Reproduction in Domestic Animals

Reprod Dom Anim doi: 10.1111/j.1439-0531.2010.01642.x
ISSN 0936-6768

Efficient Transgene Expression in IVF and Parthenogenetic Bovine Embryos by Intracytoplasmic Injection of DNA–Liposome Complexes

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Contents

Transgenic animals constitute an important tool with many biotechnological applications. Although there have been advances in this field, we propose a novel method that may greatly increase the efficiency of transgenic animal production and thereby its application. This new technique consists of intracytoplasmic injection of liposomes, in bovine oocytes and zygotes, to introduce exogenous DNA. In the first experiment, we evaluated embryo development and EGFP expression in *In Vitro* Fertilization (IVF) embryos injected with different concentrations of exogenous DNA–liposome complexes (0.5, 5, 50, 500 ng pCX-EGFP/μl). The highest EGFP-embryos rates were obtained using 500 ng pCX-EGFP/μl. In the second experiment, we evaluated embryo development and EGFP expression following the injection of DNA–liposome complexes into pre-fertilized oocytes and presumptive zygotes, 16 and 24 h post-fertilization. Approximately 70% of the cleaved embryos and 50% of the blastocysts expressed EGFP, when *egfp*–liposome was injected 16 h post-fertilization. The percentages of positive embryos for the 24-h post-fertilization and pre-fertilization groups were 30.1 and 6.3, respectively. Blastocysts that developed from injected zygotes were analysed by PCR, confirming the presence of transgene in all embryos. Finally, we examined the embryo development and EGFP expression of parthenogenetic embryos that resulted from the injection of *egfp*–liposome complexes into pre-activated oocytes, and 3 and 11 h post-activated oocytes. The group with the highest expression rate (48.4%) was the one injected 3 h post-activation. In summary, this study reports the efficient, reproducible and fast production of IVF and parthenogenetic embryos expressing EGFP, by the intracytoplasmic injection of liposomes to introduce the foreign DNA.

Introduction

Transgenic animals constitute an important tool for the pharmaceutical industry (Wilmut et al. 1990; Salamone et al. 2006), biomedicine (Stice et al. 1998; Bondioli et al. 2001) and agriculture production (Golovan et al. 2001). Currently, several methods are used for the generation of transgenic farm animals; these include direct microinjection of foreign DNA into the pronucleus of fertilized eggs (Hammer et al. 1985; Krimpenfort et al. 1991), nuclear transfer using genetically modified donor cells (Schnieke et al. 1997; Cibelli et al. 1998), viral-based constructs for the delivery of exogenous DNA into embryos and oocytes (Jaenisch 1976; Chan et al. 1998) and the utilization of sperm cells as transgene vectors (Lavitrano et al. 1989; Perry et al. 1999).

To date, one of the most widespread methods for the production of transgenic animals has been the microinjection of DNA into the pronucleus of zygotes (Gordon et al. 1980; Hofker and Breuer 1998). However, as a result of inherent technical problems, low efficiency and a high frequency of embryo mosaic expression, this strategy has had limited success in farm animals. It has been reported that expression in bovine and porcine embryos is approximately 3 and 20%, respectively, with the majority of them being mosaics (Kubisch et al. 1995a, 1995b).

As a result of these limitations, the full potential of transgenic animals has not yet been exploited. Although there have been advances in this field, we propose an alternative method that may greatly increase its application. This new technique consists of intracytoplasmic injection of liposomes, in bovine oocytes and zygotes, to introduce exogenous DNA. This method avoids the centrifugation of zygotes for pronuclei visualization and also the insertion of pipettes into the pronucleus (Chauhan et al. 1999; Murakami et al. 1999). Thus, it is technically easier than pronucleus microinjection and increases the number of zygotes that can be injected in the same period of time. Liposomes are inexpensive and easy to handle and have been used extensively for the transfection of cultured cells (Ledley 1995; Hyun et al. 2003; Jang et al. 2005) and spermatozoa (Bachiller et al. 1991). Moreover, in the mouse, GFP positive embryos have resulted from the incubation of oocytes with liposomes carrying exogenous DNA, although most of these embryos were mosaics (Carballada et al. 2000, 2002).

The objective of this work was to demonstrate the high efficiency and reproducibility of transgene expression after the intracytoplasmic injection of exogenous DNA–liposome complexes into bovine oocytes and zygotes. We examined the expression of the enhanced green fluorescent protein (EGFP) and the development of *In Vitro* Fertilization (IVF) and parthenogenetic bovine embryos injected with either *egfp*–liposome complexes or the plasmid alone carrying exogenous DNA. For these experiments, intracytoplasmic injection was performed both before and after IVF or parthenogenetic activation. The resultant embryos were analysed by PCR to determine the presence of the exogenous DNA. To our knowledge, there is no previous report of successful transgene expression in IVF and parthenogenetic bovine embryos by intracytoplasmic injection of DNA–liposome complexes.

Materials and Methods

Reagents

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

Oocyte collection and *in vitro* maturation

Ovaries were collected at a slaughterhouse and transported to the laboratory. Cumulus-oocyte-complexes (COCs) were aspirated from follicles with a diameter of 2 to 8 mm and collected into Dulbecco's phosphate buffered saline (DPBS, 14287-072; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, 10499-044; Gibco) and 2% antibiotic–antimycotic (ATB; 15240-096; Gibco). Follicular oocytes covered by at least 3 layers of granulosa cells and with an evenly granulated cytoplasm were selected for *in vitro* maturation. The maturation medium was bicarbonate-buffered TCM-199 (31100-035; Gibco) containing 2 mM glutamine (G-8540), 10% FBS, 2 μg/ml follicle-stimulating hormone (NIH-FSH-P1, Folltropin®; Bioniche, Belleville, ON, Canada), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% ATB. Oocytes were incubated in 100 μl droplets of medium covered with mineral oil (M8410) in 32 mm Petri dishes. *In vitro* maturation conditions were 6.5% CO_2 in humidified air at 39°C for 22 h. After maturation, oocytes were vortexed for 2 min in hyaluronidase (H-4272) (1 mg/ml DPBS), to remove cumulus cells, and washed three times in Hepes-buffered Tyrode's medium containing albumin, lactate and pyruvate (TALP-H; Bavister and Yanagimachi 1977).

In Vitro Fertilization (IVF)

Bovine frozen semen was thawed in a 37°C water bath for 30 s. Spermatozoa were centrifuged twice (490 g × 5 min) and resuspended in Brackett–Oliphant medium (BO, Brackett and Oliphant 1975) supplemented with 5 mM caffeine and 20 IU/ml heparin. Spermatozoa were diluted to half concentration with BO containing 10 mg/mL fatty acid-free bovine serum albumin (A6003), resulting in a final sperm concentration of 12×10^6–15×10^6/ml. Spermatozoa were coincubated with COCs in 100 ul droplets, for 5 h at 39°C in a humidified atmosphere of 5% CO_2 in air. The presumptive zygotes were then washed three times in TALP-H and cultures were continued as described as follows.

Oocyte chemical activation

Mature oocytes (MII) were activated with 5 μM ionomycin (I24222; Invitrogen, California, USA) in TALP-H for 4 min and immediately placed in 1.9 mM 6-DMAP (D2629) in Synthetic Oviductal Fluid (SOF) for 3 h. 6-DMAP was removed by washing three times in TALP-H.

DNA construction

The plasmid used was pCX-EGFP that contains an enhanced green fluorescent protein gene (*egfp*) under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control (Ikawa et al. 1995). Closed plasmid was used in this work, because we previously reported the successful production of a transgenic cow using a circular plasmid construction (Salamone et al. 2006).

Liposome–DNA coincubation

For the injection experiments, 1 μl of 4 μg/ml of DNA in combination with 3 μl of commercial liposome (Fugene; Boehringer- Manheim, Germany) was coincubated for 15 min. The liposome–DNA mixture was diluted to half concentration with 10% polyvinylpyrrolidone (PVP, 99219; Irvine Scientific, Santa Ana, CA, USA), reaching a final DNA concentration of 0.5 μg/ml. For the ZP-free oocytes and zygotes incubations, the liposome–DNA mixture was diluted in SOF medium, resulting in a final DNA concentration of 5 ng/μl, as reported by Carballada et al. (2002).

Intracytoplasmic injection of DNA–liposome complexes

After or before IVF or parthenogenetic activation, the ooplasm of the presumptive zygotes and oocytes was injected with approximately 2 pl of DNA–liposome/PVP mixture, using an injection capillary (0.7 μm in diameter) attached to a micromanipulator (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA).

ZP-free oocytes and zygotes incubations with DNA–liposome complexes

Oocytes and zygotes were treated with 1.5 mg/ml pronase (P8811) in TALP-H, for 5–10 min, using a warm plate to remove the ZP. The ZP-free oocytes and zygotes were incubated for 3 h with a DNA–liposome mixture in SOF medium (5 ng/μl), in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in air at 39°C, as reported by Carballada et al. (2002). The coincubations were carried out after or before IVF, as the injection experimental groups.

In vitro culture

Fertilized and activated oocytes were cultured in 100 μl droplets of SOF (Tervit et al. 1972; modified by Holm et al. 1999) and supplemented with 2.5% v/v FBS. The ZP-free presumptive zygotes were cultured individually in 5 μl droplets of SOF, in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in air at 39°C. Cleavage was evaluated on day 2, and the number of blastocysts on day 7, post-IVF or parthenogenetic activation.

Determination of EGFP fluorescence in IVF and parthenogenetic embryos

During *in vitro* culture, embryos were briefly exposed to blue light using an excitation filter at 488 nm and an emission filter at 530 nm to determine the expression of the *egfp* gene at different stages of development. Embryos were analysed on days 3 (cleaved) and 7 (blastocysts), after IVF or parthenogenetic activation.

Transgene detection by PCR

Day 7 blastocysts were washed in PBS, transferred to 1 μl into an eppendorf tube and incubated in 10 μl PCR

buffer containing proteinase K (2 μg/ml), at 56°C for 1 h. Proteinase K was inactivated at 95°C for 10 min. Half of the reaction was used for PCR in 10 μl final volume, containing 1× PCR buffer, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of Taq DNA polymerase and 0.5 mM of each primer. The first primer set sequences were 5′-GAAGTTCGAGGGCGACACCCTG-3′ and 5′-TCGTCCATGCCGGAGAGTGATC-3′. The first PCR product size was 369pb. One μl from the first PCR was employed as a template for the second PCR. The second primer set sequences were 5′-TGAACCG-CATCGAGCTGAAG-3′ and 5′-TCACGAACTC-CAGCAGGACCAT-3′. The primers were designed to amplify a 315 bp fragment from the pCX-EGFP plasmid. The PCR conditions consisted of a heating step (95°C for 10 min), followed by 35 amplification cycles; a second heating step (94°C, 30 s) to further denature the nucleic acid; an annealing step (65°C for 30 s); and an extension step at 72°C for 30 s. Cycle 35 contained an additional extension at 72°C for 7 min. The positive control consisted of 1 pg of pCX-EGFP plasmid and the negative control was DNA from IVF embryos that were not injected. The PCR products (5 μl) were analysed in a 2% agarose gel stained with ethidium bromide. Samples that showed a transgene-specific PCR product were classified as PCR positive for the transgene.

Experimental design

Experiment 1: Embryo development and EGFP expression in IVF embryos using different concentrations of exogenous DNA–liposome complexes.

Initially, to determine optimal conditions to produce bovine embryos expressing exogenous DNA, we evaluated four concentrations of *egfp*–liposome complexes (0.5, 5, 50, 500 ng pCX-EGFP/μl) to be injected into IVF presumptive zygotes, 16 h post-fertilization.

Experiment 2: Embryo development and EGFP expression after intracytoplasmic injection of DNA–liposome complexes in bovine MII oocytes and IVF zygotes.

Several trials were performed to establish an efficient protocol to produce transfected embryos using DNA–liposome complexes. For these, injections were given pre-fertilization and during or after pronuclear formation, 16 and 24 h post-fertilization, respectively. Oocytes and zygotes were injected with the plasmid alone or simply coincubated with *egfp*–liposome complexes at the same times as the experimental groups. The pattern of EGFP expression was evaluated on cleaved embryos and blastocysts (day 3 and 7 post-fertilization, respectively). We confirmed the viability of the embryos by comparing the development of the injected groups with a non-injected IVF control group.

In addition, blastocysts that developed from injected fertilized oocytes (16 h post-fertilization) were analysed for the presence of exogenous DNA, using PCR. The procedure was performed in embryos expressing or not expressing EGFP. For each PCR assay, the following control samples were analysed: a positive control consisting of the pCX-EGFP plasmid and a negative control of DNA from non-injected IVF embryos.

Experiment 3: Embryo development and EGFP expression after intracytoplasmic injection of DNA–liposome complexes in parthenogenetically activated oocytes.

We evaluated EGFP expression in parthenogenetic embryos that were injected either before or after activation (3 and 11 h post-ionomycin exposure). Controls injected with the plasmid alone were performed. The pattern of EGFP expression was evaluated on day 3 post-activation, and at the blastocyst stage, on day 7 of embryo culture. In addition, we compared the development of the injected groups with the parthenogenetic control.

Statistical analysis

In vitro embryo development was compared by non-parametric Fisher's exact test. For all statistical analyses, the SAS program was used (SAS Institute Inc. SAS/STAT 1989). Differences were considered significant at $p < 0.05$.

Results

Experiment 1: Embryo development and EGFP expression in IVF embryos using different concentrations of exogenous DNA–liposome complexes

We evaluated four concentrations of egfp–liposome complexes (0.5; 5; 50; 500 pCX-EGFP ng/μl) to be injected in IVF zygotes 16 h post-fertilization. The highest EGFP-embryos rates were obtained using 50 (26.6%) and 500 (38.6%) ng pCX-EGFP/μl. Nevertheless, a higher tendency of EGFP-positive blastocyst (40%) rates was noted when the 500 ng pCX-EGFP/μl mixture was used (Table 1), so we decided to use the highest concentration of *egfp* complex in the successive experiments.

Experiment 2: Embryo development and EGFP expression after intracytoplasmic injection of DNA–liposome complexes in MII oocytes and IVF bovine zygotes

The results regarding the development and the efficiency of transfection after the injection of DNA–liposome complexes into oocytes and IVF zygotes are summarized in Table 2. We detected EGFP positive embryos in all groups. The percentages of positive embryos were 68.4 and 30.1 for the 16 h and 24 h post-fertilization groups, respectively, and 6.3 for the pre-fertilization group. In our conditions, control groups of coincubated *egfp*–liposome complexes ($n = 108$) or those injected with the plasmid alone were always negative. All the positive embryos from the pre-fertilization group had mosaic expression. However, homogenic expression was seen in both of the post-fertilization groups: the embryos injected 16 h post-fertilization reached higher percentages of homogenic expression than the embryos injected 24 h post-fertilization (65.4% vs 38.1%). Expression of EGFP was also evaluated at the blastocyst stage, on day 7 of embryo culture. The positive blastocysts obtained were from the post-fertilization groups (16 and 24 h post-fertilization, 52.2% and 31.6%, respectively;

Table 1. Embryo development and EGFP expression of the *In Vitro* Fertilization (IVF) bovine embryos injected (16 h post-fertilization) with different concentrations of *egfp*–liposome complex

pCX-EGFP ng/μl	n	Cleaved (%)	+ EGFP Cleaved Embryos (%)	Blastocysts (%)	EGFP-Blastocysts (%)
0.5	105	69 (65.7)[a]	0/69 (0)[a]	27 (25.7)	0/27 (0.0)[a]
5	82	66 (80.5)[b,c]	0/66 (0)[a]	18 (22.0)	0/18 (0.0)
50	104	79 (76.0)[a,c]	21/79 (26.6)[b]	16 (15.4)	3/16 (18.7)[b]
500	104	70 (67.3)[a]	27/70 (38.6)[b]	20 (19.2)	8/20 (40.0)[b]

Three independent replicates were performed for each treatment.
[a,b]Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's test).

Table 2. Embryo development and EGFP expression of bovine oocytes and *In Vitro* Fertilization (IVF) embryos injected with *egfp*–liposome complex

Moment of transfection	Injection or coincubation of *egfp*	n	Cleaved (%)	Blastocysts (%)	+ EGFP Cleaved Embryos (%)	Homogenic Expression (%)	+ EGFP Blastocysts (%)
Pre-Fertilization	*egfp*–liposome	92	63 (68.5)[a]	20 (21.7)	4/27 (6.3)[a,b]	0/4 (0.0)[a]	0/20 (0.0)[b,d]
	egfp alone	27	19 (70.4)[a,b]	5 (18.5)	0 (0.0)[b,c]	–	0/5 (0.0)[a,b,c]
16 h post-Fertilization	*egfp*–liposome	103	76 (73.8)[a,b]	23 (22.3)	52/76 (68.4)[a,b]	34/52 (65.4)[b]	12/23 (52.2)[a]
	egfp alone	35	24 (68.6)[a,b]	8 (22.9)	0 (0.0)[b]	–	0/8 (0.0)[b,c]
24 h post-Fertilization	*egfp*–liposome	102	68 (66.7)[a]	19 (18.6)	21/68 (30.1)[a]	8/21 (38.1)[a]	6/19 (31.6)[a,c,d]
	egfp alone	29	20 (72.7)[a,b]	6 (31.3)	0 (0.0)[b,c]	–	0/6 (0.0)[b]
IVF Control	*NA*	112	93 (83.0)[b]	31 (27.7)	–	–	–

Three independent replicates were performed for each treatment.
NA, not applicable. +, positive; –, negative; EGFP, enhanced green fluorescent protein.
[a,b,c,d]Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's test).

Fig. 1), with non-fluorescing blastocysts in the pre-fertilization group. In addition to achieving embryo transfection, no differences in the rates of blastocyst development were found between injected embryos and IVF controls.

The PCR results are shown in Table 3. We evaluated 5 EGFP-positive blastocysts and 5 EGFP-negative blastocysts obtained after the injection of *egfp*–liposome complexes. The assay confirmed the presence of transgene in all embryos, as indicated by the appearance of a specific band for the gene construct. We also analysed EGFP-negative blastocysts injected with the plasmid alone and detected *egfp* in 40% of the tested embryos. This result could indicate a possible degradation of the exogenous DNA when the plasmid was injected in the absence of liposomes. No exogenous DNA was seen in the non-injected IVF control blastocysts subjected to PCR.

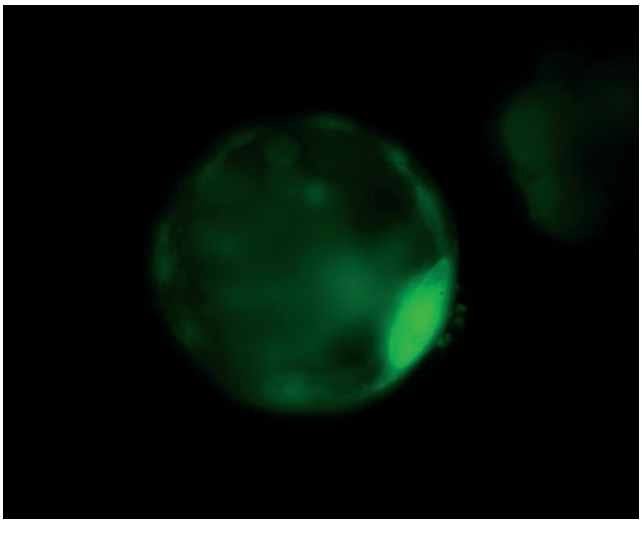

Fig. 1. Bovine EGFP-positive blastocyst produced by *In Vitro* Fertilization (IVF) and intracytoplasmic injection of egfp-liposome complex. The fluorescence was evaluated under blue light (488 nm; original magnification ×200)

Experiment 3: Embryo development and EGFP expression after intracytoplasmic injection of DNA–liposome complexes in parthenogenetically activated oocytes

In this experiment, we evaluated the EGFP expression of parthenogenetic embryos (Table 4; Fig. 2) injected with *egfp*–liposome complexes before or after oocyte activation (3 h and 11 h post-ionomycin exposure). Positive EGFP embryos were obtained in all groups when evaluated after 3 days of culture. The group with the highest expression rate was the one injected 3 h post-activation (48.4%). The other two groups (pre-activated oocytes and 11 h post-activation oocytes) showed lower percentages of EGFP expression (14.3% and 13%, respectively). The positive EGFP embryos from the pre-activation group always showed mosaic expression. However, homogenic expression was detected in both the post-activation groups. The homogenic expression rates were 53.3% and 33.3%, for the 3 and 11 h activation-groups, respectively. The EGFP expression was also evaluated at the blastocyst stage. Twenty per cent of the blastocysts were EGFP-positive, in the pre-activation group, and 60% were in the 3-h post-activation group. Blastocysts from the 11-h post-activation group did not show any transgene expression. In addition, a comparison of embryo viability between the injected groups and a parthenoge-

Table 3. Results of polymerase chain reaction (PCR) analysis for the detection of exogenous gene construct in blastocysts that developed after injection of the *egfp*–liposome complex

Category	Injected with *egfp*–liposome complex (+EGFP Embryos) n (%)	Injected with *egfp*–liposome complex (−EGFP Embryos) n (%)	Injected with *egfp* alone n (%)	Non injected embryos control n (%)
Blastocysts analyzed by PCR (n = 20)	5	5	5	5
egfp-positive Blastocysts	5 (100)	5 (100)	2 (40)	0 (0)

+, positive; −, negative; EGFP, enhanced green fluorescent protein.
All the injections were given 16 h post-fertilization.

Table 4. Embryo development and EGFP expression of bovine oocytes and parthenogenetic zygotes injected with *egfp*–liposome complex

Moment of transfection	Injection of *egfp*	n	Cleaved (%)	Blastocysts (%)	+ EGFP Cleaved Embryos (%)	Homogenic Expression (%)	+ EGFP Blastocysts (%)
Pre-Activation	*egfp*–liposome	18	14 (77.8)	5 (27.8)	2/14 (14.3)[a]	0/2 (0.0)	1/5 (20.0)[a,b]
	egfp alone	22	16 (72.7)	5 (22.7)	–	–	–
3h post-Activation	*egfp*–liposome	42	31 (73.8)	10 (23.8)	15/31 (48.4)[b]	8/15 (53.3)	6/10 (60.0)[a]
	egfp alone	24	17 (70.8)	5 (20.8)	–	–	–
11 post-Activation	*egfp*–liposome	28	23 (82.1)	5 (17.9)	3/23 (13.0)[a]	1/3 (33.3)	0/5 (0.0)[b]
	egfp alone	30	26 (86.7)	4 (13.3)	–	–	–
Parthenogenetic Control	*NA*	40	31 (77.5)	13 (32.5)	–	–	–

Two independent replicates were performed for each treatment.
+, positive; −, negative; EGFP, enhanced green fluorescent protein.
[a,b]Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's test). NA, not applicable.

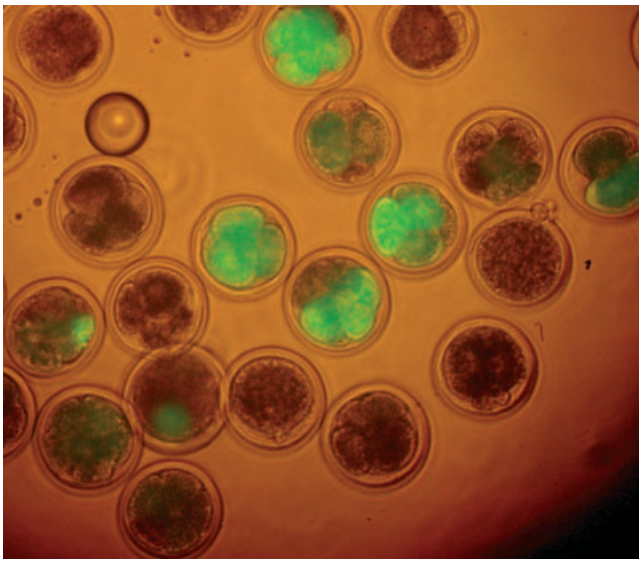

Fig. 2. Parthenogenetic EGFP-positive embryos produced by intracytoplasmic injection of egfp-liposome complex. They are shown under a combination of bright and blue light (488 nm; original magnification ×200)

netic, non-injected control group showed no differences in cleavage and blastocyst rates.

Discussion

In this study, we examined the development and EGFP expression of IVF and parthenogenetic bovine embryos injected with *egfp*–liposome complexes. Our results indicate that zygotes can be successfully and easily transfected using this new method. Approximately 70% of the cleaved embryos and 50% of the blastocysts obtained expressed EGFP when the *egfp*–liposome was injected 16 h post-fertilization, and the DNA concentration used was the highest tested (Tables 1 and 2). This contrasts with previous reports, wherein evidence of transgene expression in the bovine was displayed only in 11.9% of the embryos and 2.9% of the blastocysts after pronuclear microinjection of the same plasmid as we used in this study (Murakami et al. 1999). Low percentage of expressing blastocyst (3.2%; Kubisch et al. 1995a,b) also resulted from using a different construct (SV40-LacZ). In addition to the high expression rates obtained, we demonstrated that injected oocytes developed *in vitro* at similar rates to IVF controls (Table 2). Consistent with reports of other researchers, liposome transfection did not significantly reduce the viability of embryos and a high number of blastocysts could be generated (Carballada et al. 2000).

When the injection of *egfp*–liposome was performed prior to IVF, we obtained low percentages of EGFP-positive embryos (6.3%), and none of the blastocysts showed transgene expression. These results are not consistent with the high transfection efficiency obtained by other authors (56% positive embryos), after MII oocytes were transfected using lentivirus (Chan et al. 1998). Therefore, in some cases, not all the cells were transfected, and mosaic embryos were generated. This indicates that nuclear import or integration may occur after the first cleavage (Chan et al. 1999). Murakami et al. (1999) reported that a majority of bovine embryos were mosaic (70.3%) when microinjected with an EGFP-cDNA under control of the chicken beta-actin promoter and cytomegalovirus enhancer construct. The results of our best treatment, injecting *egfp*–liposome complexes 16 h post-fertilization, showed few mosaic expression in cleaved embryos evaluated on day 3 of culture (34.6%, Table 2).

The high rates of EGFP expression obtained when the DNA–liposome complexes were injected during pronuclear formation (16 h post-fertilization; Liu and Yang 1999) and the low rates obtained when injected after pronuclear formation (24 h post-fertilization) could be explained by the nuclear dynamics that occur after fertilization. When a sperm penetrates an oocyte, chromatin decondenses and pronuclei are formed (Xu and Greve 1988; Liu and Yang 1999). During this time, protamines dissociate from the sperm DNA, and histones associate with it, allowing replication to occur (Bedford 1983). The integration of foreign DNA may happen during this process as the DNA is relaxed and more susceptible to breakage and transcriptional activity (Kubisch et al. 1995a,b). When DNA–liposome complexes are injected into the ooplasm, the exogenous DNA must enter the nucleus for subsequent transcription to occur. We suggest that liposomes are able to deliver this exogenous DNA into the nucleus of bovine oocytes as evidenced by the high production of EGFP-positive embryos (Tables 1–4).

Parthenogenetic embryos showed similar EGFP expression patterns as IVF embryos after injection of *egfp*–liposome complexes. The percentages of expression when injected during pronuclear formation (3 h post-activation; Szöllösi et al. 1993; Liu and Yang 1999) were approximately 50% and 60% in embryos and blastocysts, respectively. These results demonstrated that transfection with exogenous DNA and its expression in resulting embryos is independent of the presence of a male pronucleus.

The results of transgene detection obtained by the PCR assay (Table 3) confirmed the presence of the pCX-EGFP plasmid in all the *egfp*–liposome injected embryos analysed, whether the blastocysts were EGFP-positive or -negative. However, in embryos injected with the plasmid alone, the exogenous DNA was detected in only 40% of the blastocysts tested. This result could indicate that in these embryos the exogenous DNA was degraded in the cytoplasm. These findings could suggest that liposomes retain and protect the exogenous DNA from endonucleases until its transcription.

The most important finding of this study consists of the efficient transgene expression in IVF embryos by intracytoplasmic injection of DNA–liposome complexes. In addition to efficient embryo development and transgene expression, it is possible that the problems associated with cloning and ICSI-mgt embryos could be avoided. In particular, the chemical assistance required for embryonic activation in these techniques generates chromatin defects (De La Fuente and King 1998; Yoo et al. 2003; Bhak et al. 2006). The methods used in this study avoid these concerns.

In summary, intracytoplasmic injection of liposomes as vectors to introduce foreign DNA into early embryos could be a useful, reproducible and faster method than pronuclear microinjection, to generate transgenic bovine embryos in large scales. Further research is required to confirm the integration efficiency and the number of exogenous DNA insertions to ensure the production of transgenic calves.

Acknowledgements

The authors thank CIALE for providing the biological material. This work was partially supported by PICT CABBIO 2008 N° 00145 and UBACYT G808.

Conflict of interest

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

Author contributions

G. Vichera has designed study, acquired and analysed the data, and drafted the paper; L. Moro acquired and analysed the data, and drafted the paper; D. Salamone designed the study, analysed the data, and drafted the paper, revising it critically.

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Submitted: 5 Feb 2010; Accepted: 9 Apr 2010

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