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High rates of bovine blastocyst development after ICSI-mediated gene transfer assisted by chemical activation

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Abstract

In order to establish conditions for intracytoplasmic sperm injection-mediated gene transfer (ICSI-MGT) in cattle, various aspects of fertilization and embryonic development were assessed after five activation treatments. Spermatozoa were co-incubated with pCX-EGFP (pCX-enhanced green fluorescent protein gene) plasmid and injected into metaphase II oocytes, which were then treated with ionomycin (Io), before further activation with the following agents: 6-dimethylaminopurine (Io-DMAP), additional Io plus DMAP (2Io-DMAP), Io alone (2Io), ethanol (Io-EtOH), or strontium chloride (Io-SrCl2). Fertilization rates at 16 h after ICSI, presence of a condensed spermatozoon head on Day 4 (Day 0 = ICSI), blastocyst and EGFP expression rates on Day 7, and Oct-4 pattern of Day 8 blastocysts were evaluated. Fertilization rates did not differ significantly among treatments. All (100%) of EGFP-positive embryos resulted from ICSI fertilization, whereas at least 60% of EGFP-negative embryos (>4 cells) had a condensed sperm head. Blastocyst rates after 2Io-DMAP were not significantly different from Io-DMAP or Io-EtOH, but they were higher than 2Io or Io-SrCl2 treatments (25.9, 18.7, 14.7, 9.4, and 10.9% respectively; $P < 0.05$). Transgene expression rates were higher for Io-DMAP, 2Io-DMAP and Io-SrCl2 than for 2Io and Io-EtOH (52.3, 53.0, 42.8, 28.2, and 29.4% respectively; $P < 0.05$). Over 80% of the blastocysts expressed egfp protein. In conclusion, ICSI-MGT was a powerful technique to produce bovine embryos that expressed the EGFP transgene. Moreover, the actual efficiency of ICSI-MGT could be readily evaluated by this method, which uses a marker expressed early in embryo development.

Keywords: EGFP; Parthenogenesis; DMAP; Strontium; Bovine embryos

1. Introduction

Intracytoplasmic sperm injection (ICSI) involves mechanical transfer of a single sperm cell into ooplasm. This procedure was first reported in mammals in 1976 [1] and, two decades later, it proved to be an efficient means to overcome male infertility problems in humans [2]. A new application has been recently found for ICSI, namely the production of transgenic animals. Perry et al [3] demonstrated that ICSI-MGT was a powerful technique to produce murine transgenic embryos and pups.

Some of the benefits associated with ICSI-MGT were that it avoids low transgenic efficiencies inherent in pronuclear microinjection [4] and that it had lower rates of imprinting defaults than somatic cell nuclear transfer (SCNT) [5]. However, for successful large scale application of ICSI-MGT in domestic species, it

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is critical to solve the ICSI fertilization problem often encountered. Although pronuclear formation of sperm-injected bovine oocytes was first reported in 1984 [6], ICSI fertilization rates have remained very low [7–9]. Chemical activation following nontransgenic ICSI has improved development of bovine embryos [8–13]; nevertheless it can result in the development of parthenogenetic as well as fertilized embryos [10].

In order to establish conditions for ICSI-MGT in cattle, this study compared various aspects of fertilization and embryonic development following five activation treatments. The early expression of a gene marker included in the pCX-EGFP (pCX-enhanced green fluorescent protein gene) plasmid [14] allowed us to identify ICSI-MGT transgene expression on Day 4, and to determine the association of EGFP expression to the presence of uncondensed sperm heads. Consequently, the actual relationship between fertilized and EGFP-expressing embryos could be determined. Transgene expression and blastocyst production rates for the five treatments were determined, as well as quality of blastocysts expressing EGFP, in terms of the day of their formation, cell number, and Oct-4 expression pattern.

2. Materials and methods

2.1. Reagents

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

2.2. Experimental design

Metaphase II bovine oocytes were injected with spermatozoa that had been co-incubated with a pCX-EGFP plasmid. Injected oocytes were randomly assigned to one of the following chemical activation treatments: 1) Io-DMAP; 2) 2Io-DMAP; 3) 2Io; 4) Io-EtOH; and 5) Io-$SrCl_2$. Their potential to assist ICSI-MGT was tested in the experiments described below. Each experiment was replicated three times.

2.2.1. Experiment 1: Evaluation of pronuclear (PN) formation after ICSI-MGT assisted by various chemical activation treatments

Injected oocytes were chemically activated and fixed (16 h after initial Io exposure) to evaluate PN formation. Oocytes with two PN and two polar bodies (2PN/2PB) were considered fertilized, whereas those with 1PN and 2PB (1PN/2PB) were considered haploid, activated, but not fertilized. Parthenogenetic embryos resulting from activation of oocytes (employing the same protocols as in ICSI) were used as controls.

2.2.2. Experiment 2: Evaluation of EGFP-expression and detection of a condensed spermatozoon head in Day 4 embryos produced by ICSI-MGT, assisted by various chemical activation treatments

Transgene expression and presence of spermatozoa were evaluated in green (EGFP-positive) and non-green (EGFP-negative) Day 4 bovine embryos produced by ICSI-MGT assisted by chemical activation. The presence of a condensed sperm head indicated parthenogenetic activation, and lack of ICSI fertilization.

2.2.3. Experiment 3: In vitro development of ICSI-MGT bovine embryos, assisted by various chemical activation treatments

Embryos produced by ICSI-MGT assisted by chemical activation were cultured *in vitro* for 8 d. Development and EGFP-expression rates were compared after various ICSI activation treatments. Polymerase chain reaction (PCR) analyses were conducted to confirm presence of the transgene.

2.2.4. Experiment 4: Quality of egfp-expressing bovine blastocysts produced by ICSI-MGT assisted by various activation treatments

Blastocysts expressing EGFP were evaluated according to the day of their formation, cell number and Oct-4 expression pattern. As controls, parthenogenetic and IVF blastocysts were also evaluated.

2.3. Oocyte collection and in vitro maturation

Ovaries were collected from cows at abbatoirs. Cumulus-oocyte-complexes (COCs) were aspirated from follicles with a diameter of 2 to 6 mm and collected in TCM-Hepes (31100-035; Gibco, Grand Island, NY, USA; H4034) containing 10% v/v fetal bovine serum (FBS, 013/07; Internegocios, Buenos Aires, Capital Federal, Argentina), and 2% v/v antibiotic-antimycotic (ATB, 15240-096; Gibco). Oocytes covered with at least three layers of granulosa cells were selected for *in vitro* maturation (IVM). The maturation medium was bicarbonate-buffered TCM-199 (31100-035; Gibco), containing 10% v/v FBS, 10 μg/mL follicle stimulating hormone (Folltropin®, Bioniche, Belleville, ON, Canada), 0.3 mmol/L sodium pyruvate (P2256), 100 μmol/L cysteamine (M9768) and 2% v/v ATB. Oocytes were incubated in 100 μL droplets of medium covered with mineral oil (M8410), in 32 mm Petri-dishes. The IVM conditions were 6.5% CO_2 in humidified air at 39 °C for 24 h. After maturation, COCs were vortexed for 2 min in hyaluronidase (H-4272; 1 mg/mL) in Dulbecco′s phos-

phate buffer saline (to remove cumulus cells) and washed three times in Hepes-buffered Tyrode's medium containing albumin, lactate and pyruvate (TALP-H). Mature oocytes, evaluated by visualizing the first polar body, were immediately used for ICSI.

2.4. DNA construction

The plasmid used was pCX-EGFP (kindly provided by Dr. Masaru Okabe) that contained an EGFP under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control [14].

2.5. Sperm-DNA coincubation

Sperm-DNA construction coincubation was carried out as reported by Perry et al [3], with slight modifications. Briefly, spermatozoa were washed and centrifuged twice (495 × g, 5 min) in 2.8% w/v sodium citrate (F71497) with 100 μmol/L EDTA (15576-028; Invitrogen, Carlsbad, California, USA). Sperm pellets were resuspended in 2.8% w/v sodium citrate with 100 μmol/L EDTA, adjusted to 20×10^6 sperm/mL and co-incubated with 0.5 μg of closed circular covalent plasmid/10^6 spermatozoa (final concentration) for 5 min at 0 °C. Spermatozoa were immediately used for ICSI.

2.6. Intracytoplasmic sperm injection (ICSI)

Sperm injection was performed in microdroplets of 20 μL of TALP-H under mineral oil (M8410) in 100 × 20 mm tissue culture dishes (430167; Corning, 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). Spermatozoa were selected according to their normal morphology, from a 4 μL droplet of Na citrate medium containing 10% v/v polyvinylpyrrolidone (PVP, 99219; Fisher Scientific, Pittsburgh, PA, USA). Each spermatozoon was immobilized by breaking its tail and aspirated tail-first into the 8 μm inner diameter injection pipette. The injection pipette was 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 ooplasm at the 3 o'clock position. Following breakage of the oolemma by aspiration, the spermatozoon and the aspirated ooplasm were expelled into the oocyte.

2.7. Oocyte chemical activation

Injected oocytes were immediately activated in TALP-H containing 5 μmol/L ionomycin (I24222; Invitrogen) for 4 min. After this initial activation, oocytes for all treatments (except for Io-SrCl2 for which it was not necessary) were placed in TCM-199 for 3 h to allow second polar body extrusion. Oocytes were subsequently treated with: a) 5 μmol/L ionomycin in TALP-H for 4 min (2Io Group); b) 1.9 mmol/L DMAP in TCM 199 (D2629) for 3 h (Io-DMAP Group); c) 5 μmol/L ionomycin, followed immediately by 1.9 mmol/L DMAP in TCM 199 for 3 h (2Io-DMAP Group); d) 7% v/v ethanol in TALP-H for 5 min (Io-EtOH Group); and e) 20 mmol/L $SrCl_2$ in TCM 199 for 5 h, immediately after ionomycin (Io-$SrCl_2$ Group).

Inhibitors were removed by washing three times in TALP-H and cultures were continued as described below.

2.8. In vitro fertilization (IVF)

The IVF procedure was previously described by Brackett and Oliphant (1975) [15]. Briefly, frozen semen was thawed in a 37 °C water bath for 30 s. Sperm were washed twice by centrifugation at 400 g with Brackett's defined medium. Sperm concentration was adjusted to 15×10^6/mL and sperm then co-incubated for 5 h with COCs in Brackett's fertilization medium. Afterward, the oocytes were washed several times in TALP-Hepes and *in vitro* cultured as described below.

2.9. In vitro culture

Presumptive zygotes were cultured in 50 μL droplets of Synthetic Oviductal Fluid (SOF) [16,17] supplemented with 2.5% v/v FBS. Culture conditions were 6.5% CO_2 in humidified air at 39 °C. Embryos were transferred to new droplets on Days 2 and 5. Cleavage was evaluated on Day 2, and the number of morulae and blastocysts on Days 5 and 8 post ICSI, respectively.

2.10. Assessment of pronuclear formation

At 16 h after initial ionomycin exposure, oocytes were fixed in acetic acid: ethanol (3:1) and stained with 1 mg/mL Hoechst 33342 (B2261) for 4 min, to examine pronuclear formation.

2.11. Determination of EGFP fluorescence and presence of spermatozoa in embryos on Day 4

After *in vitro* culture for 4 d, ICSI embryos were briefly exposed to blue light using an excitation-filter at 488 nm and an emission-filter at 530 nm to determine expression of the EGFP gene, and mosaicism patterns. In addition, embryos were mounted between coverslips,

stained with Hoechst 33342 and examined under epifluorescent microscopy, to assess cell numbers and to evaluate presence or absence of non-decondensed spermatozoa.

2.12. Polymerase chain reaction assay of embryos produced by ICSI-MGT

An analysis using PCR was performed individually on green embryos (n = 10) produced by ICSI-MGT assisted by Io-DMAP and 2Io-DMAP, and on non-green embryos (n = 15) produced by ICSI-MGT assisted by Io-DMAP, 2Io-DMAP, Io-EtOH and Io-$SrCl_2$. Green embryos consisted of blastocysts (n = 2) and day 4 (n = 8) embryos. Non-green embryos consisted of blastocysts (n = 3), embryos with a condensed sperm head (n = 9) and embryos lacking a sperm head (n = 3). Embryos expressing the transgene as well as those not expressing it, and controls produced by parthenogenetic activation (n = 15), were washed in PBS, transferred individually in 1 uL aliquots into eppendorf tubes, resuspended in 9 uL of extraction buffer (10 mM Tris-EDTA) containing proteinase K (1 mg/mL, Promega V302B, Madison, WI, USA) and incubated at 56 °C for 1 h. The samples were then heated at 95 °C for 10 min and 5 uL aliquots were used for PCR. The primer set sequences were 5′–GAAGTTCGAGGGCGACACCTG–3′ and 5′–TCGTCCATGCCGAGAGTGATC–3′ for amplifying a 269 bp GFP (green fluorescent protein) fragment. The PCR reaction conditions consisted of a heating step (95 °C for 2 min), followed by 35 amplification cycles: a heating step (94 °C for 15s); annealing (55 °C for 15 s); and extension (72 °C for 25 s). Cycle 35 contained an additional extension at 72 °C for 7 min. The positive control consisted of 3.6^{-11} g/mL of pCX-EGFP plasmid and the negative control was distilled water.

2.13. Immunocytochemistry

Whole-mount immunocytochemistry was performed on bovine blastocysts produced as a result of ICSI assisted by the five chemical activation treatments, IVF and diploid parthenogenetic activation with Io-DMAP. Briefly, embryos were fixed for 30 min in 4% v/v paraformaldehyde (F-1635) in PBS and permeabilized by 15 min incubation in PBS containing 0.2% v/v Triton X-100 (T-9284). Non-specific immunoreactions were blocked by incubation with 3% v/v FCS and 0.1% v/v Tween-20 (Promega, H5152) in PBS (blocking buffer) for 30 min. The affinity-purified primary polyclonal antibody against Oct-4 (Santa Cruz Biotechnology sc-8628, Santa Cruz, CA, USA), diluted 1:100 in PBS, was then applied for 1 h at room temperature. Following thorough washing in blocking buffer for 15 min, blastocysts were incubated with a secondary antibody [Alexa 488-donkey anti-goat IgG 2 mg/mL (A11055, Molecular Probes, Inc. Eugene, OR, USA) diluted 1:1000] for 40 min at room temperature in the dark. Embryos were then stained with propidium iodide 30 μg/mL for 10 min in the dark. Stained blastocysts were mounted on slides, in 70% v/v glycerol. Negative controls for Oct-4 were produced using only the secondary antibody.

2.14. Confocal laser scanning microscopy

Embryos were analyzed on a Nikon Confocal C.1 scanning laser microscope. An excitation wavelength of 488 nm was selected from an argon-ion laser to excite the Alexa-conjugated secondary antibody and a 544 nm wavelength to excite propidium iodide. Images of serial optical sections were recorded every 1.5–2 μm vertical step along the Z-axis of each embryo. Three-dimensional images were constructed using software EZ-C1 2.20.

2.15. Statistical analysis

In vitro embryo development, fluorescent expression, and differences between replicates were compared by Fisher's exact test. Differences in total cell number and in number of Oct-4-expressing cells were analyzed using a one-way ANOVA. For all statistical analyses, the SAS program was used [18]. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Preliminary observations

In a preliminary experiment, we compared fertilization rates of three bull donors in ICSI-MGT assisted by activation with Io-DMAP. The bull donor with the highest two pronuclear (2PN) formation and the best Day 4 EGFP expression rates was chosen for this study. A total of 865 oocytes were used for the experiments subsequently detailed.

3.2. Evaluation of pronuclear (PN) formation after ICSI-MGT assisted by various chemical activation treatments

Pronuclear formation was evaluated 16 h after initial ionomycin exposure of all activated oocytes (Table 1). All chemically activated oocytes had at least one PN. There was no difference between treatments in ICSI fertilization rates ($P > 0.05$). When only a single PN was observed, a condensed spermatozoon head was found. Zygotes with abnormal nuclear configurations (2PN/1PB/sperm head, 3PN/1PB or 1PN/1PB/sperm

Table 1
Pronuclear formation at 16 h after ICSI-mediated gene transfer (ICSI-MGT), assisted by various protocols of chemical activation in cattle.

Activation protocol	Sperm injection	No.	No. 2PN/ 2PB (%)	No. 1PN/ 2PB* (%)	No. others† (%)
Io-DMAP	+	24	12 (50.0)	2 (8.4)*[a]	10 (41.6)[a]
2Io-DMAP	+	26	20 (76.9)	4 (15.4)*[a]	2 (7.7)[b]
2Io	+	20	11 (55.0)	9 (45.0)*[b]	0 (0)[b]
Io-EtOH	+	23	17 (73.9)	5 (21.7)*[ab]	1 (4.4)[b]
Io-$SrCl_2$	+	23	18 (78.3)	4 (17.4)*[ab]	1 (4.3)[b]
Io-DMAP	−	31	1 (3.2)	17 (54.9)[c]	13 (41.9)[c]
2Io-DMAP	−	19	0 (0)	16 (84.2)[cd]	3 (15.8)[cd]
2Io	−	20	0 (0)	17 (85.0)[d]	3 (15.0)[cd]
Io-EtOH	−	22	0 (0)	18 (81.8)[cd]	4 (18.2)[cd]
Io-$SrCl_2$	−	26	0 (0)	23 (88.5)[d]	3 (11.5)[d]

Sperm injection +: ICSI assisted by activation. Sperm injection -: parthenogenetic activation.
PN, pronuclei; PB, polar body; Io, Ionomycin; DMAP, 6-Dimethylaminopurine; EtOH, Ethanol; $SrCl_2$, Strontium Chloride.
[ab] and [cd] Within a column, means without a common superscript differed for sperm injected + and sperm injected − conditions respectively ($P < 0.05$).
* Spermatozoa present in ICSI embryos.
† Others: 2PN/1PB/sperm head, 3PN/1PB, 1PN/1PB/sperm head.

head) were included as "others" in Table 1. This population was higher for ICSI assisted by Io-DMAP than for other ICSI activation treatments ($P < 0.05$).

3.3. Evaluation of EGFP-expression and detection of a condensed spermatozoon head in day 4 embryos produced by ICSI-MGT assisted by various chemical activation treatments

The presence of a condensed spermatozoon head was evaluated by Hoechst staining on Day 4 after ICSI-MGT. Two embryo groups were differentiated: green (EGFP-positive) and non-green (EGFP-negative). None of the embryos expressing EGFP had a condensed spermatozoon head (Table 2), whereas at least 50% of the non-expressing embryos had a condensed sperm head. Three of the five treatments had a 100% association between presence of sperm head and lack of transgene expression when embryos that had not been developmentally arrested (>4 cells) were evaluated (Table 2). Mosaic expression on Day 4 was similar for all treatments: 33.3, 50.0, 44.4, 35.0, and 31.5% for Io-DMAP, 2Io-DMAP, 2Io, Io-EtOH, and Io-$SrCl_2$ respectively ($P > 0.05$).

3.4. In vitro development of ICSI-MGT bovine embryos, assisted by various chemical activation treatments

A total of 485 bovine oocytes were injected with spermatozoa that had been incubated with pCX-EGFP plasmid for this experiment. All ICSI activation protocols produced blastocysts (Fig. 1). Rates of blastocyst production and also of blastocysts expressing EGFP were similar among 2Io-DMAP, Io-DMAP and Io-EtOH treatments. However, blastocysts and EGFP-blastocysts rates were higher after 2Io-DMAP than after treatment with 2Io and Io-$SrCl_2$. Transgene expression rates on Day 7 were higher for Io-DMAP, 2Io-DMAP, and Io-$SrCl_2$ than for 2Io and Io-EtOH (52.3, 53.0, 42.8, 28.2, and 29.4% respectively; $P < 0.05$). Over 80% of blastocysts expressed the transgene following ICSI activation treatments (Table 3, Fig. 1).

Twenty five ICSI-MGT embryos were analyzed by PCR. Plasmid was detected in all green (10/10) and in most non-green (13/15) embryos. Products of PCR were absent in only some of the non-green embryos that did not have a condensed sperm head (2/3). Moreover,

Table 2
Sperm head presence on EGFP-expressing and non-expressing ICSI-mediated gene transfer (ICSI-MGT) Day 4 bovine embryos.

Activation protocol	No. oocytes injected	No. 2–16 cell embryos analyzed (%)	Embryos expressing EGFP	No. sperm head present/ total embryos (%)	No. sperm head present/ >4 cell embryos (%)
Io-DMAP	54	41 (75.9)	+	0/27 (0)[a]	0/22 (0)[a]
			−	7/14 (50.0)[b]	3/5 (60)[b]
2Io-DMAP	69	52 (75.3)	+	0/34 (0)[a]	0/19 (0)[a]
			−	11/18 (61.1)[b]	4/5 (80)[b]
2Io	62	31 (50.0)	+	0/8 (0)[a]	0/2 (0)[a]
			−	14/23 (60.8)[b]	4/4 (100)[a]
Io-EtOH	47	28 (59.5)	+	0/20 (0)[a]	0/9 (0)[a]
			−	5/8 (62.5)[b]	2/2 (100)[b]
Io-$SrCl_2$	48	34 (70.8)	+	0/19 (0)[a]	0/5 (0)[a]
			−	9/15 (60)[a]	2/2 (100)[a]

Io, Ionomycin; DMAP, 6-Dimethylaminopurine; EtOH, Ethanol; $SrCl_2$, Strontium Chloride.
[ab] Within a column, means without a common superscript differed ($P < 0.05$).

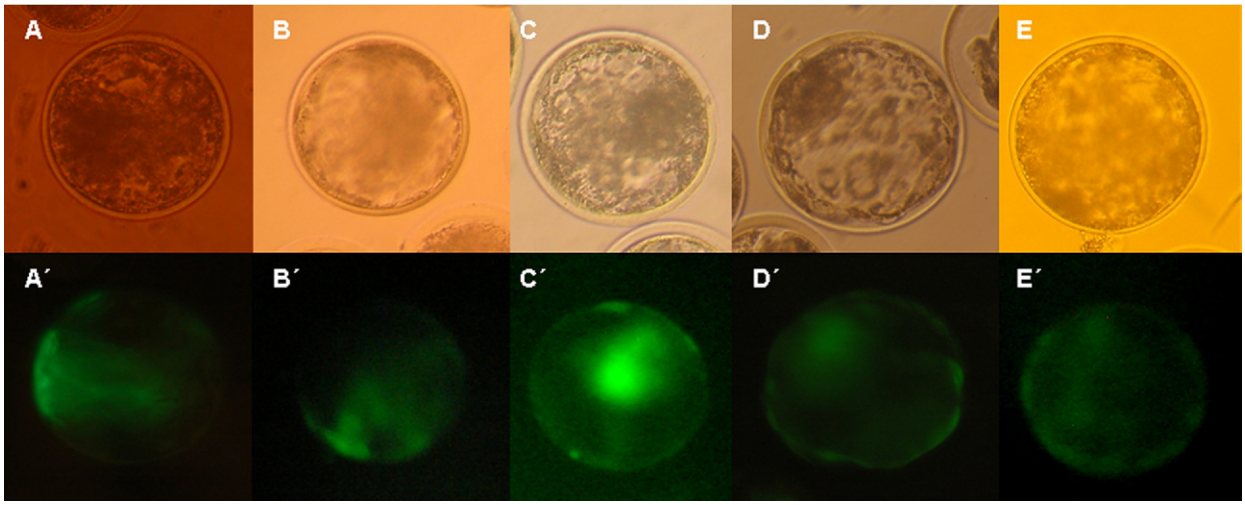

Fig. 1. Expression of EGFP by bovine blastocysts produced by ICSI-MGT assisted by: A–A′) Io-DMAP; B–B′) 2Io-DMAP; C–C′) 2Io, D–D′) Io-$SrCl_2$; and E–E′) Io-EtOH. A–E) Under bright light. A′–E′) blue light (488 nm). (Original magnification × 200).

PCR products were also absent in parthenogenic control embryos (15/15).

3.5. Quality of EGFP-expressing bovine blastocysts produced by ICSI-MGT assisted by various activation treatments

To compare the quality of EGFP-expressing blastocysts produced after various ICSI-MGT activation treatments, day of blastocyst formation, total cell number and Oct-4 expression patterns were evaluated (Table 4). Blastocysts expressing EGFP produced by ICSI-MGT assisted by activation with Io-DMAP, 2Io-DMAP, Io-EtOH, and Io-$SrCl_2$ appeared in higher proportions on Day 7; this was not significantly different from IVF control blastocysts. However, there was a delay in blastocyst development after ICSI-MGT assisted by 2Io ($P < 0.05$). Mean cell numbers of EGFP-expressing blastocysts were not significantly different among ICSI-MGT activation treatments, IVF, or parthenogenetic activation with Io-DMAP.

The expression of Oct-4 was not restricted to the inner cell mass (ICM) in any of the blastocysts analyzed (Fig. 2), but was also distributed in the trophoectoderm. In most cases, Oct-4 colocalized to the nuclei (Table 4, Fig. 2).

4. Discussion

In this study, all five activation treatments induced high pronuclear formation (range 50–78%) after ICSI-MGT. Three of these activation treatments, 2Io-DMAP, 2Io, and Io-$SrCl_2$, had never before been previously employed to assist bovine ICSI. In previous reports, ICSI using a piezo-electric actuator was more effective than conventional ICSI [19]. Nevertheless, in most cases, piezo-electric ICSI did not generate a sufficient

Table 3
In vitro development and EGFP-expression in bovine embryos produced by ICSI-mediated gene transfer (ICSI-MGT) assisted by various protocols of chemical activation.

Activation protocol	No.	No. cleaved (%)	No. morulae (%)	No. blastocysts (%)	No. blastocysts expressing *egfp*/	
					No. (%)	No. blastocysts (%)
Io-DMAP	80	57 (71.2)^{ab}	17 (21.2)^{ab}	15 (18.7)^{ab}	15/80 (18.7)^{ab}	15/15 (100.0)
2Io-DMAP	81	67 (82.7)^{a}	26 (32.0)^{a}	21 (25.9)^{b}	20/81 (24.6)^{b}	20/21 (95.2)
2Io	85	51 (60.0)^{b}	8 (9.4)^{c}	8 (9.4)^{a}	7/85 (8.2)^{a}	7/8 (87.5)
Io-EtOH	102	66 (64.7)^{b}	21 (20.5)^{ab}	15 (14.7)^{ab}	13/102 (12.7)^{ab}	13/15 (86.6)
Io-$SrCl_2$	119	76 (63.8)^{b}	16 (13.4)^{bc}	13 (10.9)^{a}	11/119 (9.2)^{a}	11/13 (84.6)

Io, Ionomycin; DMAP, 6-Dimethylaminopurine; EtOH, Ethanol; $SrCl_2$, Strontium Chloride.
$^{a-c}$ Within a column, means without a common superscript differed ($P < 0.05$).

Table 4
Quality of EGFP expressing bovine blastocysts produced by ICSI-mediated gene transfer (ICSI-MGT) in terms of formation day, cell number and Oct 4 expression.

Activation protocol	Early formation (≤Day 7) (%)	Late formation (>Day 7) (%)	No.	Cell number (mean ± SD)	Oct-4$^+$cells (mean ± SD)
Io-DMAP	13/15 (86.7)^a	2/15 (13.3)^a	3	68.33 ± 12.0	56.3 ± 10.0
2Io-DMAP	16/20 (80.0)^{ab}	4/20 (20)^{ab}	4	91.5 ± 30.7	71.7 ± 26.0
2Io	3/8 (37.5)^b	5/8 (62.5)^b	2	70.0 ± 22.6	38 ± 1.4
Io-EtOH	8/13 (61.6)^{ab}	5/13 (38.4)^{ab}	4	69.2 ± 25.4	50.2 ± 15.5
Io-$SrCl_2$	8/11 (72.7)^{ab}	3/11 (27.3)^{ab}	4	69.2 ± 14.9	52.5 ± 16.0
PA DMAP	8/10 (80)^{ab}	2/10 (20)^{ab}	5	68.4 ± 10.8	48.8 ± 16.4
IVF	6/6 (100)^a	0/6 (0)^a	4	104.8 ± 54.4	75 ± 32.5

Io, Ionomycin; DMAP, 6-Dimethylaminopurine; EtOH, Ethanol; $SrCl_2$, Strontium Chloride; PA, Parthenogenetic Activation; IVF, *in vitro* fertilization.
$^{a-b}$ Within a column, means without a common superscript differed ($P < 0.05$).

calcium response when bovine or mouse sperm were injected into bovine oocytes [20]. In order to duplicate the activating effect produced by piezo-electric [12], all treatments in the present study included an initial incubation in Io. In ICSI assisted by Io-DMAP, there was an increased presence of "other" nuclear configurations at the pronuclear stage, with many of the fertilized oocytes exhibiting a decondensed spermatozoon. These results were consistent with previous reports that DMAP-related noxious nuclear affected both partheno-

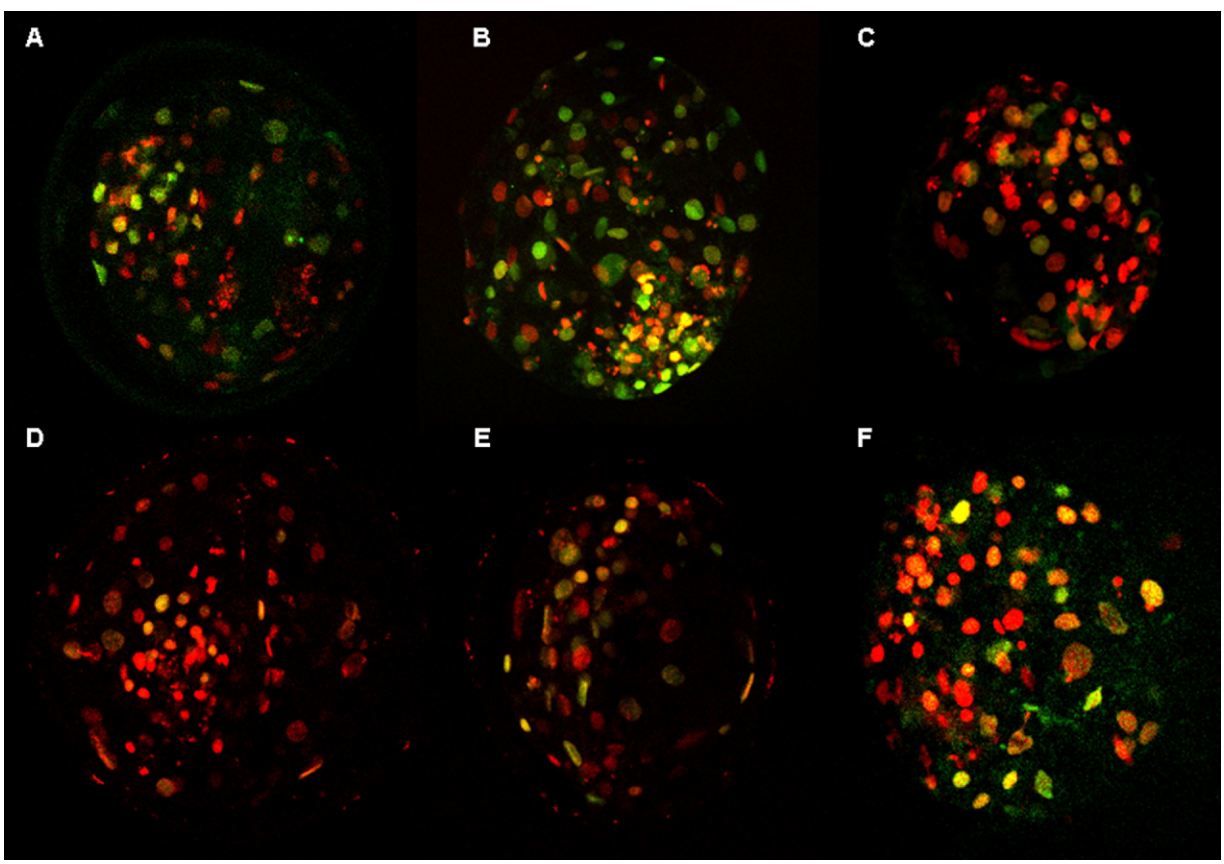

Fig. 2. Oct-4 expression pattern of EGFP-expressing bovine blastocysts produced by ICSI-MGT assisted by: A) Io-DMAP; B) 2Io-DMAP; C) 2Io; D) Io-SrCl2, E) Io-EtOH; and F) IVF control (Original magnification × 200).

genetic and cloned embryos [21,22]. Coincidently, embryos produced by ICSI assisted by activation with Io-DMAP had poor viability after transfer to recipient heifers [8,13,23]. Surprisingly for ICSI-MGT, the addition of a second Io immediately before DMAP decreased the incidence of abnormal nuclear species at the pronuclear stage. The second calcium peak and consequent decrease in Mitosis Promoting Factor (MPF) activity produced by the second Io incubation just before DMAP could have decreased the abnormal nuclear species at 16 h after Io-DMAP.

Sperm decondensation was fundamental for ICSI-MGT; there was not a single condensed sperm head in any green embryo analyzed. In all cases where the spermatozoon was condensed, the transgene was not expressed. A fraction of the non-green embryos also did not exhibit condensed spermatozoa. This could be attributed to transgenesis expression failure resulting from: 1) developmental arrest before maternal embryonic transition (MET) [24]; 2) integration in non-transcriptionally active genome loci [25]; 3) active silencing due to repetitive transgene insertion [26]; or 4) failure in sperm deposition. The present observations were consistent with previous reports that ICSI-MGT transgenic embryos were a product of fertilization and therefore, were not parthenogenetic [3,27,28]. Based on the present study, transgene expression could be considered a marker of fertilization when an early expressed gene marker was used.

The five activation treatments evaluated in the present study induced high blastocyst development after ICSI-MGT. Treatment with 2Io-DMAP tended to improve blastocyst rates. Treatment of ICSI embryos with Io-DMAP, 2Io-DMAP, and Io-$SrCl_2$ had higher EGFP expression rates. However, in our experience, strontium was not capable of sustaining development as efficiently as the treatments that incorporated DMAP or ethanol; the latter induced developmental rates similar to those resulting from DMAP treatment, but was unable to sustain high transgene expression rates. Treatments capable of inducing the highest blastocyst rates also induced the highest EGFP blastocysts rates. Calcium dynamics differed after ethanol, ionomycin and strontium parthenogenetic activation; the ethanol-induced calcium peak was longer than that stimulated by ionomycin [29]. Conversely, strontium promoted Ca^{+2} increases in multiple pulses [30], resembling calcium behavior following conventional fertilization. Strontium is part of an oocyte activation procedure commonly employed in mice [31], but it is not routinely used in cattle [32].

In the present study, EGFP expression rates exceeded 50%, with >80% of blastocysts expressing the transgene. The PCR analysis detected EGFP amplification in all green embryos analyzed and in all non-green embryos with a condensed sperm head. This was consistent with high PCR sensitivity that is capable of amplifying low plasmid copies bound to sperm membranes. Conversely, no PCR product was obtained from two of three embryos without a condensed sperm head, perhaps due to failure in sperm deposition or to free plasmid degradation [33]. Results for ICSI-MGT bovine blastocyst production were comparable and seemed even better than previous reports using SCNT [34,35] or pronuclear microinjection [4]. However, both for ICSI-MGT and for pronuclear microinjection, integration of the transgene can occur after first genomic DNA replication, resulting in chimeric animals [36,37]. This problem was not detected when transgenic cells stably transfected are used as donor nuclei for SCNT. Nevertheless, in the case of SCNT, a selection marker gene is required. In future studies, transgenic offspring production should be evaluated to assess the efficacy of ICSI-MGT in cattle, using activation treatments such as those used in this study.

The detection of transgene expression at the blastocyst stage may indicate transgene integration. In previous reports involving similar work in mice and pigs, there was no discussion regarding the state of the transgene after ICSI-MGT. In both species, 5–25 transgene copies were integrated in one or two host genome loci [3,36,38–40]. The size of the transgene (5.5 kb) and the amount of DNA in a bovine blastocyst (≤6 pg) made it very difficult to confirm transgene integration using techniques such as FISH or Southern blots.

There was high EGFP-expressing blastocyst formation by Day 7, for Io-DMAP, 2Io-DMAP, Io-EtOH, and Io-$SrCl_2$, with no significant differences from IVF controls. With the exception of the 2Io treatment, these results disagreed with a previous report of a delay in Days 7 to 8 blastocyst formation following ICSI [41]. In addition, EGFP-expressing blastocysts had similar mean cell numbers and Oct-4 expression patterns to those produced by IVF, indicating that neither the ICSI-activation procedure nor the presence of the transgene affected these aspects of embryo quality. Colocalization of Oct-4 was not restricted to the ICM, but to the trophoectoderm also. This was in contrast to mice [42], but consistent with previous reports that Oct-4 cannot be considered a marker of pluripotency in cattle [43].

Based on the present study, we inferred that ICSI-MGT may be useful for large scale transgenic bovine

offspring production. Although the possibility of obtaining transgenic offspring by ICSI-MGT in ruminants was proposed previously [44], the lack of basic work in this area has made it difficult to apply.

In summary, five chemical activation treatments were used to assist bovine ICSI-MGT with high efficiency. In addition, fertilization was crucial for transgene expression; it was affected by various treatments designed to assist ICSI and can be considered a marker of fertilization. These activation treatments should be considered for future application in ICSI-MGT.

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