Journal of Reproduction and Development, Vol. 57, No. 2, 2011

—Original Article—

Efficiency of Sperm-Mediated Gene Transfer in the Ovine by Laparoscopic Insemination, *In Vitro* Fertilization and ICSI

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Abstract. Transgenesis constitutes an important tool for pharmacological protein production and livestock improvement. We evaluated the potential of laparoscopic insemination (LI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) to produce *egfp*-expressing ovine embryos, using spermatozoa previously exposed to pCX-EGFP plasmid in two different sperm/DNA incubation treatments: "Long Incubation" (2 h at 17 C) and "Short Incubation" (5 min at 5 C). For LI, Merino sheep were superovulated and inseminated with treated fresh semen from Merino rams. The embryos were recovered by flushing the uterine horns. For IVF and ICSI, slaughterhouse oocytes were fertilized with DNA-treated frozen/thawed sperm. All recovered embryos were exposed to blue light (488 nm) to determine green fluorescent morulae and blastocysts rates. High cleavage and morulae/blastocysts rates accompanied the LI and IVF procedures, but no *egfp*-expressing embryos resulted. In contrast, regardless of the sperm/plasmid incubation treatment, *egfp*-expressing morulae and blastocysts were always obtained by ICSI, and the highest transgenesis rate (91.6%) was achieved with Short Incubation. In addition, following the incubation of labeled plasmid DNA, after Long or Short exposure treatments, with fresh or frozen/thawed spermatozoa, only non-motile fresh spermatozoa could maintain an attached plasmid after washing procedures. No amplification product could be detected following PCR treatment of LI embryos whose zonae pellucidae (ZP) had been removed. In order to establish conditions for transgenic ICSI in the ovine, we compared three different activation treatments, and over 60% of the obtained blastocysts expressed the transgene. For ICSI embryos, FISH analysis found possible signals compatible with integration events. In conclusion, our results show that in the ovine, under the conditions studied, ICSI is the only method capable of producing exogenous gene-expressing embryos using spermatozoa as vectors.
Key words: Green fluorescent protein, Sheep, Transgenesis

(**J. Reprod. Dev. 57:** 188–196, 2011)

Transgenesis in mammals constitutes an important tool for recombinant protein production [1, 2] and also for livestock improvement [3]. Currently, the challenge is to find a high efficiency and low-cost technique for producing transgenic animals. Microinjection of foreign DNA into the male pronuclei of zygotes [4] and nuclear transfer using genetically modified somatic donor cells [5], are two commonly used methods for the generation of transgenic animals. Transgenesis by microinjection is used in mice mainly for research purposes. This procedure has also proven to be efficient in rabbits, sheep and pigs [6], but it is quite dependant on proper visualization of the male pronucleus. In addition, nuclear transfer, using genetically modified somatic donor cells is not very efficient, which can be partly explained by epigenetic reprogramming failure [7].

Evidence of heterologous DNA interaction with mammalian spermatozoa and subsequent delivery into the oocyte during fertilization was first reported in 1971 [8]. Sperm-mediated gene transfer has opened the possibility for large-scale transgenic animal production both for basic research and biotechnological applications. Several authors have published alternative procedures to obtain transgenic embryos and offspring by means of LI [9], IVF [10] and ICSI [11]. These techniques are collectively called "sperm-mediated gene transfer" (SMGT). By LI and IVF, the generation of transgenic animals could be remarkably simple. However, results obtained so far in mice and domestic animals have been inconsistent [10, 12–16]. On the other hand, ICSI has resulted in the production of transgenic offspring in murine and porcine species, while Southern Blot and FISH analyses in resultant embryos and offspring have proven efficient in demonstrating successful gene integration [11, 17]. However, for successful large-scale application of transgenic ICSI to domestic species, it is critical to solve the ICSI fertilization problem associated with activation failure [18].

The ovine is a good model in the field of animal transgenesis and biotechnology due to its relatively short pregnancy period compared with the bovine. Moreover, its significant saliva and milk production make the salivary and mammary glands interesting targets for recombinant protein expression [19]. Nevertheless, to our knowledge, no work has explored the different variants of SMGT in the ovine. The objective of the present study was to produce exogenous gene-expressing sheep embryos by LI, IVF and ICSI

Received: April 16, 2010
Accepted: October 5, 2010
Published online in J-STAGE: November 10, 2010

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techniques using sperm incubated with DNA for a long (2 h to 17 C) or short (5 min to 0–5 C) period of time. In addition, the interaction between sperm and labeled DNA was analyzed by fluorescent microscopy in order to interpret differences between treatments. Presence of exogenous plasmid DNA in LI, IVF and ICSI embryos was determined by PCR, and localization of exogenous DNA into their nuclei was confirmed by FISH in ICSI-derived embryos. Chemical activation following nontransgenic ICSI has been reported to improve bovine embryo development [20–24]. We also demonstrated that the activation in transgenic ICSI improves embryo development [25], showing different outcomes according to the chemical activation treatments [26]. For this reason we compared three different activation treatments to assist the transgenic ICSI technique in the ovine.

Materials and Methods

Animal care and governmental authorization

Animal usage and care treatments were approved by the Institutional Committee of Care and Use of Laboratory Animals of the Buenos Aires University (Resolution No. 2007/15). The Secretariat of Agriculture, Livestock, Fisheries and Foods of Argentina, as counseled by the Comisión Nacional de Biotecnología Argentina (CONABIA), approved the animal confinement conditions for LI (Resolution No. 250).

Chemicals and media

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

DNA construction

The plasmid used was pCX-EGFP (kindly provided by Dr M Okabe, Osaka University, Osaka, Japan) that contains an enhanced green fluorescent protein gene (*egfp*) under chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control [27]. The same stock of plasmid was used for all the experiments.

Sperm/DNA incubation

Sperm/pCX-EGFP incubation was carried out in accordance with two procedures 1) In the first procedure, long sperm/DNA incubation (Long Incubation), fresh or frozen/thawed semen (pooled from eight rams) was treated as previously described [28], with slight modifications. Briefly, collected (Fresh) or thawed semen straws (Frozen/Thawed) were diluted 1:10 v/v in a pre-warmed extender SFM [28] (5 ml of fresh semen in 45 ml or 500 μl of frozen/thawed semen in 4.5 ml). Seminal plasma (fresh semen) or cryoprotectant (frozen/thawed semen) were removed by centrifugation at 800 × *g* in extender medium for 10 min (fresh semen) or at 495 × *g* for 5 min (frozen/thawed semen), at room temperature in both cases. Then, the supernatant was aspirated, and the sperm cells were resuspended in the same volume to repeat centrifugation under the same conditions. Finally, the spermatozoa were resuspended in 2 ml of extender medium at a concentration of 2500 $\times 10^6$ cells/ml (fresh semen) or in 200 μl of extender medium at a concentration of 100 $\times 10^6$ cells/ml (frozen/thawed semen) and mixed with pCX-EGFP plasmid (final concentration: 0.5 μg/million spermatozoa). The spermatozoa and pCX-EGFP plasmid were incubated at 17 C for 2 h. The flasks were inverted every 20 min to prevent sedimentation of sperm 2) In the second procedure, short sperm/DNA incubation (Short Incubation), sperm/plasmid incubations were carried out following a previously reported method [11] with slight modifications. Briefly, the pooled semen samples (fresh or frozen/thawed) were diluted and centrifuged as previously described, in 2.8% sodium citrate (F71497) medium containing 100 μM EDTA (15576-028, Invitrogen). The spermatozoa were resuspended in 2 ml sodium citrate medium at a concentration of 2500 $\times 10^6$ cells/ml (fresh semen) or in 200 μl of sodium citrate medium at a concentration of 100 $\times 10^6$ cells/ml (frozen/thawed semen) and incubated with 0.5 μg of the DNA construct/million spermatozoa (final concentration) for 5 min at 5 C (following cooling at a rate of 2 C/3 min). The same collected Fresh or Frozen/Thawed semen samples were used for the different treatments. The semen straws were frozen and thawed by standard procedures. Both, long and short sperm/DNA incubation (without previous washing procedures) and control spermatozoa (without plasmid incubation) were used for the LI, IVF or ICSI techniques as described below.

Experimental design

Three independent assays were carried out in order to evaluate exogenous gene-expressing ovine embryo production following the treatments described above. For LI, we used fresh sperm subjected to long or short sperm/DNA incubation to fertilize superovulated sheep. For the IVF and ICSI experiments, frozen/thawed sperm subjected to the same sperm-plasmid incubation treatments were used to fertilize oocytes collected from slaughterhouse ovaries. In order to analyze sperm/exogenous DNA interaction, we tested labeled-DNA adhesion to sperm membranes after Long and Short Incubation with fresh and frozen/thawed sperm. We used PCR and FISH analyses to determine transgene presence and localization in these embryos. Finally, in order to establish conditions for ICSI in the ovine, this study compared three different activation treatments to assist the ICSI technique. Each experiment was replicated at least three times.

In vivo embryo production

Superovulation treatments: Donor adult Merino sheep (n=17) were synchronized by insertion of an intravaginal sponge (60 mg of medroxyprogesterone acetate, Syntex; Lab. Syntex, Buenos Aires, Argentina) for 14 days. During days 12–14 of treatment, donor ewes were superovulated with six decreasing doses of FSH i.m. (18 mg × 2, 14 mg × 2, 8 mg × 2, NIH-FSH-P1, Folltropin®, Bioniche, Armidale, NSW, Australia) administered twice a day starting on the morning of Day 12, 48 h before pessary removal, and finishing 12 h after progestagen withdrawal. Progestagen removal was performed on Day 14, coinciding with the fifth FSH treatment, and a single dose of eCG (200 UI Novormon 5000, Lab. Syntex) was administered. All ewes were checked twice daily for the onset of estrus 24 h after sponge withdrawal using an adult ram.

Laparoscopic insemination (LI): Ewes in estrus were fasted 12 h prior to artificial insemination and randomly assigned to LI with fresh semen from the Long Incubation (n=9), Short Incubation (n= 8) or control (n=1) treatments. Merino rams (n= 8) of proven fertil-

ity were utilized as sperm donors. Ejaculates were collected using an artificial vagina, pooled in a water bath at 36 C and treated as described previously. LI was performed 12 h after estrus detection. Ewes were placed on a standard cradle for laparoscopic procedures. The surgical field, cranial to the udder, was shaved and disinfected. General anesthesia was administered with xylazine (0.5 mg/10 kg of Kensol 2%, Konig, Buenos Aires, Argentina) and ketamine (25 mg/10 kg of Ketalar; Parke-Davis, Buenos Aires, Argentina) and local anesthesia was applied (lidocaine, Frankaina 2%; FatroVon-Frankel, Buenos Aires, Argentina). Thereafter, an endoscope (Richard Wolf, Knittlingen, Germany) was inserted into the abdominal cavity through a trocar approximately 10 cm cranial to the udder and 5 cm to the left side of the midline for visualization of the uterine horns. A second trocar was inserted in the right side of the abdominal wall and used to deliver 200 μl of previously incubated or not (control) fresh sperm (500×10^6/ewe final concentration) into the uterine horns (100 μl each) using a cannula (Aspic for pellet insemination in sheep, 23-gauge needle, IMV; L'Aigle, France). Finally, the trocar orifices were treated with a local antibiotic solution (Young Plata, Quimagro, Buenos Aires, Argentina), and oxytetracycline (1 ml/10 kg, Hostaciclina LA; Hoechst, Dublin, Ireland) was given as preventative treatment.

Embryo recovery: Six days after estrus detection, embryos were collected from donors placed under general anesthesia, as described above. In brief, embryos were surgically obtained through prepubian laparotomy. Both uterine horns were flushed using a Foley catheter. The collection medium was prewarmed (38 C) commercial Complete Flush ViGro (Bioniche, Bogart, GA, USA). All the morulae and blastocysts collected from the different groups were kept separated for examination by fluorescence microscopy, as described below.

In vitro embryo production

Oocyte collection and *in vitro* maturation: Ovaries were collected from slaughterhouses and transported to the laboratory at ambient temperature. Cumulus-oocyte-complexes (COCs) were aspirated with 21-gauge needles from follicles with a diameter of 2 to 5 mm. They were collected into Dulbecco's phosphate buffered saline (DPBS, 14287-072; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, 013/07; Internegocios, Buenos Aires, Argentina) and 2% antibiotic-antimycotic (ATB, 15240-096; Gibco). Oocytes covered with at least 3 layers of granulosa cells were selected for *in vitro* maturation (IVM) in bicarbonate-buffered TCM-199 (31100-035; Gibco) containing 2 mM glutamine (G-8540), 10% FBS, 10 μg/ml follicle stimulating hormone (NIH-FSH-P1, Folltropin®, Bioniche), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% ATB. Oocytes were incubated in 500 μl of medium under mineral oil (M8410) in 4-well dishes (Nunclon®, Nunc, Naperville, IL, USA) in an atmosphere of 6.5% CO_2 in humidified air at 39 C for 24 h.

In vitro fertilization (IVF): Semen was collected from two Merino rams using an artificial vagina, and the samples were frozen by standard procedures [29] with Triladyl extender (Minitub GmBH, Landshut, Germany). Frozen semen was thawed in a 37 C water bath for 30 sec and treated as described in the "Sperm/DNA incubation" section. After incubation, an aliquot of sperm/DNA incubation mix was diluted (without any selection process) in Brackett-Oliphant (BO) medium [30] supplemented with 2.5 mM caffeine (C4144), 10 IU/ml heparin (H3149) and 5 mg/ml fatty acid-free bovine serum albumin (FAF-BSA) (A6003) at 20×10^6/ml. A sperm control group, without DNA incubation, was carried out in parallel. COCs were washed twice with BO medium containing 5 mg/ml FAF-BSA and subsequently exposed to the sperm suspension for 5 h in a 100 μl droplet at 39 C in a humidified atmosphere of 6.5% CO_2 in air. Presumptive zygotes were then washed tree times in TALP-H, and culture was continued as described below.

Intracytoplasmic sperm injection (ICSI): For ICSI, oocytes were vortexed (to remove cumulus cells) for 2 min [1 mg/ml hyaluronidase (H-4272) in DPBS] and washed tree times in HEPES-buffered (H4034) TCM-199. Mature oocytes were evaluated by visualizing the first polar body and immediately used for ICSI. Frozen semen were thawed in a 37 C water bath for 30 sec and subjected to either the Long or Short Incubation described above. After incubation, less than 0.05 μl of spermatozoa was transferred to a 4-μl droplet of TALP-H with 10% v/v of polyvinylpyrrolidone (PVP, 99219; Fisher Scientific, Pittsburgh, PA, USA) a used directly for ICSI, which was performed as described previously [25]. Briefly, the ICSI was performed in microdroplets of 20 μl of TALP-H under mineral oil (M8410) in 100 × 20 mm tissue culture dishes (430167; Corning, NY, USA) using Narishige hydraulic micromanipulators (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA). Each selected spermatozoon was immobilized by breaking its tail and aspirated tail-first into an 8-μm inner diameter injection pipette. The injection pipette was immediately passed through a microdroplet of TALP-H to remove any attached spermatozoa and then transferred to a droplet containing an oocyte. Metaphase II oocytes were held under negative pressure in the holding pipette, with the polar body at the 6 or 12 o'clock position. The microinjection pipette was pushed through the zona pellucida and into the cytoplasm of the oocyte at the 3 o'clock position. Aspiration was used to break the oolemma. The spermatozoon and aspirated ooplasm were then expelled into the oocyte with a minimal volume (less than 10 pl) of PVP. A sperm control group, without DNA incubation, was carried out in parallel.

Oocyte chemical activation

Injected oocytes were immediately activated in TALP-H with 5 μM ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) for 4 min and placed in TCM-199 for 3 h to allow second polar body extrusion (except for Io-EtOH activation, where the 3-h window is not necessary for polar body emission). Oocytes were subsequently treated with a) TCM-199 with 1.9 mM DMAP (D2629) for 3 h (Io-DMAP Group); b) 5 μM ionomycin followed immediately by TCM-199 with 1.9 mM DMAP for 3 h (2Io-DMAP Group); or c) TALP-H with 7% (v/v) ethanol for 5 min (Io-EtOH Group). In all the cases, inhibitors were removed by washing three times in TALP-H, and culture was continued as described below.

In vitro embryo culture

Presumptive IVF and ICSI zygotes (15–30 per group) were cul-

tured in 50-μl droplets of SOF medium [31] containing 2.5% FBS and incubated at 39 C in 6.5% CO_2 in air. Embryos were moved to a new droplet every 48 h. Cleavage was evaluated on day 2, and the numbers of morulae and blastocysts were evaluated on day 7 postfertilization.

Evaluation of EGFP fluorescence in embryos

On day 7 postfertilization, all embryos recovered *in vivo* and produced *in vitro* were briefly exposed to blue light using an excitation filter at 488 nm and an emission filter at 530 nm to determine *egfp* gene expression.

Sperm/exogenous DNA (eDNA) interaction

In order to analyze sperm/eDNA interaction, pCX-EGFP plasmid was labeled with Alexa fluor (F6257) by the Nick Translation System (18160-010; Invitrogen). Label eDNA was incubated in accordance with the long and short treatments described above. Afterwards, an aliquot of each labeled-DNA/spermatozoa complex was diluted (10 μl in 90 μl of prewarmed TALP-H medium) and centrifuged 5 min at 490 × *g*. Then, the supernatant was removed carefully, and the pellets were resuspended in 100 μl of TALP-H (washing procedure). Immediately, 10 μl of each sample (Fresh or Frozen-Thawed and washed or non-washed spermatozoa) were mounted between coverslips to observed under blue light the presence of label eDNA in sperm cells in order to distinguish between motile and immotile ones. Autoflorescence was discarded by checking the spermatozoa before incubation treatments and after unlabelled bovine genomic DNA fragment incubation treatments. A second experiment was performed by labeling bovine genomic DNA with rhodamine by the Nick Translation System (0.1 to 1 Kb fragments) and using it for both sperm incubation treatments. All experiments were repeated at least two times using pooled semen from two different rams.

Determination of sperm viability before and after exogenous DNA incubation

Fresh semen (from two rams) that had been treated according to the Long Incubation protocol were stained by incubation in TCM-199 containing 1 mg/ml of Hoechst 33258 (861405) for 2 min, both before and after pCX-EGFP addition. Then, a 10-μl aliquot was placed between coverslips to visualize spermatozoa. At least 100 spermatozoa were observed under epifluorescent microscopy (UV 380) and classified as i) damaged membrane (stained sperm cells) motile and immotile or ii) undamaged membrane (unstained sperm cells) motile and immotile. Control spermatozoa were coincubated with Hoechst, but not with plasmid.

PCR analysis

Morulae and blastocysts from LI, IVF and ICSI Short Incubation with and without zonae pellucidae (ZPs) were washed in PBS, transferred as 1-μl aliquots into an eppendorf tube, resuspended in 9 μl of extraction buffer (1 mg/ml proteinase K in 10 mM Tris–EDTA, V302B, Promega, Madison, WI , USA) and incubated at 56 C for 1 h. The samples were then heated at 95 C for 10 min, and 5-μl aliquots were used for PCR. The primer set sequences were 5–GAAGTTCGAGGGCGACACCTG–3 and 5–TCGTCCATGCCGAGAGTGATC–3 for amplifying a 269 bp fragment of EGFP. The PCR reaction conditions consisted of denaturation at 95 C for 2 min, followed by 35 amplification cycles of denaturation at 94 C for 15 sec; annealing at 55 C for 15 sec and extension at 72 C for 25 sec. Cycle 35 contained an additional extension at 72 C (7 min). The positive control consisted of 3.6^{-11} g/ml of pCX-EGFP plasmid, and the negative control was distilled water. The ZP was removed by 5 min incubation in 1.5 mg/ml pronase (P8811) in TALP-H, followed by careful washing five times in 3 ml of TALP-H.

Determination of the cell number in blastocysts or spermatozoa bound to embryos

Embryos were stained (2 min) in TCM-199 containing 1 mg/ml of Hoechst 33342 (B2261) and immediately mounted between coverslips to visualize spermatozoa bound to the ZP (LI embryos) or to count total nuclei (ICSI blastocysts) using epifluorescent microscopy (UV 380).

Fluorescence in situ hybridization (FISH)

Ovine 8-cell embryos were incubated for 20 h with 0.1 μg/ml demecolcine (D1925). Afterwards, the embryos were treated in a hypotonic solution (1% Na citrate in distilled water for 10 min) and fixed on a poly-L-lysine-coated slide with 3:1 methanol-acetic acid solution. Indirect labeled signals of FITC anti-mouse (F6257) and anti-digoxigenin (D8156), which bind the digoxigenin-labeled pCX-EGFP (5.5 kb) probe, were labeled using the Nick Translation System (18160-010; Invitrogen). The total DNA was counterstained with DAPI. Images of each cell and their signals were recorded with an Optronics camera (CCD; Optronics, Goleta, CA, USA).

Data analysis

Embryo development and fluorescent expression were compared by Fisher's exact test analysis. Differences in total cell number or stained sperm were analyzed using a one-way ANOVA test. For statistical analyses, the SAS program was used [32]. Differences were considered to be significant at $P<0.05$.

Results

Laparoscopic insemination, in vitro fertilization and intracytoplasmic sperm injection

A pool of semen collected from eight rams and incubated (long or short treatment) with pCX-EGFP plasmid was used to inseminate seventeen ewes. After six days, LI embryos were recovered through laparotomy and uterine flushing. Fertilization rates were not statistically different betweens Long and Short Incubation treatments and the control group (90.4, 98.5 and 100% respectively). None of the 78 collected embryos (38 and 40 for the long and short procedures, respectively) were fluorescent after observation under blue light (Table 1).

One hundred and sixty-two oocytes were fertilized by IVF with spermatozoa previously incubated with pCX-EGFP plasmid in the long or short treatments. Plasmid incubation groups were not different from the controls in terms of cleavage (long, short and

Table 1. Development and transgenesis rates after laparoscopic insemination (LI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with semen previously incubated with pCX-EGFP plasmid in the ovine

Incubation sperm/pCX-EGFP	Technique	Animals (n)	Oocytes[1] (n)	Morulae and blastocysts (%)	*Green* morulae and blastocysts (%)
Long	LI	9	42	38 (90.4)[a]	0 (0)[a]
	IVF	*na*	91	35 (38.4)[bc]	0 (0)[a]
	ICSI	*na*	25	6 (24.0)[c]	4/6 (66.6)[b]
Short	LI	8	44	40 (90.9)[a]	0 (0)[a]
	IVF	*na*	71	27 (38.0)[bc]	0 (0)[a]
	ICSI	*na*	82	36 (43.9)[b]	33/36 (91.6)[c]
Control [2]	LI	1	14	12 (85.7)[a]	*na*
	IVF	*na*	69	35 (50.7)[b]	*na*
	ICSI	*na*	44	20 (45.5)[b]	*na*

na: Not applicable. [1] Total structure recovered. [2] Without sperm/DNA incubation. [a, b and c] Percentages within columns with different superscripts are statistically different (Fisher's test; $P<0.05$). Within each group, no significant differences were observed between replicates ($P>0.05$, Fisher's test).

control: 86.8, 87.3 and 88.4%, respectively) or embryonic development (Table 1). Approximately 40% of the embryos reached the morula and blastocyst stages. Nevertheless, as in LI, no embryo showed *gfp* expression under blue light (Table 1).

A total of 107 oocytes were injected with spermatozoa incubated with pCX-EGFP plasmid. Independent of incubation treatment, high embryo development and transgene expression rates were obtained (Table 1). Although no differences were observed in cleavage rates (long, short and control: 80.0, 79.2 and 86.3%, respectively), a significant decrease in embryo development was found after the ICSI Long Incubation treatment compared with the Short Incubation treatment and control groups ($P<0.05$). Furthermore, the short sperm/DNA incubation treatment showed the highest percentage of exogenous gene-expressing embryos ($P<0.05$). Figure 1 shows three *egfp*-expressing blastocysts produced by the ICSI Short Incubation treatment.

Sperm/exogenous DNA interaction

Plasmid DNA attachment to the plasma membrane was observed in most of the frozen-thawed spermatozoa subjected to the Long and Short (Fig. 2A) Incubation treatments. Nevertheless, sperm/DNA cell complexes were quite labile, and specific fluorescence signals disappeared after washing (Fig. 2A'). Exogenous labeled-DNA attachment was observed in all fresh spermatozoa from the Long and Short (Fig. 2B) Incubation treatments. However, in contrast to frozen-thawed spermatozoa, labeled DNA could not be removed from nonmotile fresh spermatozoa by washing (Fig. 2B'). The same patterns of interaction were observed with labeled bovine genomic DNA fragments (Fig. 2C; 2C'). No fluorescence signals were detected in negative control groups, and no autofluorescence was found in untreated sperm.

Determination of sperm viability before and after exogenous DNA incubation

The percentage of Fresh spermatozoa with a damaged membrane did not differ before (33/110; 33.0%) or after pCX-EGFP addition (43/110; 39.0%) for the Long Incubation treatment. However, both results differed significantly with respect to the control group (26/120, 21.6%; $P<0.05$). All stained sperm cells (membrane damaged) were immotile.

PCR analyses

PCR analysis showed and *egfp* amplification product for LI whole embryos (with a ZP). Nevertheless, when LI embryos were released from their ZPs by pronase treatment, no *egfp* PCR product was visible in any of the embryos analyzed. For IVF, no embryos (with or without a ZP) showed PCR amplification products. On the other hand, all ICSI embryos showed amplification of the transgene (Table 2). We observed numerous spermatozoa (>25) adhered to the ZP in stained recovered LI embryos (8/8).

In vitro development of transgenic-ICSI ovine embryos assisted by different chemical activation treatments

In total, 304 ovine oocytes were injected with spermatozoa incubated with pCX-EGFP plasmid. All ICSI activation protocols were capable of producing blastocysts (Table 3). The rate of blastocysts and of *egfp* expressing-blastocysts (*Green* blastocysts) following ICSI activation with 2Io-DMAP was not different from treatment with Io-DMAP and Io-EtOH. Transgene embryo expression rates were not statistically different between groups. Over 60% of blastocysts expressed the transgene following the ICSI activation treatments (Table 3). We could not detect mosaicism at the blastocyst stage, probably due to homogenous expression at that embryo stage or intracellular protein migration. Fluorescent (n=5) and non-fluorescent (n=4) ICSI-derived blastocysts from the Io-DMAP activation protocol were selected and stained with Hoechst 33342 to count the number of nuclei. Mean cell numbers were not different between the *egfp*-expressing and non-expressing blastocysts (61.6 ± 14.0 *vs*. 70.3 ± 2.5; mean ± S.D.) respectively.

FISH analyses

FISH analysis displayed various possible integration loci in eight of twelve (66.6%) ovine 8-cell stage embryos (Fig. 3).

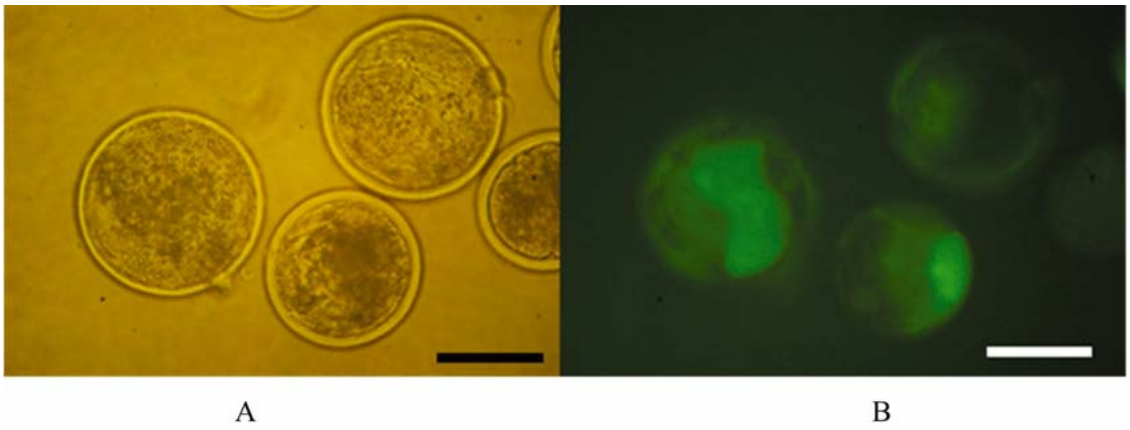

Fig. 1. EGFP-expressing embryos were produced by ICSI, injecting spermatozoa previously incubated with pCX-EGFP plasmid in a Short Incubation treatment (5 min at 0–5 C). A: This figure shows three ovine blastocysts under bright light. B: The same three blastocysts are shown under blue light (488 nm; original magnification, ×200). The scale bars represent 100 μm.

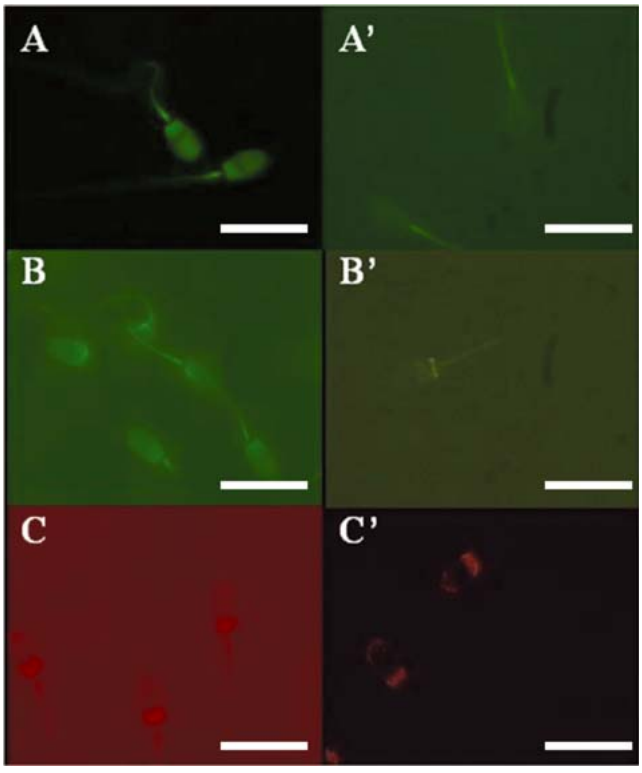

Fig. 2. Frozen-thawed and fresh ovine sperm cells were incubated with labeled plasmid or DNA fragments. Frozen-thawed labeled spermatozoa showed fluorescence signals on the entire sperm surface after a short exposure period (A, exposure 1/15) and did not show signals after washing treatment (A', exposure 1/4). In contrast, fresh labeled spermatozoa showed fluorescence signals after short procedure (B, exposure 1/4) and after washing treatment only in nonmotile sperm cells (B', exposure 1/4). Fresh labeled rhodamine bovine spermatozoa after Short Incubation (C, exposition 1/15) showed the same patterns of fluorescence signals after a washing procedure (C'. exposure 1/15; original magnification, ×1000). The scale bars represent 10 μm.

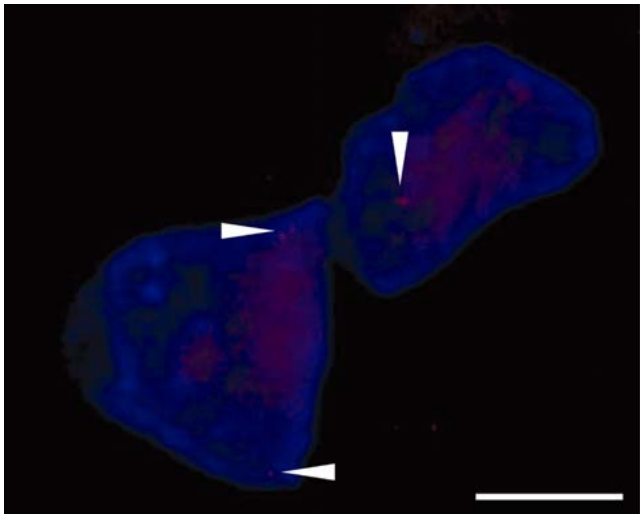

Fig. 3. FISH analysis of ovine interphase eight-cell embryos with the pCX-EGFP plasmid as a probe. The white arrows indicate various signals compatible with various putative integration loci (original magnification, ×1000). The scale bar represents 5 μm.

Table 2. PCR analysis of laparoscopic insemination (LI), *in vitro* fertilization (IVF) and intracitoplasmic sperm injection (ICSI) in ovine embryos with or without a zona pellucida (ZP)

Technique	GFP detection in embryos	
	With ZP (n)	Without ZP (n)
LI	+ (4/4)	– (4/4)
IVF	– (10/10)	– (10/10)
ICSI	+ (10/10)	+ (10/10)

Table 3. *In vitro* development and *egfp*-expression in ovine embryos produced by transgenic ICSI assisted by different protocols of chemical activation

Chemical activation	No. of oocytes injected	Cleaved (%)	*Green* embryos (%)	Morulae (%)	Blastocysts (%)	*Green* blastocysts (%)
Io + DMAP	106	78 (73.5)[a]	60 (56.6)	38 (35.8)[a]	14 (13.2)	12/14 (85.7)
2Io + DMAP	71	67 (94.3)[b]	33 (46.4)	24 (33.8)[a]	13 (18.3)	8/13 (61.5)
Io+ EtOH	127	114 (89.7)[a,b]	59 (46.4)	21 (16.5)[b]	18 (14.1)	12/18 (66.6)

Io, Ionomycin; DMAP, 6-Dimethylaminopurine; EtOH, Ethanol. [a, b and c] Percentages within columns with different superscripts are statistically different (Fisher's test; $P<0.05$). Within each group, no significant differences were observed between replicates ($P>0.05$, Fisher's test).

Discussion

After both the long and short sperm/exogenous DNA (eDNA) incubation treatments, LI embryos did not express *egfp* transgene (Table 1), even though seminal plasma was removed immediately after semen collection and eight sperm donors were pooled in order to minimize the "male factor" described in the porcine [28]. When the long and short sperm/eDNA incubations were used in IVF, spermatozoa were capable of fertilization, and the resulting embryos developed as well as the controls. However, no *egfp*-expressing embryos were generated (Table 1). These results agree with previous reports for mice [12, 16]. In contrast, low production of exogenous gene-expressing embryos has been reported in bovine using different sperm/DNA incubation conditions [33]. In the present study, exogenous gene-expressing embryos were always obtained by ICSI after sperm/DNA incubation treatments. During Long Incubation, sperm cells are exposed to particular conditions for a long period of time, which could induce apoptosis mechanisms by active endonucleases into the spermatozoa [28], causing lower rates of embryo development. Embryos that express *egfp* were generated after short exposure (Table 1) in greater percentages that in previous reports [25]. A plausible explanation could be that low temperatures of short sperm/DNA incubation enhance DNA binding [34].

Several aspects of spermatozoa/DNA interaction were studied. Both fresh and frozen/thawed spermatozoa bound eDNA, as has been found in mice and some domestic species [35–37; for a review, see 38]. Labeled DNA signals were detected on the surfaces of all fresh and frozen-thawed sperm after both treatments. However, in agreement with observations in the bovine [39], only nonmotile fresh spermatozoa maintained adhered eDNA after washing (Fig. 2). These results demonstrate a stronger interaction between eDNA and nonmotile fresh spermatozoa than between eDNA and motile fresh or frozen-thawed spermatozoa. The reversible interaction between eDNA and frozen-thawed sperm was confirmed using washed frozen-thawed spermatozoa in ICSI, where no *egfp*-expressing embryos were produced (n=30, data not shown). One difference between fresh and frozen-thawed spermatozoa is the removal of seminal plasma from the former (see Materials and Methods). Some pioneer studies in pigs and mice have demonstrated that elimination of seminal plasma is necessary for a strong interaction between eDNA and sperm membranes [28, 36, 40]. On the other hand, when the sperm membrane integrity of Fresh sperm cells incubated for a long time was analyzed, we observed that all the nonmotile spermatozoa showed membrane damage. It has been reported that sperm membrane rupture improves the binding of exogenous DNA to sperm [11]. In view of these findings, absence of fluorescence in LI embryos could be explained by the lack of motility of fresh sperm carrying eDNA. On the other hand, the absence of fluorescence in embryos generated by IVF could be explained by the reversible interaction between frozen-thawed sperm and eDNA detected in our study. Consequently, we propose that during IVF, plasmid may be removed from spermatozoa by dilution in IVF media or during ooplasm penetration, as has been observed previously in monkeys [41]. We consider that using fresh spermatozoa and solving the lack of motility of sperm carrying eDNA are key factors for successful SMGT by LI or IVF in the ovine. Future research could investigate changes in incubation conditions (shorter time or different sperm/DNA exposure medium) and/or the addition of liposomes [14, 42]. In contrast, ICSI bypasses the natural process of gamete fusion and lack of sperm motility; therefore, the characteristics described for sperm/DNA interactions are not an impediment for the generation of *egfp*-expressing embryos by this method of fertilization.

We saw that the fresh spermatozoa that maintained exogenous attached DNA were nonmotile. Unexpectedly, embryos produced by LI (with fresh sperm) showed amplification of the transgene by PCR analysis. Nevertheless, when the PCR analysis was performed on ZP-free embryos, we did not find a specific transgene band. In order to clarify these results, we stained LI embryos with Hoechst. We observed numerous spermatozoa adhered to the ZP in 100% of the stained recovered embryos, verifying that PCR amplification was derived from spermatozoa attached to the ZP and not from the embryos. This raised the question of how spermatozoa carrying the transgene arrived at the ZP. We propose two hypotheses. 1) The first is that a small proportion of motile spermatozoa carrying the transgene were able to reach the oocytes, but none of them were able to fertilize it, and 2) the second is that nonmotile spermatozoa carrying the transgene remained in the uterine horns and could adhere to the ZP during embryo migration to the uterus. The first hypothesis has been reported in pigs, where a low percentage of motile spermatozoa (1–3%) carried eDNA, although non transgene-expressing-embryos were detected [43]. Perhaps differences in motility between sperm cells with and without attached eDNA (membrane integrity) could explain the absence of transgene-expressing embryos. In our work, we have never seen an ovine motile spermatozoon carrying label eDNA. For this reason, we are leaning towards the second hypothesis, although additional studies should be done to confirm these hypotheses. In the case of PCR analyses of embryos produced by IVF and ICSI, the results were negative and positive, respectively, and as it was expected,

the results were independent of ZP presence.

The three different activation treatments evaluated induced blastocyst development after ICSI. In order to replace the activating effect produced by piezoelectric microinjection [20], all treatments in our work included an initial incubation in ionomycin. The second chemical stimuli by ethanol or ionomycin induced a longer calcium peak, resembling the calcium behavior following conventional fertilization [44]. The ICSI embryos activated with ethanol were particularly interesting because, at least in the bovine, activation with DMAP in non-transgenic ICSI has been shown to result in poor viability after transfer to recipient heifers in comparison with ethanol activation [23, 24, 45]. Our results for exogenous gene-expressing ICSI ovine blastocyst production are comparable and even improved over previous reports of transgenic SCNT [5, 46] or transgenic pronuclear microinjection [47]. Transgenic ICSI in the ovine introduces an easy DNA construct delivery system and is interesting for implementation in early reprograming events and genetic therapy research. On the other hand, the results obtained in this work suggest that transgenic ICSI might be useful for large-scale transgenic ovine offspring production. In addition, in our recent report in the bovine, ICSI-derived green embryos and blastocysts showed normal parameters, cell numbers and Oct-4 expression that were indicative of normal embryonic development [26]. The possibility of obtaining transgenic offspring by ICSI in ruminants has been anticipated [48], but the absence of basic work in this area has made it difficult to realize. However, if necessary transfer this embryos into the recipient ewes for confirm this suggestions.

Simple PCR amplification or RT-PCR can be used to detect the presence of a transgene, but they cannot determine its localization. An alternative technique, fluorescent in situ hybridization (FISH), can be used for this purpose. For ICSI embryos, FISH analysis at interphase found possible signals inside the nucleus compatible with at least two or four integration loci (Fig. 3). In mice and pigs, there has been no discussion regarding the state of the transgene after transgenic ICSI. In both species, it has been demonstrated that 5–25 transgene copies are integrated in one or two host genome loci [11, 17]. Unfortunately, the size of transgene (5.5 kb) and amount of DNA in a blastocyst (<6 pg) make it very difficult to confirm transgene integration through techniques such as FISH or Southern blot in our experience. However, the results obtained in our interphase FISH analysis confirm that sperm can import eDNA into the nuclei of ICSI-derived ovine embryos and suggest that the ICSI technique can be used in transgenic ovine production.

In conclusion, under the conditions studied, ICSI was the only method of fertilization to successfully produce exogenous gene-expressing ovine embryos, and the Short Incubation treatment enhanced transgene expression percentages. In addition, we found it necessary to carefully remove the ZP from embryos in order to avoid false positives by PCR. To our knowledge, this is the first study to explain possible failures involved in SMGT by LI or IVF in the ovine. The results obtained in this work demonstrated that sperm-mediated gene transfer by ICSI could be useful for inducing exogenous gene expression in ovine embryos.

Acknowledgments

The authors are grateful to B Romina for critical suggestions and revision of the manuscript. The authors are grateful to Mr F and A Gonzales (El Ombu) for providing biological materials, to D Comas and D Moreno for their collaboration management of the rams and to DrA Rincón for technical assistance. Part of this work was financed by the Instituto Nacional de Tecnología Agropecuaria (AEGR 3424) and PICT CABBIO 00145.

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