc* tetraploid embryo.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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Author contribution statement

M D R, R F M and D F S designed the experiments. M D R performed domestic cat oocyte retrieval, collections, IVF and tetraploid aggregation. A S provided the *Leopardus Geoffroyi* sperm. M D R, R F M and D F S analyzed the data. L D R and S B R performed qPCR experiments. C G and P D C performed karyotype experiments. M D R, A G, and O B performed immunofluorescence assays. M D R, A G, R F M and D F S wrote the manuscript. Funding Acquisition by D F S. All authors reviewed and approved the final manuscript.

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