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Tiger, Bengal and Domestic Cat Embryos Produced by Homospecific and Interspecific Zona-Free Nuclear Transfer

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Contents

The aim of this study was to evaluate three different cloning strategies in the domestic cat (*Felis silvestris*) and to use the most efficient to generate wild felid embryos by interspecific cloning (iSCNT) using Bengal (a hybrid formed by the cross of *Felis silvestris* and *Prionailurus bengalensis*) and tiger (*Panthera tigris*) donor cells. In experiment 1, zona-free (ZP-free) cloning resulted in higher fusion and expanded blastocyst rates with respect to zona included cloning techniques that involved fusion or injection of the donor cell. In experiment 2, ZP-free iSCNT and embryo aggregation (2X) were assessed. Division velocity and blastocyst rates were increased by embryo aggregation in the three species. Despite fewer tiger embryos than Bengal and cat embryos reached the blastocyst stage, Tiger 2X group increased the percentage of blastocysts with respect to Tiger 1X group (3.2% vs 12.1%, respectively). Moreover, blastocyst cell number was almost duplicated in aggregated embryos with respect to non-aggregated ones within Bengal and tiger groups (278.3 ± 61.9 vs 516.8 ± 103.6 for Bengal 1X and Bengal 2X groups, respectively; 41 vs 220 ± 60 for Tiger 1X and Tiger 2X groups, respectively). OCT4 analysis also revealed that tiger blastocysts had higher proportion of OCT4-positive cells with respect to Bengal blastocysts and cat intracytoplasmic sperm injection blastocysts. In conclusion, ZP-free cloning has improved the quality of cat embryos with respect to the other cloning techniques evaluated and was successfully applied in iSCNT complemented with embryo aggregation.

Introduction

Interspecific SCNT (iSCNT) involves embryo reconstruction using the recipient ooplast from one species and the donor cell from another one. This technique has been used with various purposes among which are included: nucleocytoplasmic interaction research (Thongphakdee et al. 2008), derivation of human embryonic cell lines using non-human oocytes (Chen et al. 2003) and production of embryos from species from which oocytes are very difficult to obtain (Lagutina et al. 2013; Moro et al. 2015). Interspecific SCNT has been evaluated for wild species conservation, and several animals were born by this technique (Lanza et al. 2000; Loi et al. 2001; Janssen et al. 2004). Specifically on felids, the successful application of iSCNT was confirmed after birth of African wild cats (*Felis silvestris lybica*; Gómez et al. 2004) and sand cats (*Felis margarita*; Gómez et al. 2008), and pregnancies obtained in leopard cats (*Prionailurus bengalensis*, Yin et al. 2006). Despite these achievements, it is still very difficult to obtain pregnancies and births after iSCNT, as was shown in several reports (Thongphakdee et al. 2010; Gómez et al. 2011; Imsoonthornruksa et al. 2012). The causes of its inefficiency are many, and they could be related to failures in nuclear-cytoplasmic communications (St John et al. 2004) and to the basis of nuclear transfer itself (Niemann et al. 2008; Palmieri et al. 2008). The SCNT technique has evolved since the birth of Dolly (Wilmut et al. 1997), and different alternatives have been developed to improve it by making it more efficient, easier or less dependent on expensive equipment. Some of these methods included the intracytoplasmic injection of the donor cell (Wakayama et al. 1998; Ideta et al. 2005), handmade cloning (Vajta et al. 2001) and embryo aggregation (Boiani et al. 2003; Ribeiro et al. 2009). This last strategy consists on culturing several zona-free zygotes together that would be integrated into one single embryo. Embryo aggregation allows the complementation of clones with equal genomic characteristics but that have been reprogrammed differently, improving embryo development of clones and birth rates in different mammalian species (Zhou et al. 2008; Ribeiro et al. 2009; Gambini et al. 2012, 2014).

Most of the 36 species of wild felids are threatened with extinction, and the possibility to restore lost genetic, relocate parentally unrelated individuals or produce specie-specific embryonic stem cells by cloning makes it a valuable tool for contribution in species conservation and research. The tiger (*Panthera tigris*) is one of the world's most endangered species, and without conservation measures, tigers will soon become extinct in the wild (Chundawat et al. 2011; Cho et al. 2013). It has been reported that the tiger and the domestic cat have similar genome architectures, based on proteomic, tandem repeats and transposable elements (Cho et al. 2013). In order to avoid invasive procedures to obtain tiger oocytes and because of the difficulty to gather many females, we propose to use domestic cat ooplasts to reprogram tiger cells and generate tiger embryos by iSCNT. This objective was previously achieved by our group using cheetah cells as nuclear donors and preimplantation cheetah embryos could be generated successfully using the zona-free iSCNT (Moro et al. 2015).

In this study, we evaluated the developmental ability of domestic cat embryos produced by three different

SCNT techniques, one of which had not been previously reported in this species. The best strategy was chosen for iSCNT, using domestic cat ooplasts and Bengal or tiger donor cells. The Bengal cat is a hybrid feline, result of the cross between the domestic cat and the Asian leopard cat (*Prionailurus bengalensis*), and was used in this work to determine the effect of the phylogenetic distance between the donor cell and the recipient ooplast in iSCNT. Moreover, we evaluated whether embryo aggregation improves the developmental competence in these both species.

Materials and Methods

Ethics for use of research animals

Animal manipulation was performed according to the rules of the Direction of National Wildlife. The standards established by the code of ethics of ALPZA (Latin American Association of Zoological Parks and Aquariums) were followed. The study design was approved by the Ethics and Animal Welfare Committee for the Faculty of Agriculture University of Buenos Aires under number CEyBAFAUBA2014/3.

Reagents

Except otherwise indicated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Media were prepared weekly and filtered through 0.22-μm membranes (#4192 Acrodisc; Pall Corp., Ann Arbor, MI, USA) into sterile tubes.

Experimental design

In the first experiment, embryo development and blastocyst quality were evaluated using different SCNT strategies in the domestic cat: (i) oocyte enucleation with zona pellucida (ZP) followed by intracytoplasmic injection of the donor cell (*Injection ZP* group); (ii) oocyte enucleation with ZP followed by cell transfer to the perivitelline space and fusion (*Fusion ZP* group); and (iii) oocyte enucleation without ZP followed by adhesion and fusion of the donor cell (*Fusion ZP-free* group). Three replicates were included for each experimental group. As controls, oocytes with ZP and ZP free were parthenogenetically activated and cultured in the same conditions as the SCNT reconstructed embryos (*PA ZP* and *PA ZP-free* groups). In the second experiment, the *Fusion ZP-free* strategy was chosen for iSCNT. Zona-free SCNT in the domestic cat and zona-free iSCNT using domestic cat ooplasts and Bengal or tiger donor cells were performed. Moreover, the reconstructed embryos obtained in this experiment were cultured in microwells individually (1X) or in pairs together (2X or aggregated embryos). Three replicates were performed for each experimental group, and the embryos were assigned randomly for each culture system. The experimental groups were as follows: *Tiger 1X, Tiger 2X, Bengal 1X, Bengal 2X, Cat 1X and Cat 2X*. Embryo development, cleavage dynamics, total cell number and OCT4 expression in blastocysts were analysed. One control group with fertilized cat oocytes by intracytoplasmic sperm injection (ICSI) was included.

Oocyte collection and *in vitro* maturation

Domestic cat ovaries were recovered from queens subjected to ovariectomy and transported to the laboratory within 2 h in physiological solution at room temperature. They were washed in Tyrode's albumin lactate pyruvate buffered with HEPES (TALP-H, Bavister and Yanagimachi 1977), and the cumulus–oocyte complexes (COCs) were released from the follicle by repeatedly puncturing and scraping the ovaries. The maturation medium used was TCM 199 (31100-035; Gibco, Grand Island, NY, USA) supplemented with 1 IU/ml hCG (Ovusyn, Syntex SA, Ciudad Autónoma de Buenos Aires, Argentina), 10 ng/ml eCG (Novormon 500, Syntex SA), 2.2 mM calcium lactate (L2000), 0.3 mM pyruvate (P2256), 0.3% wt/vol BSA (A6003) and 3% vol/vol antibiotic–antimycotic (ATB; penicillin, streptomycin and amphotericin B; 15240-096; Gibco). *In vitro* maturation conditions were 6.5% CO_2 in humidified air at 39°C. The oocytes were incubated in 100 μl of medium droplets covered with mineral oil (M8410) for 22 h.

Somatic cell culture

Adult fibroblasts were obtained through culture of minced skin from the inguinal area of one domestic cat and one Bengal cat, both adult and male. Tiger samples were obtained from ear cartilage of new born animals with perinatal death. Domestic and Bengal cat samples were cultured in Dulbecco's modified Eagle's Medium (DMEM, 11885, Gibco) with 10% foetal bovine serum (FBS) and 1% ATB, in 5% CO_2 in humidified air at 39°C. The tiger sample was cultured in DMEM medium supplemented with 10% FBS (10499-044, Gibco), 0,292 mg/ml L-Glutamine (25030-149, Gibco), 2.5 μg/ml Fungizone Amphotericin B (15290-018, Gibco) and penicillin-streptomycin 100 μg/ml and 100 μg/ml, respectively. Culture conditions were 5% CO_2 in humidified air at 39°C. After the primary culture was established, cells were either subcultured every 4–6 days or expanded and frozen in DMEM with 10% FBS and 10% DMSO, and stored in liquid nitrogen. Quiescence of donor cells was induced by growth to confluence for 2–3 days prior to SCNT or iSCNT.

Preparation of the oocytes

After 22 h of IVM, the oocytes were denuded of cumulus cells by pipetting in hyaluronidase (H4272, 1 mg/ml TALP-H) for 1 min and washed three times

with TALP-H. Two different techniques were used for oocyte enucleation.

Enucleation with ZP

Prior to enucleation, mature oocytes were incubated in 100 µl microdroplets with 4 µM Demecolcine (D1925) for 1 h to protrude the chromosome plate, and they were stained with 20 µg/ml Hoechst bisbenzimide 33342 (H33342) for 15 min. The metaphase plate was aspirated using a spiky pipette (20 µm inner diameter), and a holding pipette to support the oocyte during the enucleation. Enucleated oocytes were kept in 100 µl droplets of synthetic oviductal fluid medium (SOF; Tervit et al. 1972; Holm et al. 1999) supplemented with 2.5% v/v FBS (10499-044; Gibco) until nuclear transfer. Some mature oocytes that were not subjected to enucleation were used for parthenogenetic activation (PA) as control.

Enucleation ZP free

Before enucleation, mature oocytes were incubated in 1.5 mg/ml pronase (P-8811) in TALP-H for 3–8 min on a warm plate to remove the ZP. After that, they were individually incubated with Demecolcine and Hoechst bisbenzimide 33342 as described above. Unlike enucleation with ZP, the metaphase plate was aspirated using a blunt pipette (20 µm inner diameter) and a closed holding pipette (150 µm diameter), which works as a support to manipulate the oocyte without aspiring it. Enucleated oocytes were individually kept in SOF until nuclear transfer. Some mature ZP-free oocytes that were not subjected to enucleation were used for PA as control.

In both procedures, enucleation was confirmed by observing the stained metaphase inside the pipette under UV light.

Nuclear transfer

Embryo reconstruction was achieved using three different strategies described below

Injection ZP

A single cell was drawn in and out of the injection pipette (10 µm diameter) to break the cell membrane. After that, the oocyte cytoplasm was aspirated until the ooplasm was broken and the cell was injected intracytoplasmatically into the enucleated oocyte with ZP. The reconstructed embryos were then placed in 100 µl SOF droplets.

Fusion ZP

A single cell was introduced into the perivitelline space of an enucleated oocyte with ZP using the same pipette as for the enucleation procedure. Afterwards, the couplets were placed in fusion medium [0.3 M mannitol (M9546), 0.1 mM MgSO4 (A665286 525; Merck, Darmstadt, Germany), 0.05 mM $CaCl_2$ (C7902) and 1 mg/ml polyvinyl alcohol (P8136)] for 30 s and then moved to a fusion chamber containing 2 ml of fusion medium at 30°C. Membrane fusion was performed with two 30 µs pulses of 2.4 kV/cm, 0.1 s apart. After giving the pulses, the couplets were placed in 100 µl SOF droplets.

Fusion ZP free

Zona pellucida-free enucleated oocytes were individually transferred to 50 µl drops of 1 mg/ml phytohemagglutinin (PHA; L8754) dissolved in TCM-199. After a few seconds, they were quickly dropped over a single donor cell (a tiger, Bengal or domestic cat cell); consequently, these two structures were paired. Fusion was induced with two 30 µs pulses of 1.4 kV/cm, 0.1 s apart. After giving the pulses, the couplets were placed individually in SOF.

Fusion was assessed after 20 min by confirming the presence or absence of the cell into the perivitelline space or attached to the ZP-free enucleated oocyte. Refusion was performed when necessary. Two hours after injection or fusion, the reconstructed embryos were activated with 5 µM ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) in TALP-H for 4 min followed by culture in 1.9 mM 6-dimethylaminopurine (6-DMAP; D2629) in SOF for 3 h. As controls, mature oocytes with ZP and ZP free were parthenogenetically activated using the same activation protocol (PA ZP and PA ZP-free groups).

Intracytoplasmic sperm injection

The ICSI was performed as previously described by our group (Moro et al. 2014). Briefly, frozen–thawed epididymal domestic cat spermatozoa were resuspended in Brackett–Oliphant medium (BO, Brackett and Oliphant 1975) supplemented with 5 mM caffeine (C4144) and 20 IU/ml heparin (H3149) and washed by centrifugation (490 ***g*** for 5 min). The spermatozoa were microinjected into domestic cat mature oocytes using a Narishige hydraulic micromanipulator (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA). The presumptive zygotes were immediately cultured as described below.

Embryo culture

Reconstructed, parthenogenetic and ICSI embryos with ZP were cultured in groups of 20 in 100 µl SOF droplets supplemented with 2.5% v/v FBS covered with mineral oil (Fisher Chemical, Pittsburgh, PE, USA). ZP-free reconstructed and parthenogenetic embryos were cultured using the same medium and in microwells (20 microwells per 100 µl droplet), as was previously described by our group (Moro et al. 2015). ZP-free

reconstructed zygotes were cultured individually (1X, one zygote per microwell) or in pairs together (2X or aggregated embryos, two zygotes per microwell) for the second experiment. Culture conditions were humidified gas mixture of 5% CO_2, 5% O_2 and 90% N_2 at 39°C. Culture medium was changed on day 2 and then supplemented with 10% FBS on day 5. Cleavage rates and the number of cell divisions were assessed 43 h after activation, morula formation on day 5 and blastocyst formation on day 8.

Immunocytochemistry

The blastocysts generated by iSCNT (Tiger 1X, Tiger 2X, Bengal 1X and Bengal 2X groups) and domestic cat ICSI blastocysts (Control) were analysed by immunocytochemistry to determine OCT4 expression. Embryos were fixed for 20 min in 4% v/v paraformaldehyde (F1635) in DPBS (14287-072, Gibco) 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 with 3% v/v FBS and 0.1% v/v Tween 20 (Promega, Madison, WI, USA; H5152) in DPBS (blocking solution). Incubation with the primary antibody against OCT4 (goat polyclonal IgG, SC-8628 Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 100 in blocking solution was performed for 1 h at room temperature. Embryos were then rinsed in blocking solution for 15 min. Incubation with the secondary antibody (Alexa 488-donkey anti-goat IgG, A11055; Molecular Probes Inc., Eugene, OR, USA) diluted 1 : 1000 in blocking solution was performed for 45 min at room temperature in the dark. Nuclei were counterstained with 30 μg/ml propidium iodide (P4170) for 20 min in the dark. Stained blastocysts were mounted on glass slides, in 70% v/v glycerol under a cover slip and stored at 4°C for 24 h before fluorescence microscopic evaluation. Negative controls for OCT4 were generated using only the secondary antibody.

Confocal laser scanning microscopy

Blastocysts were analysed on a Nikon Confocal C.1 scanning laser microscope. An excitation wavelength of 488 nm was selected from an argon ion laser to excite the Alexa-conjugated secondary antibody and an excitation wavelength of 544 to excite propidium iodide. Complete Z series of 13–18 optical sections at 3- to 4-μm intervals were acquired from each blastocyst, and three-dimensional images were constructed using the software EZ-C1 2.20. Total cell number and OCT4-positive cells (OCT4+) were counted. In these cases, the mean and the standard error of the mean were calculated. The proportion of OCT4+ cells was determined by dividing total OCT4+ cells by total cells in each blastocyst.

Statistical analysis

In vitro embryo development was compared by non-parametric Fisher's exact test. Differences in total cell numbers and OCT4+ cells were analysed using Proc Mixed, considering heterogeneity of variances and setting degrees of freedom by Kenward–Roger method. For these statistical analyses, the SAS program was used (SAS 1989). The proportion of OCT4-expressing cells over total cell numbers was analysed by 'difference of proportions test' using InfoStat software 2007 version. In all cases, differences were considered significant at $p < 0.05$.

Results

In vitro development of domestic cat embryos produced by three different SCNT procedures

The results of this experiment are detailed in Table 1. Fusion rates were higher in the *Fusion ZP-free* group with respect to the *Fusion ZP* group ($p < 0.05$). Significant differences were also observed in the percentage of reconstructed embryos that were cultured after each cloning procedure, obtaining the lowest number of cultured embryos in the *Fusion ZP* group and the highest one in the *Fusion ZP-free* group. The *Injection ZP* group showed lower cleavage rates than the other two cloning groups ($p < 0.05$), but this difference was not observed in morula nor in blastocyst rates. Both parthenogenetic activation control groups had similar

Table 1. *In vitro* development of domestic cat embryos reconstructed by three different SCNT techniques

Groups	n	Fusioned (%)	Cultured (%)	Cleaved (%)	Morulae* (%)	Blastocysts* (%)	Expanded blastocysts† (%)	Blastocyst cell number ± SEM
Fusion ZP	71	18 (25.4)^{a}	18 (25.4)^{a}	17 (94.4)^{a}	4 (23.5)^{a}	2 (11.8)^{a}	0.0^{a}	89.0^{ab}
Injection ZP	75	–	45 (60.0)^{b}	26 (57.8)^{b}	10 (38.5)^{a}	5 (19.2)^{a}	1 (16.2)^{b}	77.2 ± 6.0^{a}
Fusion ZP free	98	81 (82.7)^{b}	71 (72.5)^{c}	55 (77.5)^{a}	23 (41.8)^{a}	9 (16.4)^{a}	7 (77.8)^{c}	177.9 ± 52.6^{b}
PA ZP	88	–	88 (100.0)^{d}	72 (81.8)^{a}	54 (75.0)^{b}	42 (58.3)^{b}	22 (52.4)^{c}	221 ± 57.0^{b}
PA ZP free	77	–	77 (100.0)^{d}	60 (77.9)^{a}	46 (76.7)^{b}	38 (63.3)^{b}	26 (68.4)^{c}	240 ± 77.0^{b}

ZP, zona pellucida; ZP free, without zona pellucida; PA, parthenogenetic embryos.
(a, b, c, d) Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's exact test).
*Calculated with respect to cleaved embryos.
†Calculated with respect to total blastocysts.

cleavage rates as the SCNT fusion groups, but higher morulae and blastocysts formation ($p < 0.05$) were observed. Despite it was possible to obtain blastocysts using the three different SCNT protocols, the *Fusion ZP-free* group showed higher fusion rates, higher expanded blastocyst rates and higher blastocyst cell number.

In vitro embryo development of tiger, Bengal and domestic cat embryos produced by ZP-free SCNT and embryo aggregation

The results of this experiment are shown in Table 2. High cleavage rates were obtained in all the experimental groups, irrespective of the donor cell source or embryo aggregation. However, the division velocity was positively influenced by embryo aggregation as the proportion of embryos with more than three cell divisions (more than eight blastomeres for 1X embryos and more than 16 blastomeres for 2X embryos) was increased by this strategy within each species (Fig. 1). Furthermore, morula formation tended to be positively influenced by embryo aggregation, and higher percentage of compacted morula was obtained in the 2X groups. Despite this observation, morula rates were lower in the tiger groups with respect to the Bengal and domestic cat groups. In correlation with the low rates of morula formation, we observed that fewer tiger embryos reached the blastocyst stage with respect to the other groups comparing the different species. In contrast, Bengal embryos developed similarly as cat embryos. All the aggregated groups showed an increase in the blastocyst rates compared to non-aggregated groups within each species, on a per-well basis.

Cell number and OCT4 expression in tiger, Bengal and cat blastocysts generated by iSCNT or ICSI

Total cell number and OCT4 expression were evaluated by immunocytochemistry to determine the effect of interspecific cloning and embryo aggregation in nuclear reprogramming and pluripotency (Table 3). In Fig. 2, the OCT4 expression pattern of tiger, Bengal and domestic cat blastocysts is shown. Aggregated embryos showed almost double cell number than non-aggregated embryos in each species. However, no differences in the proportion of OCT4+ cells were observed between aggregated and non-aggregated embryos within each species. Tiger blastocysts showed higher proportion of OCT4+ cells with respect to Bengal and cat ICSI blastocysts.

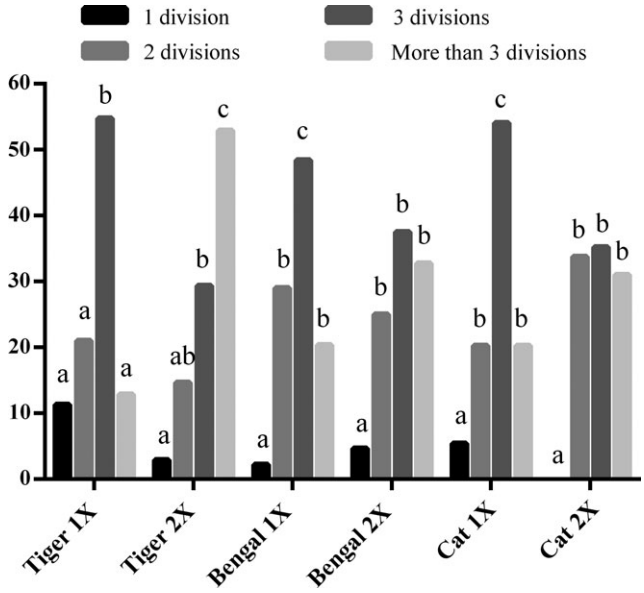

Fig. 1. Number of cell divisions in tiger, Bengal and cat cleaved embryos, aggregated or not, after 43 h of Interspecific SCNT (iSCNT) or SCNT. (a, b, c) Values with different letters in a column are significantly different within each group ($p < 0.05$, Fisher's exact test). 1X, embryos cultured individually; 2X, aggregated embryos

Discussion

In the present study, we first compared three different methods of nuclear transfer in the domestic cat, and we chose the best one to evaluate the developmental competence of Bengal and tiger embryos generated by iSCNT using domestic cat cytoplasts as recipients.

Until now, most of the nuclear transfer methods reported to produce feline clones were based on that described by Shin et al. (2002). This method is equivalent to the one we called 'Fusion ZP' and basically consists on a first step of enucleation, a second step of cell transfer to the perivitelline space, a third step of donor cell fusion and finally the activation of the

Table 2. Effects of interspecific SCNT and aggregation on *in vitro* development of tiger, Bengal and domestic cat embryos

Groups	Reconstructed embryos	Cultured embryos	Cleavage (%)	Morulae (%)*	Blastocysts (%)*
Tiger 1X	63	63	59 (93.7)^{abc}	11 (18.6)^{a}	2 (3.4)^{a}
Tiger 2X	132	66	63 (95.9)^{ac}	16 (25.4)^{ac}	8 (12.7)^{a}
Bengal 1X	154	154	129 (83.8)^{b}	49 (38.0)^{cd}	48 (37.2)^{bc}
Bengal 2X	210	105	98 (93.3)^{abc}	52 (53.1)^{b}	46 (46.9)^{b}
Cat 1X	101	101	92 (91.1)^{a}	39 (42.4)^{bd}	24 (26.1)^{c}
Cat 2X	96	48	48 (100.0)^{c}	22 (45.8)^{bd}	23 (47.9)^{b}

(a, b, c, d) Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's exact test). 1X, embryos cultured individually; 2X, aggregated embryos.
*Calculated with respect to cleaved embryos.

Table 3. Cell number and OCT4 expression in domestic cat, Bengal and tiger blastocysts

Groups	Blastocyst cell number (Mean ± SEM)	OCT4+ cells	OCT4+ cells/blastocyst cell number
Tiger 1X	41	40	97.6[a]
Tiger 2X	220 ± 60	190 ± 63	86.4[b]
Bengal 1X	278 ± 62	129 ± 23	46.3[c]
Bengal 2X	517 ± 104	234 ± 60	45.4[c]
Cat intracytoplasmic sperm injection	106 ± 16	42 ± 9	39.7[d]

(a, b, c, d) Values with different superscripts in a column are significantly different. For blastocyst cell number and OCT4+ cells, Proc Mixed was applied ($p < 0.05$). For OCT4+ cells/cell number, the difference of proportions test was applied ($p < 0.05$).

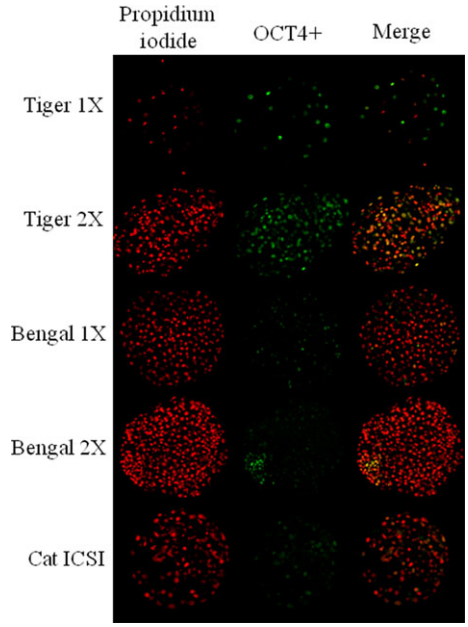

Fig. 2. OCT4 expression pattern of tiger, Bengal and domestic cat blastocysts generated by cloning (with or without aggregation) and intracytoplasmic sperm injection (ICSI). Each picture represents one section of the total blastocyst. The nuclei are shown in red (propidium iodide), and OCT4 is shown in green (Alexa Fluor 488). 1X, embryos cultured individually; 2X, aggregated embryos. Zoom 20×

reconstructed embryo. We compared this method with two others, one of them involved the injection of the donor cell instead of fusion (Injection ZP group) and the other one, the enucleation and fusion of ZP-free oocytes (Fusion ZP-free group). The advantage of injecting the donor cell instead of fusing it relies on the possibility to obtain embryos and viable offspring from dead cells. That is the case of viable mice that were produced by cloning using dead cells frozen without any cryoprotectant (Wakayama et al. 1998; Li and Mombaerts 2008). This advantage of the technique is very useful in interspecific cloning as extinct species might be revived using the nuclear genome of tissue samples frozen without a conventional cryopreservation protocol. However, the oocyte undergoes more handling by this strategy as it is necessary to disrupt the oolemma twice, first for enucleation and then for donor cell injection. In our experience, 60% of the reconstructed embryos remained intact after the injection. Despite developmental rates to blastocyst were similar among the three cloning groups, the high number of discarded oocytes decreased the overall efficiency of this technique.

While for the ZP-included procedures, it is necessary to enucleate first and then transfer the donor cell by micromanipulation, in the ZP-free method both steps can be performed simultaneously. In addition, enucleation can be faster using a blunt pipette because it is not necessary to pass across the ZP. These differences, with respect to the ZP-included procedures, make the ZP-free method an easier technique. Moreover, fusion rates were much higher in the Fusion ZP-free group than in the Fusion ZP group, as was previously reported for other mammalian species (Lagutina et al. 2007). These results can be related with an increased contact between the cell and oocyte caused by the employment of phytohemagglutinin in cell adhesion (Vajta et al. 2001). In the Fusion ZP-free group, embryo development was also improved and higher expanded blastocyst rates were obtained. However, both parthenogenetic control groups showed similar rates of expanded blastocyst regardless of the presence or absence of the ZP; thus, we attributed this result to higher embryo quality. Parthenogenetic controls were also very important to elucidate whether the individual culture in microwells was detrimental or beneficial in cat embryos. In our hands, no differences were seen neither in embryo development nor in embryo quality with respect to blastocyst expansion rates and blastocyst cell number, indicating no apparent detrimental effect in ZP-free embryos, as was previously observed in other species (Lagutina et al. 2007). In addition, blastocyst cell numbers in ZP-free clones were also higher than in the other cloning groups. This increase in the number of cells was previously observed in the cat after the enzymatic removal of the ZP in day 6 embryos (Freistedt et al. 2001). Despite the ZP remains in cat embryos until day 10 *in vivo* (Thatcher et al. 1991), Freistedt et al. (2001) suggested that the release of the embryo from the ZP might improve developmental potential. Until now, it has not been demonstrated in the cat or other feline species whether the ZP is critical

for implantation or embryo development *in vivo*. In other mammalian species, such as bovine (Oback et al. 2003; Vajta et al. 2004), equine (Galli et al. 2003; Lagutina et al. 2005; Gambini et al. 2012), murine (Ribas et al. 2005) and porcine (Du et al. 2007), offspring was successfully obtained by transferring ZP-free embryos. The only requirement for this technique is the need to transfer embryos at the morula or blastocyst stages as the absence of the ZP might cause disaggregation of blastomeres at earlier stages.

In the next experiment, we decided to use the ZP-free method for interspecific cloning due to the technical advantages and higher embryo quality obtained with this strategy in the first assay. In addition, we determined whether embryo aggregation improves *in vitro* development, as was previously demonstrated in cheetah embryos by our group (Moro et al. 2015). We obtained tiger, Bengal and cat embryos at the blastocyst stage by this technique. In addition, blastocyst production in the tiger was enhanced using embryo aggregation. Despite this improvement, fewer tiger embryos reached the blastocyst stage with respect to the other two groups. We observed that embryo arrest mainly occurred after cleavage and before morula formation, in concordance with one previous report of interspecific Siberian tiger embryos generated with porcine oocytes (Hashem et al. 2007). As was previously suggested for interspecific embryos, major limitations are first observed at the time of embryonic gene activation (Beyhan et al. 2007), when the zygote ceases to use maternal transcripts and begins to generate their own (Memili and First 2000). It was suggested that one of the reasons for interspecific embryo block might be some differences in gene structure, but it should be investigated more deeply (Lagutina et al. 2010). The presence of OCT4 in tiger blastocysts demonstrated the capacity of the domestic cat oocyte to reprogram tiger nucleus, but other pluripotent genes should be studied to determine whether the overexpression of this gene in tiger embryos is the consequence of an inefficient process that prevents a correct regulation. In a previous report, we produced cheetah blastocysts by iSCNT but with lower relative expression of pluripotent and trophoblastic genes than IVF embryos, suggesting that nuclear reprogramming could be incomplete (Moro et al. 2015). However, intergenus SCNT embryos derived from Asian leopard cat (*Prionailurus bengalensis*) cells, transferred to domestic cat oocytes, were able to implant and form foetuses (Yin et al. 2006), which demonstrates the potential of interspecific cloning in felids.

Unlike tiger embryos, Bengal embryos did not show differences in *in vitro* development with respect to the domestic cat. It seems like the hybrid embryos behaved as the homospecific ones. This result was previously observed in gaur-cattle embryos produced by iSCNT after fusion of gaur-cattle hybrid cells with cattle oocytes. In this case, their developmental potential was similar to cattle control embryos (Mastromonaco et al. 2007). This reported work and our results suggest that the recognition of cytoplasmic components by the nucleus is essential for embryo development, and it is enough that only a portion of the nuclear genome corresponds to the inherited cytoplasm.

To better understand the effects of aggregation on embryo development, we determined the number of blastomeres at day 2 of culture and we observed that cleavage velocity was influenced by the aggregation. Most of the embryos from the 1X groups had undergone three cell divisions by the time of evaluation, whereas this dynamic differed in aggregated embryos in which more embryos with more than three cell divisions were observed. Our hypothesis refers to the capacity of the aggregated embryos to reach 5–8 blastomeres before the non-aggregated ones, moment in which embryonic gene activation takes place (Hoffert et al. 1997) and could augment cell division rates. This tendency was also observed in blastocysts. The increase in blastocyst cell numbers shows the complementary effect of aggregated embryos, which improves embryo quality by correcting gene expression (Balbach et al. 2010; Moro et al. 2015) and enhances pregnancy and birth rates in other species (Boiani et al. 2003; Pedersen et al. 2005; Ribeiro et al. 2009; Gambini et al. 2012).

In summary, this is the first time that three cloning techniques were compared in the cat resulting all of them efficient to generate cat embryos. In spite of this, ZP-free cloning was found to enhance fusion rates, expanded blastocyst rates and blastocyst cell number in this species with potential to be used in wild felid cloning. Moreover, we demonstrated that Bengal and tiger cells can be reprogrammed using domestic cat oocytes by ZP-free iSCNT, obtaining embryos at the blastocyst stage with detectable expression of OCT4. In addition, the developmental ability and blastocyst cell number were improved in the tiger by embryo aggregation. With these achievements, it would be possible to isolate wild felid embryonic stem cells from interspecific embryos and contribute to species conservation.

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Conflict of interest

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

Author contributions

We declare that all listed authors have made substantial contributions to the research design, or the acquisition, analysis or interpretation of data; and to drafting the paper or revising it critically and that all authors have approved the submitted version. We also declare that

nobody who qualifies for authorship has been excluded from the list of authors. L.N. Moro designed the study, acquired and analysed the data, and drafted the paper; J. Jarazo and C. Buemo collaborated with the cloning experiment, A.J Sestelo obtained and frozen the cell lines, and D. Salamone designed the study, analysed the data and drafted the paper, revising it critically.

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