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Evaluation of Cheetah and Leopard Spermatozoa Developmental Capability after Interspecific ICSI with Domestic Cat Oocytes

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Contents
The ICSI procedure is potentially of great value for felids, and it has not been extensively studied in these species. The objectives of this work were to determine the best conditions for ICSI in the domestic cat (DC) to generate interspecific embryos by injecting cheetah (Ch) and leopard (Leo) spermatozoa. Firstly, DC oocytes were matured with insulin–transferrin–selenium (ITS) or without it (MM) and cultured using atmospheric (21%) or low (5%) oxygen tension after ICSI. The group ITS-5%O_2 showed the highest blastocyst rate ($p < 0.05$), 20.9% vs 8.7%, 7% and 6.5%, for MM-21%O_2, MM-5%O_2 and ITS-21%O_2, respectively. The best conditions were used to generate the interspecific embryos, together with ionomycin activation (Io) after ICSI. Interspecific embryos resulted in high rates of blastocysts that were not positively affected by Io activation: 32.6% vs 21% for Ch and Ch-Io, 9.8% vs 21% for Leo and Leo-Io, and 20% vs 17.4% for DC and DC-Io. We also evaluated DNA-fragmented nuclei of experiment 1 and 2 blastocysts, using TUNEL assay. The fragmented nucleus proportion was higher in the ITS-5%O_2 group, 67.6%. Surprisingly, interspecific blastocysts showed the lowest fragmented nucleus proportion: 27% and 29.9% for Ch and Leo, respectively. We concluded that ITS and 5%O_2 improve blastocyst formation in DC, although with a concomitant increase in DNA fragmentation. Most importantly, cheetah and leopard spermatozoa were able to generate blastocysts without artificial activation, which suggests that developmental capacity of wild felid spermatozoa can be evaluated by interspecific ICSI. This technique should be used to assist wild felid reproduction.

Introduction

Assisted reproductive techniques (ART) have been widely used in many mammalian species with different objectives. One of these techniques is intracytoplasmic sperm injection (ICSI, Palermo et al. 1992), which has improved fertilization rates when the quality of semen is poor (Donoghue et al. 1992; Penfold et al. 2003). Low semen quality is observed in several wild felids (Wildt et al. 1988) and limits the application of artificial insemination or *in vitro* fertilization (IVF, Wildt et al. 1992). Two of the species with this problem are the cheetah (*Acinonyx jubatus*) and the leopard (*Panthera pardus*). High incidence of structural pleomorphism and abnormal spermatozoa was observed in both species, caused by a genomic homozygosity among individuals. It was shown that cheetah and leopard ejaculates contained 71% and 80% morphologically abnormal spermatozoa, respectively, compared with 29.1% in the domestic cat (DC, *Felis catus*, Wildt et al. 1983, 1988). As the ICSI allows the selection of the spermatozoon that would be injected, morphologically normal spermatozoa can be selected, even from teratospermic samples (Penfold et al. 2003). For this reason, ICSI could be relevant to assist reproduction and preserve the existing genetic biodiversity in these species and in other wild felids.

Due to the similarities between wild felid species and the DC (*Felis catus*), the improvement of embryo production with ICSI in the DC or the generation of interspecific embryos using DC oocytes, may contribute to the preservation of wild felid species, most of which are considered vulnerable, threatened or endangered (Nowell and Jackson 1996).

It is well established that DC oocytes can be fertilized by ICSI with testicular or ejaculated sperm (Comizzoli et al. 2006). The first studies published on ICSI in cats demonstrated that the zygotes generated can develop to the blastocyst stage *in vitro* and can result in the birth of normal kittens after embryo transfer (Pope et al. 1998; Gomez et al. 2000). However, there are still opposing opinions about the need to induce artificial activation of the oocytes after ICSI in DC. Some authors have reported that artificial activation is necessary to restart the cell cycle of the oocytes (Bogliolo et al. 2001; Comizzoli et al. 2006), whereas others have observed embryo development without any activation treatment (Pope et al. 1998; Penfold et al. 2003).

Maturation medium and culture conditions are other parameters that need further study for felids. To optimize embryo production *in vitro*, oocytes and embryos need to be protected against oxidative stress (Thompson et al. 1990; Johnson and Nasr-Esfahani 1994). Previous studies in pigs have indicated that the reduction of oxygen tension from 20% to 10% or 5% during *in vitro* culture decreases the formation of reactive oxygen species, thus improving *in vitro* embryo development to the blastocyst stage (Karja et al. 2004; Kitagawa et al. 2004).

Another strategy is the supplementation of maturation and culture media with insulin–transferrin–selenium (ITS). Insulin is a hormone that promotes the uptake of glucose and amino acids. Transferrin serves as a carrier for iron, and it helps to reduce toxic levels of oxygen radicals and peroxide. Selenite is a cofactor for glutathione peroxidase that is used as an antioxidant in media (Ebert et al. 2006). ITS supplementation has been widely used in pigs (Lee et al. 2005; Jeong et al. 2008) and bovines (Córdova et al. 2010), but not yet in DC.

The objective of this work was first to determine the optimal conditions for ICSI in the DC, to use this technique and evaluate the developmental competence of interspecific ICSI embryos produced by the injection

of cheetah and leopard spermatozoa in DC oocytes. Moreover, leopard oocytes were injected by ICSI to generate homospecific leopard embryos. In this way, we could demonstrate the potential of the ICSI technique in wild felids.

Materials and Methods

Reagents

Except otherwise indicated, all chemicals were obtained from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Media were prepared weekly and filtered (22 μm, #4192 Acrodisc; Pall Corp., Ann Arbor, MI, USA).

Ethics for use of research animals

Animal manipulation was performed accordingly to the rules of the Direction of National Wildlife. The standards established by the code of ethics of ALPZA (American Association of Zoological Parks and Aquariums) were followed.

Experimental design

In the first experiment, we evaluated the development of DC embryos after ICSI or SHAM using four different conditions: (i) maturation medium without ITS (MM) and 21% oxygen tension during embryo culture (21%O_2), (ii) MM and 5% oxygen tension during embryo culture (5%O_2), (iii) MM supplemented with ITS and 21%O_2 and (iv) MM supplemented with ITS and 5%O_2. The ICSI groups were the following: MM-21%O_2, MM-5%O_2, ITS-21%O_2 and ITS-5%O_2. The control SHAM groups were the following: MM-21%O_2^{CT}, MM-5%O_2^{CT}, ITS-21%O_2^{CT} and ITS-5%O_2^{CT}. In the second experiment, we evaluated *in vitro* development of interspecific ICSI embryos generated by the injection of cheetah or leopard spermatozoa in DC oocytes, using the best conditions according to the results of experiment 1, and with ionomycin activation (Io) assistance after sperm injection. The experimental groups were Ch and Ch-Io (when cheetah spermatozoa were used), Leo and Leo-Io (when leopard spermatozoa were used), ITS-5%O_2 and ITS-Io-5%O_2 (when DC spermatozoa were used), ITS-5%O_2^{CT} and ITS-Io-5%O_2^{CT} (control sham groups). We also determined total cell numbers and DNA fragmentation by the terminal deoxynucleotidyl transferase (TdT) nick end labelling assay (TUNEL) of DC ICSI blastocysts from all treatments evaluated in experiment 1, and using cheetah and leopard spermatozoa. Finally we had a clinical case of a female leopard that had to be subjected to ovariohysterectomy, we recovered the oocytes from the ovary and performed homospecific ICSI in this specie using the same semen as in experiment 2.

Oocyte collection and *in vitro* maturation

Domestic cat ovaries were recovered from queens subjected to ovariectomy and transported to the laboratory within 2 h. They were washed in Tyrode's albumin lactate pyruvate buffered with HEPES (TALP-H, Bavister and Yanagimachi 1977). Domestic cat cumulus–oocyte complexes (COCs) were released from the follicles by repeatedly puncturing and scraping the ovaries. Leopard ovaries were obtained from a female with endometrial hyperplasia that was subjected to ovariohysterectomy after unsuccessful antibiotic treatments. Leopard COCs were released from visible antral follicles by aspiration and from non-visible follicles by puncturing and scraping the ovaries. The maturation medium was TCM 199 (31100-035; Gibco, Grand Island, NY, USA) containing 1 IU/ml HCG, 10 ng/ml ECG, 2.2 mM calcium lactate, 0.3 mM pyruvate (P2256), 0.3% wt/vol BSA and 3% vol/vol antibiotic–antimycotic (ATB; penicillin, streptomycin and amphotericin B; 15240-096; Gibco). Depending on the experimental group, supplementation was carried out with 1 μl/ml ITS (0.00067 g/l sodium selenite, 11.00 g/l sodium pyruvate, 1 g/l insulin and 0.55 g/l transferrin; 51300-044 Gibco). *In vitro* maturation conditions were 6.5% CO_2 in humidified air at 39°C. The oocytes were incubated in 100 μL droplets of medium covered with mineral oil (M8410). After 22–24 h of IVM, the oocytes were denuded of cumulus cells by vortexing for 2 min in hyaluronidase (H4272, 1 mg/ml TALP-H), and nuclear maturation was assessed by observation of the first polar body. Only oocytes with a first polar body were used for the experiments.

Semen collection and processing

Domestic cat spermatozoa were collected from the epididymides of adult cats. Briefly, the epididymides were removed from the testis, repeatedly cut and submerged into cryopreservation medium (AndroMed; 13503-0200; Minitube, Tiefenbach, Germany) for 30 min. The supernatant was recovered and centrifuged once at 490 G for 5 min. The pellet was removed, diluted with cryopreservation medium, stocked in straws of 0.25 ml and then frozen in N_2 vapour for 7 min before storing in N_2 liquid. Sperm motility was evaluated before and after freezing, and only those samples with more than 60% of progressive motility were used. Cheetah and leopard samples were obtained from the genetic bank of the Buenos Aires Zoo and were stored in liquid N_2 for several years until they were used in this work. These samples were obtained by electroejaculation. Semen collection was performed under surgical anaesthesia using xilazine (Xilazine 100, Richmond Vet Pharma, Buenos Aires, Argentina) plus ketamine hydrochloride (Ketonal 100, Richmond Vet Pharma, Buenos Aires, Argentina). Xilazine effects were reversed by injection of yohimbine hydrochloride 1% (Reverze, Vetcross, Portinco SA, Montevideo, Uruguay). The dosages used for the leopard were xilazine 100 mg IM, ketamine 180 mg IM, yohimbine 0.125 mg/kg half EV and half IM. The dosages used for the cheetah were xilazine 80 mg IM, ketamine 120 mg IM, yohimbine 0.125 mg/kg half EV and half IM. Electroestimulation was carried out as described previously by Howard 1993 with some modifications. Two different rectal probes were used, one (no. 3.5) with 17.5 cm length and 13.25 mm diameter (P.T.

Electronics, Boring, OR, USA) with three longitudinal electrodes (36.4 mm) for cheetah, and other (# C) with 23.0 cm length and 15.0 mm diameter with two circular electrodes (15.0 mm each, 20.0 mm from tip and separated 18.0 mm each other) for leopard. The rectal probes were placed in the rectum above the prostate and bulbourethral glands and voltage applied. A total of 80 electrical stimuli were delivered using a 50/60 Hz, 220 Volt AC, stimulator (P.T. Electronics). Stimulations from 2 V to 5 V were delivered in sets of 10 each, and a total sequence was divided into three series. Semen was collected in warmed sterile polypropylene 25 ml cups. The use of these big cups made the process easier and more efficient. For sperm cryopreservation, two different cryodiluents were used: (i) TEST, which consists of 4.83% Tes (Sigma, T-1375), 1.15% Tris (Gibco, 15504-0125), 0.4% glucose (Mallinckrodt, Dublin, Ireland, 4912), 200 UI penicillin/ml, 200 μg streptomycin/ml, 20% egg yolk and 4% glycerol (Merck, Darmsdadt, Germany, 4096) was used for Cheetah and (ii) PDV-62, which contains 11% lactose (Mallinckrodt, 5652), 200 UI penicillin/ml, 200 μg streptomycin/ml, 20% egg yolk and 4% glycerol was used for leopard. For refrigeration, temperature was lowered from 20°C to 5°C over a period of 120 min. After refrigeration period, straws were frozen in N_2 vapour for 10 min before storing in N_2 liquid.

Intracytoplasmic sperm injection (ICSI)

For ICSI procedures, straws of frozen sperm were thawed by immersion in a 37°C water bath for 30 s. 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). After centrifugation, spermatozoa were resuspended with BO containing 10 mg/ml fatty acid-free bovine serum albumin (A6003). Sperm was microinjected into 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 injection pipettes used had an inner diameter of 7 μm. One droplet of sperm-polyvinylpyrrolidone (PVP, 99219; Irvine Scientific, Irvine, CA, USA) suspension and one droplet of 80 μl of TALP-H with the denuded oocytes were placed in a 100 × 20 mm petri dish (Corning, New York, NY, USA 430167) under mineral oil (M8410). A DC, cheetah or leopard spermatozoon with progressive motility was immobilized by breaking the tail; it was aspirated tail-first into the injection pipette and moved to the drop with the oocytes. The microinjection pipette was pushed through the zona pellucida into the cytoplasm of a DC or leopard oocyte with the polar body at the 12 O'clock position, and the aspiration was used to break the oolemma. The spermatozoon and aspirated ooplasm were then expelled into the oocyte with a minimal volume of PVP. As negative controls, we performed the same injection but without any spermatozoon (SHAM). For experiment 2, after ICSI or SHAM, one group of presumptive zygotes was immediately cultured as described below, and another group was activated by incubation with 5 μM Io (I24222; Invitrogen, Carlsbad, California, USA) in TALP-H for 4 min before culture.

In vitro culture

ICSI and SHAM presumptive zygotes were cultured in 50 μL droplets of Synthetic Oviductal Fluid (SOF, Tervit et al. 1972; Holm et al. 1999) supplemented with 2.5% v/v foetal bovine Serum (FBS), in a humidified atmosphere of 21% O_2 and 5% CO_2 in air, or 5% O_2, 5% CO_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 was evaluated on day 2 and blastocyst formation on day 7 and 8 post-injection.

TUNEL and confocal microscopy

DNA fragmentation was detected by TUNEL assay (DeadEnd™ Fluorometric TUNEL System; Promega G3250, Madison, WI, USA). Following fixation for 20 min in 4% (v/v) paraformaldehyde (F-1635), blastocysts were washed for 30 min in 0.4% (v/v) BSA in PBS and then permeabilized by 15 min of incubation in 0.2% (v/v) Triton X-100 (T-9284) in PBS. The next step consisted of 15 min of incubation in the equilibration buffer contained in the kit and then 2 h at 39°C in incubation buffer (equilibration buffer, nucleotide mix and terminal deoxynucleotidyl transferase enzyme, rTdT). 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 coverslip and stored at 4°C for 24 h before fluorescence microscopic evaluation. Positive controls were generated by incubation in 50 U/ml DNase (prior to permeabilization) to induce DNA fragmentations, and negative controls were generated by omitting the rTdT enzyme from the reaction. Embryos 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 detect green fluorescence of fragmented nuclei (fluorescein-12-dUTP) 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 embryo, and three-dimensional images were constructed using the software EZ-C1 2.20. Total cell numbers and TUNEL positive cells (TUNEL+) were counted. To determine the DNA-fragmented nucleus index, the mean TUNEL+ cell number of each group was divided by the mean blastocyst cell number.

Statistical analysis

In vitro embryo development was compared by non-parametric Fisher's exact test. Differences in total cell numbers were analysed using Proc Mixed, considering heterogeneity of variances and setting degrees of freedom by Kenward-Roger. For these statistical analyses, the SAS programme was used (SAS Institute 1989). The proportion of fragmented nuclei over total cell numbers (DNA-fragmented nucleus index) was analysed by "Difference of proportions test" using InfoStat

software 2007 version. In all cases, differences were considered significant at $p < 0.05$.

Results

Preliminary observations

We compared the nuclear maturation efficiency of the MM and the MM supplemented with ITS. No differences were observed between them in the percentage of matured oocytes, 47.4% for MM (n = 348) and 46.1% for MM supplemented with ITS (n = 308). A total of 1567 oocytes were used for the experiments subsequently detailed.

In vitro development of domestic cat embryos after ICSI and SHAM treatments

Table 1 shows the development of ICSI and SHAM embryos exposed to different conditions during oocyte maturation and embryo culture. The cleavage rates were lower ($p < 0.05$) in the ITS-21%O_2 group (35.7%) respect to the other three ICSI groups (52.2%, 55.6% and 56.8% for MM-21%O_2, MM-5%O_2 and ITS-5%O_2, respectively). The SHAM groups MM-5%O_2^{CT} and ITS-5%O_2^{CT} showed statistically lower cleavage rates (10.5% and 28.4%) than the ICSI groups treated with the same conditions. Respect to blastocyst development, all the groups showed blastocyst formation and the highest blastocyst rates (36.7% respect to cleaved embryos and 20.9% respect to total embryos) were observed in the group matured with ITS supplementation and cultured with low oxygen tension. Otherwise, no blastocyst development was observed in any of the SHAM groups.

In vitro development of interspecific ICSI embryos assisted or not with ionomycin activation

The results regarding the development of interspecific ICSI embryos using cheetah and leopard spermatozoa assisted or not with ionomycin activation, are summarized in Table 2 (Fig. 1). The interspecific groups showed similar or higher cleavage rates than the homospecific control group using DC spermatozoa. We observed that Io activation increased cleavage ($p < 0.05$) in Leo-Io (73.7%) and ITS-Io-5%O_2 (69.7%) groups respect to Leo (35.3%) and ITS-5%O_2 (43.5%) groups. The same effect was observed for control SHAM groups, and cleavage rates were 19.2% vs 65.3% for ITS-5%O_2^{CT} and ITS-Io-5%O_2^{CT}, respectively. Interspecific embryos using cheetah spermatozoa showed similar cleavage rates regardless Io activation, 66.3% (Ch) vs 73.6% (Ch-Io). Respect to blastocyst rates, it was notorious that interspecific embryos reached the blastocyst stage likewise DC embryos, 32.6%, 9.8% and 20% for Ch, Leo and ITS-5%O_2 groups, respectively. Despite the observed differences in cleavage, blastocyst formation was not positively affected by Io activation in any of the ICSI groups, 21%, 21% and 17.4% for Ch-Io, Leo-Io and ITS-Io-5%O_2 groups, respectively. However, blastocyst formation was observed when SHAM embryos were assisted with Io after injection, 0% vs 6.9% for ITS-5%O_2^{CT} and ITS-Io-5%O_2^{CT} groups, respectively.

Total cell numbers and DNA fragmentation in ICSI blastocysts evaluated by TUNEL assay

Blastocysts obtained after homospecific and interspecific ICSI were evaluated to determine blastocyst cell numbers and the presence of fragmented nuclei by TUNEL assay (Table 3, Fig. 2). DC blastocyst cell numbers were similar among the different treatments evaluated, although the group MM-21%O_2 showed the highest one (177.8 ± 28.7, 105.9 ± 16.7, 128.6 ± 18.3 and 129.4 ± 17.9 for MM-21%O_2, MM-5%O_2, ITS-21%O_2 and ITS-5%O_2 groups, respectively). Moreover, interspecific blastocysts showed no differences respect to DC blastocysts generated by the same treatment, 174.6 ± 22.8 and 100.2 ± 12.6 for Ch and Leo groups, respectively. With respect to DNA fragmentation, the DNA-fragmented nucleus index was statistically higher in the DC blastocysts matured with ITS supplementation and cultured with low oxygen tension, respect to the other treatments (43.5%, 36.5%, 34% and 67.6% for MM-21%O_2, MM-5%O_2, ITS-21%O_2 and ITS-5%O_2 groups, respectively). Interspecific blastocysts showed statistically lower DNA fragmentation than the DC without statistical differences between them, despite having received the same treatment (27% and 29.9% for Ch and Leo groups, respectively).

Table 1. Development of domestic cat embryos after ICSI and SHAM treatments

Methods	Groups	n	Cleaved (%)	Blastocyst production (%) from	
				Cleaved embryos	Total embryos
ICSI	MM-21%O_2	138	72 (52.2)^{ac}	12 (16.7)^{a}	(8.7)^{a}
	MM-5%O_2	142	79 (55.6)^{a}	10 (12.7)^{a}	(7)^{a}
	ITS-21%O_2	154	55 (35.7)^{b}	10 (18.2)^{a}	(6.5)^{a}
	ITS-5%O_2	206	117 (56.8)^{a}	43 (36.7)^{b}	(20.9)^{b}
SHAM	MM-21%O_2^{CT}	60	23 (38.3)^{bc}	0^{c}	0^{c}
	MM-5%O_2^{CT}	76	8 (10.5)^{d}	0^{c}	0^{c}
	ITS-21%O_2^{CT}	73	25 (34.2)^{b}	0^{c}	0^{c}
	ITS-5%O_2^{CT}	67	19 (28.4)^{b}	0^{c}	0^{c}

MM, maturation medium without ITS; ITS, maturation medium supplemented with ITS; 21%O_2, embryo culture with atmospheric oxygen tension; 5%O_2, embryo culture with 5% oxygen tension.
(a,b,c,d) Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's exact test).

Table 2. *In vitro* development of interspecific ICSI embryos assisted or not with ionomycin activation

Methods	Groups	N	Cleaved (%)	Blastocysts (%)
ICSI	Ch	98	65 (66.3)[a]	32 (32.6)[a]
	Ch-Io	91	67 (73.6)[a]	19 (21)[ab]
	Leo	51	18 (35.3)[b]	5 (9.8)[bc]
	Leo-Io	57	42 (73.7)[a]	12 (21)[ab]
	ITS-5%O_2	85	37 (43.5)[b]	17 (20)[ab]
	ITS-Io-5%O_2	109	76 (69.7)[a]	19 (17.4)[b]
SHAM	ITS-5%O_2^{CT}	52	10 (19.2)[c]	0[d]
	ITS-Io-5%O_2^{CT}	72	47 (65.3)[a]	5 (6.9)[cd]

Ch, interspecific embryos generated with cheetah spermatozoa; Leo, interspecific embryos generated with leopard spermatozoa; MM, maturation medium without ITS; ITS, maturation medium supplemented with ITS; Io, ionomycin activation after sperm injection; 21%O_2, embryo culture with atmospheric oxygen tension; 5%O_2, embryo culture with 5% oxygen tension.
(a,b) Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's exact test).

Oocyte recovery and embryo development after ICSI using leopard oocytes

A total of 9 COCs (grade 1 = 5, grade 2 = 3 and grade 3 = 1) were recovered from 10 visible antral follicles, and six more (grade 1 = 1, grade 2 = 1 and grade 3 = 4) were obtained after scraping the ovaries. Maturation rate was very low if we consider total oocytes (4/15, 27%), but maturation rate was similar to the DC (4/10, 40%) if we consider only grade 1 and 2 oocytes (as we do for the DC). After ICSI, 2 of the 4 injected oocytes extruded the second polar body and one of them was cleaved on day 2. A condensed sperm head was observed inside the other two injected oocytes.

Discussion

This work showed significant findings to be considered for the ICSI technique in cats and wild felids. Firstly, we used different conditions to generate domestic cat embryos by ICSI to evaluate the best protocol for *in vitro* embryo development. To our knowledge, ITS supplementation had never before been tested in cat oocytes and only one report has evaluated the variation in gas composition during cat embryo culture (Johnston et al. 1991). Studies in pigs and buffalo oocytes have shown that ITS addition during oocyte maturation improves the developmental competence of the embryos (Raghu et al. 2002; Jeong et al. 2008). Respect to oxygen tension, little information is available on its influence on *in vitro* development of felid embryos. Our experiments showed that ICSI blastocyst rate was enhanced when the oocytes were matured with ITS, and the embryos were cultured in low oxygen tension (ITS-5%O_2 group), suggesting a synergistic effect between ITS and 5%O_2. It is important to note that the absence of blastocysts in the SHAM groups allowed us to ensure that the blastocysts obtained after ICSI were the consequence of sperm fertilization and not the result of parthenogenetic activation. Besides being an antioxidant agent, the ITS has demonstrated to facilitate sperm chromatin decondensation (Jeong et al. 2008). An increase on male pronucleus formation rate following IVF was observed in pigs when ITS was used. This result was attributed to an increase in glutathione concentration (Jeong et al. 2008), which is an agent that reduces the sperm nuclear disulphide bonds after sperm penetration. Moreover, higher blastocyst rates were obtained when the intracellular glutathione content of the oocytes was increased during maturation (De Matos and Furnus 2000).

In the second experiment, we generated interspecific embryos by injecting cheetah and leopard spermatozoa in DC oocytes using the best conditions according to the results previously obtained. The objective of this experiment was to demonstrate the cheetah and leopard spermatozoa developmental capability after ICSI and the potential of this technique in wild felids. Moreover, we evaluated whether ionomycin activation post-injection

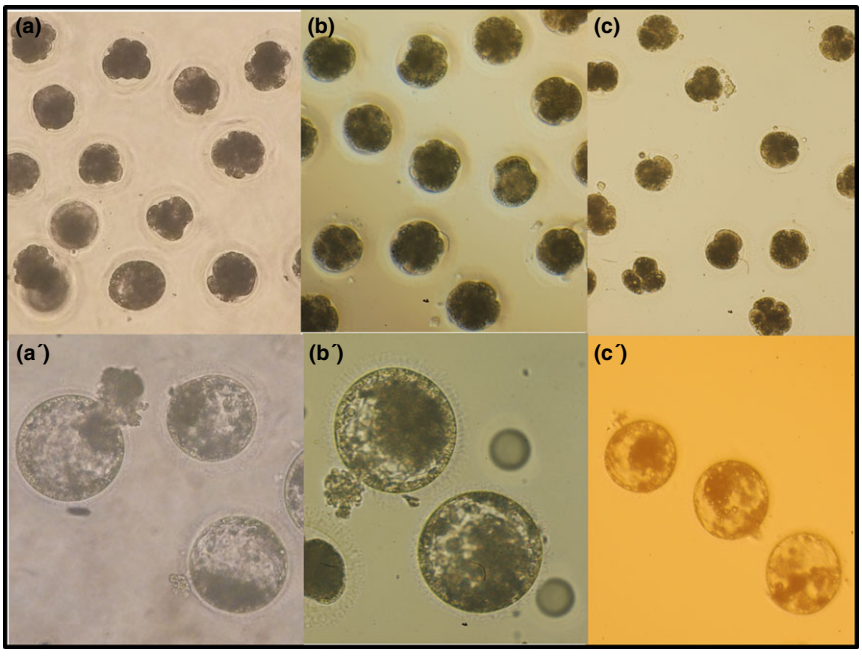

Fig. 1. (a) day 2 interspecific embryos generated by ICSI with cheetah spermatozoa and DC oocytes, (a′) day 7 interspecific embryos generated by ICSI with cheetah spermatozoa and DC oocytes, (b) day 2 interspecific embryos generated by ICSI with leopard spermatozoa and DC oocytes, (b′) day 7 interspecific embryos generated by ICSI with leopard spermatozoa and DC oocytes, (c) day 2 DC ICSI embryos (c′) day 7 DC ICSI embryos

Table 3. Evaluation of cell number and DNA-fragmented nuclei by the TUNEL assay in ICSI blastocysts obtained by different treatments

Groups	n	Blastocyst cell number Mean ± SEM	TUNEL+ cells Mean ± SEM	DNA-fragmented nucleus index*
MM-21%O_2	6	177.8 ± 28.7^{a}	77.3 ± 12.2^{a}	43.5^{a}
MM-5%O_2	7	105.9 ± 16.7^{b}	38.7 ± 3.1^{b}	36.5^{b}
ITS-21%O_2	10	128.6 ± 18.3^{ab}	43.7 ± 8.5^{b}	34^{be}
ITS-5%O_2	9	129.4 ± 17.9^{ab}	87.4 ± 11.4^{a}	67.6^{c}
Ch	9	174.6 ± 22.8^{a}	47.2 ± 5.9^{b}	27^{d}
Leo	5	100.2 ± 12.6^{b}	30 ± 7^{b}	29.9^{de}

MM, maturation medium without ITS; ITS, maturation medium supplemented with ITS; 21% O_2, embryo culture with atmospheric oxygen tension; 5% O_2, embryo culture with 5% oxygen tension; Ch, interspecific embryos generated with cheetah spermatozoa; Leo, interspecific embryos generated with leopard spermatozoa. (a,b,c,d,e) Values with different superscripts in a column are significantly different. For blastocyst cell number and TUNEL cells, Proc Mixed was applied ($p < 0.05$). For the DNA-fragmented nucleus index, the difference of proportions test was applied ($p < 0.05$).
*To calculate the DNA-fragmented nucleus index, the mean TUNEL+ cell number of each group was divided by the mean blastocyst cell number.

improved embryo development to the blastocyst stage. While ionomycin is an effective activation stimulator of mammalian oocytes, it has not been previously used to assist ICSI in cats, for which ethanol has most commonly been used to activate development following injection (Bogliolo et al. 2001; Comizzoli et al. 2006; Waurich et al. 2010; Ringleb et al. 2011). In some mammalian species such as rabbits, mice and humans, sperm injection is sufficient to activate oocytes and initiate embryo development (Keefer 1989; Van Steirteghem et al. 1993; Kimura and Yanagimachi 1995). In others such as bovines and porcines, sperm pretreatments or artificial activation after sperm injection are needed to induce sperm head decondensation and subsequent embryo development (Chung et al. 2000; Tian et al. 2006). In the DC, it is still controversial whether activation is necessary to improve embryo development. In the first experiment, we elucidated that there was no need to activate DC embryos after ICSI to obtain blastocysts in our hands, but we did not know whether cheetah or leopard spermatozoa would need extra stimulation to fertilize DC oocytes or DC oocytes would need extra stimulation to be activated by heterospecific spermatozoa. Surprisingly, interspecific embryos developed as well as DC embryos without any artificial assistance. After this essay, we concluded that chemical activation was not necessary neither for DC ICSI nor interspecific ICSI. These results are of considerable interest, demonstrating that the spermatozoon alone is enough to activate domestic cat oocytes by ICSI and has the capability to form blastocysts. In addition, Ch-DC embryos had higher blastocyst rates than Leo-DC embryos. This observation could be attributed to a differential fertilizing capability of each species, may be given by distinct PLCZ activities (Bedford-Guaus et al. 2011), or to the differential phylogenetic distance between the cheetah vs the DC respect to the leopard vs the DC (Johnson et al. 2006). The good results obtained using leopard spermatozoa for interspecific ICSI, allowed us to assume that the lower rates of development using leopard oocytes resulted from a maternal factor, may be given by the poor clinical conditions of the animal. Despite this, it was possible to fertilize leopard oocytes by ICSI and get a cleaved embryo.

To determine the quality of the homospecific and interspecific ICSI blastocysts generated, we evaluated the total cell numbers and the proportion of fragmented

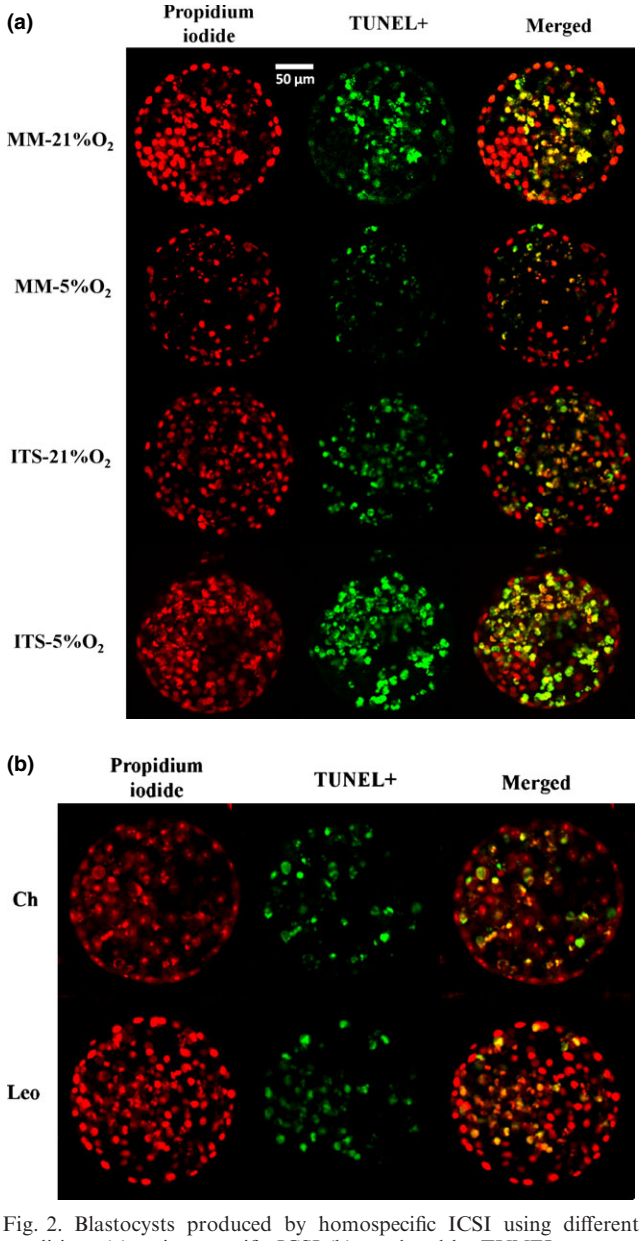

Fig. 2. Blastocysts produced by homospecific ICSI using different conditions (a) or interspecific ICSI (b), analysed by TUNEL assay to determine the presence of DNA fragmentation. TUNEL positive cells (TUNEL+) are labelled with fluorescein-12-dUTP (green), and nuclei are counterstained with propidium iodide (red)

nuclei, related to the apoptosis process. All the blastocysts evaluated showed TUNEL+ labelling with different fragmented nucleus index. It has been demonstrated that spontaneous apoptosis has certain roles during pre-implantation embryo development (Hardy 1997; Betts and King 2001) and occurs normally in *in vitro* produced blastocysts cultured either in high or low oxygen tension (Byrne et al. 1999; Matwee et al. 2000), but inadequate or exacerbated apoptosis at pre-implantation stages could affect the post-implantation developmental potential. Among the ICSI groups, the DNA-fragmented nucleus index was higher in the ITS-5%O_2 group. It is noteworthy that this group showed the highest blastocyst rates. These results indicate that ITS and 5%O_2 better promote blastocyst formation, but induce more DNA fragmentation. However, interspecific embryos that were equally treated with these conditions showed the lowest fragmented nucleus index among all the groups. This was an unexpected observation. Although DNA fragmentation is not a determinant of developmental incompetence, it is an indicative of cell stress. We believe that the study of *in vivo* cat embryo development could contribute to an understanding of the normal pattern of DNA fragmentation in domestic cat blastocysts and the reasons, which lead to an increase in this parameter.

In summary, ITS supplementation and low oxygen tension enhance the developmental capacity of DC ICSI embryos *in vitro*, although with an accompanying increase in DNA fragmentation. The most important finding of this work was that cheetah and leopard spermatozoa were able to fertilize DC oocytes, obtaining good rates of blastocysts without any chemical assistance. This study shows that fertilizing capability of wild felid spermatozoa can be evaluated using ICSI with *in vitro*-matured DC oocytes. Finally, results suggest that ICSI is a promising technique to assist felid reproduction, especially applicable for inbred species with low-quality sperm.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

L.N Moro designed the study, acquired and analysed the data and drafted the paper; A.J Sestelo acquired the data and drafted the paper, and D. Salamone designed the study, analysed the data and drafted the paper, revising it critically.

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