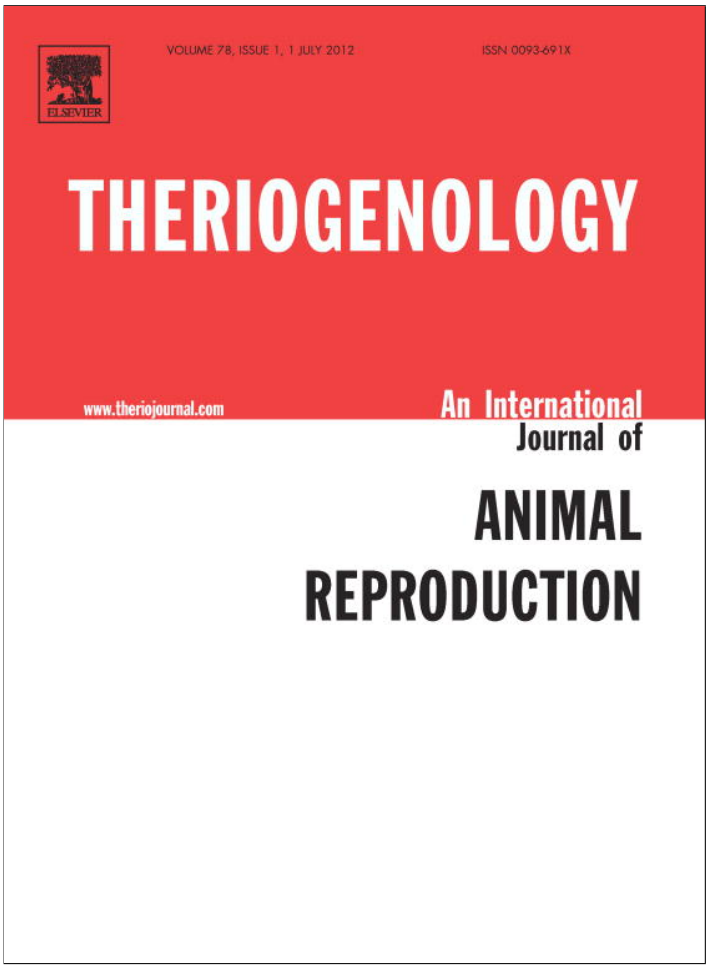

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Production of IVF transgene-expressing bovine embryos using a novel strategy based on cell cycle inhibitors

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Abstract

The objective was to evaluate the effects of cell cycle inhibitors (6-dimethylaminopurine [DMAP], and dehydroleukodine [DhL]) on transgene expression efficiency and on mosaic expression patterns of IVF bovine zygotes cytoplasmically injected with oolema vesicles coincubated with transgene. The DNA damage induced by the transgene or cell cycle inhibitors was measured by detection of phosphorylated histone H2AX foci presence (marker of DNA double-stranded breaks). Cloning of *egfp* blastomeres was included to determine continuity of expression after additional rounds of cellular division. The pCX-EGFP [enhanced green fluorescent protein gene (EGFP) under the chimeric cytomegalovirus IE-chicken-β-actin enhancer promoter control] gene plasmid (50 ng/μL) was injected alone (linear or circular exogenous DNA, *leDNA* and *ceDNA*, respectively) or associated with ooplasmic vesicles (*leDNA-v* or *ceDNA-v*). The effects of 2 mM DMAP or 1 μM DhL for 6 h (from 15 to 21 h post IVF) was evaluated for groups injected with vesicles. The DMAP increased ($P < 0.05$) *egfp* homogenous expression relative to transgene alone (21%, 18%, and 11% for *leDNA-v* + DMAP, *leDNA-v*, and *leDNA*, respectively) and also increased ($P < 0.05$) the phosphorylated histone H2AX foci area. Expression of *egfp* was higher ($P < 0.05$) for linear than for circular pCX-EGFP, and *egfp* blastocyst rates were higher ($P < 0.05$) for groups injected with linear transgene coincubated with vesicles than for linear transgene alone (95%, 77%, 84%, and 52% for *leDNA-v* + DMAP, *leDNA-v* + DhL, *leDNA-v*, and *leDNA*, respectively). Moreover, DMAP tended to improve *egfp* blastocysts rates for both circular and linear transgenes. Based on fluorescent in situ hybridization (FISH) analysis, there was evidence of integration in *egfp* embryos. Finally, clones derived from *leDNA-v* + DMAP had the highest *egfp* expression rates (96%, 65%, and 65% for *leDNA-v* + DMAP, *leDNA-v*, and *leDNA*, respectively). Transgenesis by cytoplasmic injection of *leDNA-v* + DMAP is a promising alternative for transgenic animal production.

Keywords: 6-Dimethylaminopurine; Dehydroleukodine; Transgenesis; Phosphorylated; Histone H2AX; Cattle

1. Introduction

Several techniques are currently used to produce transgenic mammalian embryos, including pronuclear microinjection [1], SCNT [2], sperm mediated gene transfer [3], and intracytoplasmic sperm injection (ICSI)-mediated gene transfer [4]. However, mechanisms involved are not yet fully understood. The first critical step for stable transgenesis is introduction of exogenous DNA into the host genome. After transgene incorporation into a pronucleus, exogenous DNA (eDNA) can be integrated into the host genome mainly as result of the activity of DNA repair machinery associated with replication [5,6]. It has been suggested that during pronuclear microinjection, sponta-

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neous chromosomal ruptures, exacerbated by the micromanipulation procedure, become eDNA integration sites by ligation reaction [7]. In addition, using sperm-mediated transgenesis, the interaction between transgene and the spermatozoon is responsible for an increase in paternal chromosome ruptures, which are proportional to transgenesis efficiency [8]. All of these observations indicate that chromosomal ruptures and integration rates are closely related.

The early integration of the eDNA into the host genome is a critical step for homogeneous transgenic embryo production. The chemical agents 6-dimethylaminopurine (DMAP) and dehydroleukodine (DhL) are used for parthenogenic activation, SCNT, and ICSI [9–14]. In addition, effects of DMAP on the inhibition of DNA synthesis [15] and on cell synchronization [16] are also known.

Dehydroleukodine was originally discovered as an agent capable of arresting the cell cycle of smooth muscle vascular cells at the G2 stage [17]. Treatment with these agents during the first pronuclear phase of zygotes injected with transgene could arrest the cell cycle and provoke DNA ruptures, increasing the activity of the repairing mechanisms associated with replication. Consequently, they could improve incorporation of transgenes into the bovine genome.

A group of cellular proteins are responsible for arresting the cell cycle and for activating DNA repair pathways in response to DNA double-stranded breaks (DSBs) or other DNA damage. The phosphorylation of histone H2AX (γH2AX) is a marker of DNA DSBs; [18] it plays a key role in DNA damage checkpoint activation by recruiting numerous repairing proteins to the vicinity of DNA lesions [19,20]. To date, γH2AX has apparently not been measured in bovine zygotes exposed to exogenous DNA and to various agents during the first embryonic S phase.

Our group recently developed a new technique for transgene-expressing embryo production, involving generation of small oolema vesicles by oocyte microsurgery, their short coincubation with eDNA, and finally their injection into the cytoplasm of presumptive zygotes generated by IVF [21]. This method was efficient for bovine and ovine transgene-expressing embryo production; however, as was the case for pronuclear microinjection and ICSI-mediated gene transfer, mosaic expression was observed [1,4].

In the present study we exploited these observations as opportunities to design strategies which could be applied to improve transgenesis efficiency and to reduce mosaic expression patterns of bovine transgene-expressing embryos. The effects of two cell cycle synchronizers (DMAP and DhL) incubated with zygotes on the expression of eDNA and on the induction of DNA breaks (measured by immunocytochemistry against γH2AX), were evaluated. Cytoplasmic injection of plasmid alone or coincubated with vesicles (both linear and circular eDNA) were also tested. The integration status of the transgene was assessed by fluorescent in situ hybridization (FISH) analyses. Finally, cloning of transgene expressing blastomeres was included to evaluate homogenous transgene expression after additional rounds of cellular division.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: effect of cell cycle inhibitors (DMAP and DhL) during first pronuclear phase on IVF zygotes development

The IVF-derived presumptive zygotes were incubated in DMAP or DhL for 9 h (from 15 to 24 h post IVF) or for 6 h (15 to 21 h post IVF). First cellular division and development to blastocysts were evaluated.

2.1.2. Experiment 2: effect of cell cycle inhibitors (DMAP and DhL) on transgene expression of bovine embryos produced by vesicle-mediated transgenesis

Exogenous DNA alone (eDNA alone) or coincubated with vesicles (eDNA-v) was injected into bovine presumptive zygotes immediately after IVF. A group of IVF presumptive zygotes injected with vesicles + eDNA was incubated in DMAP (eDNA-v + DMAP) or DhL (eDNA-v + DhL) for 6 h (from 15 to 21 h post IVF). All of these treatments were repeated for circular (*ceDNA*) and linear (*leDNA*) structures of the transgene. Transgene expression was evaluated daily.

2.1.3. Experiment 3: FISH analysis of bovine embryos produced by vesicle and by transgene alone-mediated transgenesis

Bovine *egfp*-expressing blastocysts produced by IVF, followed by injection with linear transgene alone (*leDNA* alone) or coincubated with vesicles (*leDNA-v*), were subjected to FISH analysis to evaluate transgene integration status.

2.1.4. Experiment 4: effect of transgene and cell cycle inhibitors on DNA damage measured by γH2AX in the pronuclei of presumptive transgene expressing zygotes

Bovine presumptive zygotes produced by groups *leDNA* alone, *leDNA-v, leDNA-v* + DMAP, and *leDNA-v* + DhL were fixed at 16 h post IVF and subjected to immunocytochemistry against histone γH2AX, a marker of DNA double-stranded breaks, to measure the number of DNA ruptures after various treatments. Control groups lacking transgene were also included; these consisted of injection of sham vesicles (vesicles lacking transgene) or sham injection (medium alone) and IVF.

2.1.5. Experiment 5: cloning of egfp-expressing blastomeres produced by the different treatments

Day 3 *egfp* blastomeres produced by IVF followed by injection with *leDNA* alone, *leDNA-v*, or *leDNA-v* + DMAP were used as donors for zona-free cloning.

Homogeneous expression was evaluated before and after cloning.

2.2. Chemicals

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

2.3. DNA construction

The plasmid used was pCX-EGFP [enhanced green fluorescent protein gene (EGFP) under the chimeric cytomegalovirus IE-chicken-β-actin enhancer promoter control] kindly provided by Dr Masaru Okabe (Osaka University, Osaka, Japan) that contained the enhanced green fluorescent protein gene (*egfp*) under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control [22]. The plasmid pCX-EGFP employed was either in circular or linear (by *HindIII* digestion) form.

2.4. Oocyte collection and in vitro maturation

Bovine ovaries were collected from abattoirs and transported to the laboratory at 25 °C to 30 °C. Cumulus-oocyte-complexes (COCs) were aspirated (with a 21-gauge needle) from ovarian follicles 2 to 5 mm in diameter, into HEPES-buffered Tyrode's albumin lactate pyruvate (HEPES-TALP). Oocytes with at least three layers of granulosa cells were selected for in vitro maturation (IVM). The maturation medium was bicarbonate-buffered TCM-199 (31 100-035; Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS, 013/07; Internegocios, Buenos Aires, Argentina), 10 ug/mL follicle-stimulating hormone (NIH-FSH-P1, Folltropin, Bioniche, Caufield Junction Caufield North, Victoria, Australia), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768), and 2% antibiotic-antimycotic (ATB, 15 240-096; Gibco). The oocytes were incubated for 24 h under mineral oil (M8410) in 100 μL droplets, in 6.5% CO_2 in humidified air at 39 °C.

2.5. IVF procedure

Frozen semen was thawed in a 37 °C water bath for 30 sec. Spermatozoa were then centrifuged twice (490 × *g* for 5 min) in Brackett-Oliphant medium (BO) [23] and resuspended in BO supplemented with 5 mM caffeine (C4144) and 20 IU/mL heparin (H3149). Spermatozoa were adjusted to 40 × 10^6/mL and diluted to half concentration (20 × 106/mL) with BO containing 10 mg/mL fatty acid-free bovine serum albumin (FAF-BSA) (A6003). The cumulus-oocyte-complexes were washed twice with BO medium plus 5 mg/mL FAF-BSA and subsequently exposed to the sperm suspension for 5 h in a 100 μL drop at 39 °C under 5% CO_2 in humidified air. Presumptive zygotes were then washed three times in HEPES-TALP. After IVF, cumulus cells were removed from presumptive zygotes by vortexing for 2 min in hyaluronidase (H-4272; 1 mg/mL in Dulbecco's PBS). Then, presumptive zygotes were washed in HEPES-TALP, selected by visualization of at least one polar body, and immediately injected with vesicles or DNA alone.

2.6. Vesicle production and incubation with exogenous DNA

Oocytes to be used as vesicle donors were subjected to IVM, IVF, and hyaluronidase treatment. Then, presumptive zygotes were transferred to 20-μL droplets of HEPES-TALP. Each presumptive zygote was held under negative pressure with a holding pipette while a 9-μm pipette was passed through its zona pellucida until it contacted the ooplasm. A small fraction of the ooplasm (<10 μm) was then aspirated by negative pressure, avoiding plasma membrane breakage. Approximately 15 vesicles were obtained from each donor presumptive zygote. Vesicles that formed inside the pipette were transferred into a 3-μL droplet of 10% polyvinylpirrolidone containing 50 ng/μL circular or linear pCX-EGFP and left there for 5 min (plasmid incubation). Finally, vesicles were aspirated into the 9-μm pipette and directly injected into the denuded fertilized oocytes.

Presumptive zygotes were subsequently cultured as described below.

2.7. Injection of exogenous DNA alone

Fertilized oocytes subjected to hyaluronidase treatment were transferred to 20 uL droplets of HEPES-TALP and injected, using a 9-μm pipette, with 10% polyvinylpirrolidone containing 50 ng/μL pCX-EGFP in a volume equivalent to that used for the vesicles injection (<10 pL). Presumptive zygotes were subsequently cultured as described below.

2.8. Incubation in DMAP or DhL

Presumptive zygotes subjected to vesicle-mediated transgenesis were incubated for 6 h (from 15 to 21 h post IVF) or 9 h (from 15 to 24 h post IVF) in 1.9 mM DMAP (D2629) in a 100-μL droplet of synthetic oviductal fluid (SOF) [24] or in 1 μM DhL (Instituto de Embriologia e Histologia, Mendoza, Argentina) diluted in TCM-199 medium. After treatments, oocytes were thoroughly washed in HEPES-TALP and cultured as described below.

2.9. SCNT

2.9.1. Enucleation procedure

After 21 h of IVM, MII oocytes were subjected to hyaluronidase treatment and stained with 1 μg/mL of Hoechst bisbenzimide 33342 for 10 min. Oocytes were then immediately transferred into 50 μL microdroplets of HEPES-TALP supplemented with 0.3 g/mL BSA, under mineral oil, in 100 × 20 mm tissue culture dishes (430167; Corning, Horseheads, NY, USA). Then, stained oocytes were mechanically enucleated using a Narishige hydraulic micromanipulator (Narishige Sci., Tokyo, Japan) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA). Enucleation was performed using a 20-μm internal diameter pipette. Metaphase chromosomes were visualized under ultraviolet light (<10 sec) and aspirated into the pipette with a minimal volume of oocyte cytoplasm. Chromosome removal was confirmed by the presence of stained MII chromosomes inside the pipette.

2.9.2. Donor cell preparation

Day 3 EGFP-expressing embryos produced by *leDNA-v* + DMAP, *leDNA-v* or *leDNA* alone were treated with 1.5 mg/mL pronase (P8811) dissolved in HEPES-TALP to remove the zona pellucida (ZP). Gentle pipetting was applied to disaggregate blastomeres from these embryos. Blastomeres expressing EGFP were selected under blue light using an excitation filter at 488 nm and an emission filter at 530 nm and then used as donor cells for cloning.

2.9.3. Fusion procedure

To fuse EGFP-expressing blastomeres to enucleated oocytes, the latter were first incubated in 1.5 mg/mL pronase to remove the ZP. These ZP-free enucleated oocytes were then individually transferred to a drop of 1 mg/mL phytohemagglutinin (L8754) dissolved in TCM-199 without serum, where they remained for a few seconds. Following this, they were quickly dropped over a single blastomere resting on the bottom of a 100 μL HEPES-TALP drop. Following attachment, the ZP-free enucleated oocyte/*egfp*-blastomere pair was picked up, transferred to fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 1 mg/mL polyvinyl alcohol), for 2 to 3 min and then to a fusion chamber (BTX Instrument Division, Harvard Apparatus, Holliston, MA, USA) containing 2 mL of the same warm medium. Fusion was performed with a double direct current (dc) pulse of 75 V, each pulse for 30 ms 0.1 sec apart. The reconstructed zygotes were then carefully transferred to SOF culture droplets for 2 h to allow reprogramming.

2.9.4. Chemical oocyte activation

Embryos produced by SCNT were activated with 5 μL ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) in HEPES-TALP for 4 min and subsequently transferred individually to 1.9 mM DMAP (D2629) in SOF droplets for 3 h. Embryos were then washed three times in HEPES-TALP to remove the inhibitor, and cultures were continued as described below.

2.10. In vitro embryo culture

Presumptive zygotes derived by vesicle and by free transgene mediated transgenesis were cultured in 50-μL droplets of SOF medium supplemented with 2.5% FBS at 39 °C in 6.5% CO_2 in humidified air. The embryos were transferred to a new droplet every 48 h. Cleavage was evaluated on Day 2 and the number of blastocysts on Day 7. Reconstructed SCNT embryos were cultured in SOF medium in a system similar to the Well of the Well (WOW) [25], whereby microwells were produced using a heated glass capillary slightly pressed to the bottom of a petri dish and then covered with a 100 μL microdrop of SOF medium (20 to 30 WOW in each microdrop, one embryo per WOW). During cloned embryo culture, the medium was supplemented with 7.5% FBS on Day 5. Cleavage was evaluated on Day 2 and blastocyst formation on Day 7 (after fusion).

2.11. Immunocytochemistry

Bovine presumptive zygotes produced by *leDNA-v* + DMAP, *leDNA-v* + DhL, *leDNA*-v, *leDNA* alone and sham vesicles, sham injection and IVF controls were treated to remove ZP and fixed 21 h post fertilization with 4% paraformaldehyde (F-1635) in PBS for 30 min. Briefly, whole-mount immunocytochemistry consisted of permeabilization by 15-min incubation in PBS containing 0.2% vol/vol Triton-X 100 (T-9284). Nonspecific immunoreactions were blocked by incubation with 3% vol/vol fetal calf serum and 0.1% vol/vol Tween 20 (Promega, Madison, WI, USA; H5152) in PBS (blocking buffer) for 30 min. After this pretreatment, the antiphospho-histone H2AX (Ser 139) clone JBW 301 (mouse monoclonal IgG1; 1039, Millipore, Temecula, CA, USA) diluted 1:100 in PBS was applied for 2 h at 37 °C. Presumptive zygotes at the pronuclear stage were washed extensively in blocking buffer for 15 min. Then, samples were incubated with secondary goat anti-mouse fluorescein isothiocianate (FITC)-IgG 2 mg/mL (1709, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200, for 45 min at 37 °C in the dark. After additional washing, the embryos were incubated in PBS containing propidium iodide (PI) (5 μg/mL) for 10 min in the dark. Embryos were mounted on slides using 70% vol/vol glycerol. Negative controls were produced using only the secondary antibody.

2.11.1. Confocal laser scanning microscopy

The 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 FITC secondary antibody and a 544-nm wavelength to excite PI. Images were scanned in 2048 × 2048 for a resolution of 0.15 × 0.15-μm pixel size. Three-dimensional images were constructed using software EZ-C1 2.20 (Nikon Corporation, Melville, NY, USA).

2.11.2. Quantification of γH2AX

Three-dimensional images were imported into ImageJ v 1.42 (National Institutes of Health, Bethesda, MD, USA). Images were converted into an 8-bit grayscale image and the Threshold tool in ImageJ was used to adjust all the pictures to the minimal threshold level of 30. The minimum size of a particle was defined as the size of one pixel in um^2, and the pronuclear area was delimited for analysis.

2.12. Evaluation of EGFP fluorescence in embryos

All embryos produced 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.

Embryos obtained by the different transgenesis strategies were evaluated daily. Cloned embryos were evaluated five and seven days post-chemical activation.

2.13. Fluorescence in situ hybridization (FISH)

Embryos from *leDNA-v* and free *leDNA* that expressed *egfp* were subjected to FISH using the pCX-EGFP plasmid as a probe. Embryos were incubated for 20 h with 0.1 μg/mL demelcochine (D1925). Afterward, embryos were placed on poly-L-lysine coated slides, previously treated with hypotonic solution (1% Na citrate in distilled water for 10 min) and fixed in situ with 3:1 methanol-acetic acid. The pCX-EGFP was labeled with Rhodamine-5-dUTP (RU-013-0135; eENZYME, Montgomery Village, MD, USA) by Nick Translation System (18 156-010; Invitrogen). The DNA probe was denatured for 10 min at 85 °C in a hybridization mix (H. Mix) containing 50% formamide (Merck, Darmstadt, Germany), 40% sulfate dextrane (D8906), 20× sodium chloride-sodium citrate (SSC) 10%, and 0.03% herring sperm DNA. The proportion used was 5:1 (hybridization mix: labeled DNA). Before application of the denatured probe, fixed embryos on slides were denatured with 70% formamide in 2× SSC for 2 min at 72 °C and dehydrated by successive passage through 70%, 95%, and 100% ethanol.

The probe was incubated overnight in a moist dark chamber at 37 °C. After incubation the coverslips were detached with a short incubation in 2× SSC, the slides were washed at 72 °C for 2 min in 0.4× SSC with 0.3% Tween and then washed at room temperature in 2× SSC with 0.1% Tween. The total DNA was counterstained with 4′,6-diamidino-2-phenylindole and the coverslips were applied. Images of each cell and their signals were recorded with an Optronics camera.

2.14. Statistical analysis

In vitro embryo development and fluorescent expression were compared by Fisher's exact test analysis. Histone γH2AX foci number and area were compared by one-way ANOVA and Tukey's post test. All statistical analyses were done with SAS [26] and differences were considered significant at $P < 0.05$.

Table 1
Effect of cell cycle inhibitors (DMAP and DhL) on transgene expression of bovine embryos produced by vesicle-mediated transgenesis.

Treatment	N	Blastocyst (%)	*egfp* dynamics		*egfp* blastocysts/blastocysts (%)
			Day 3 (%)	Day 4 (%)	
ceDNA-v + DMAP	72	26 (36)[a,b]	4 (5)[a]	19 (26)[a,b]	16/26 (61)[a,c]
ceDNA-v + DhL	72	23 (31)[a,b]	6 (8)[a]	22 (30)[a]	11/23 (47)[a]
ceDNA-v	93	28 (30)[a,b]	7 (7)[a]	25 (26)[a,b]	13/28 (46)[a]
ceDNA	73	18 (24)[a]	5 (6)[a]	14 (19)[b]	8/18 (44)[a]
leDNA-v + DMAP	81	22 (27)[a]	55 (67)[b]	61 (75)[c]	21/22 (95)[b]
leDNA-v + DhL	68	22 (32)[a,b]	39 (57)[b,c]	42 (61)[c,d]	17/22 (77)[b,c]
leDNA-v	84	26 (31)[a,b]	45 (53)[b,c]	54 (64)[c,d]	22/26 (84)[b,c]
leDNA	63	19 (30)[a,b]	30 (47)[c]	32 (50)[d]	10/19 (52)[a]
Control DMAP	116	39 (33)[a,b]	NA	NA	NA
Control DhL	67	24 (35)[a,b]	NA	NA	NA
Control	130	55 (42)[b]	NA	NA	NA

Within a column, percentages without a common superscript differed ($P < 0.05$). *leDNA-v* + DMAP, *leDNA-v* + DhL, *leDNA-v*, and *leDNA*; the treatments previously described, but injected with linear pCX-EGFP.
ceDNA, circular exogenous DNA alone injection; *ceDNA-v*, circular exogenous DNA coincubated with vesicles; *ceDNA-v* + DhL, circular exogenous DNA coincubated with vesicles followed by dehydroleukodine (DhL) incubation; *ceDNA-v* + DMAP, circular exogenous DNA coincubated with vesicles cytoplasmically injected into IVF presumptive zygotes followed by dimethylaminopurine (DMAP) incubation; NA, not applicable.

3. Results

3.1. Experiment 1: effect of cell cycle inhibitors (DMAP and DhL) during first pronuclear phase on IVF zygotes development

In a first experiment, the effect of incubation in DMAP and DhL for 9 h (from 15 to 24 h post IVF) was evaluated. Incubation in DMAP or DhL inhibited cleavage (0/83, 0% and 6/66, 9% cleavage rates for IVF plus DMAP and IVF plus DhL, respectively; vs. 45/70, 64% for the IVF control) at 24 h post IVF. After DMAP or DhL removal, in vitro culture was continued and more than 50% of the oocytes cleaved at 48 h post IVF. However, development to blastocysts was affected (3/37, 6% and 3/30, 10% blastocyst rates for DMAP and DhL, respectively; vs. 12/24, 50% for the control). Consequently, DMAP and DhL 6-h incubation (from 15 to 21 h after IVF) was evaluated. Blastocyst rates did not differ from the control group in this case (40%, 41%, and 50% for DMAP, DhL, and control, respectively). Based on these results, we decided to use this 6-h incubation window for DMAP or DhL as the conditions for the remaining work.

3.2. Experiment 2: effect of cell cycle inhibitors (DMAP and DhL) on transgene expression of bovine embryos produced by vesicle-mediated transgenesis

Transgene expressing embryos were produced by injection of oolema vesicles coincubated with circular or linear pCX-EGFP or by injection with pCX-EGFP alone in IVF presumptive zygotes (Table 1, Fig. 1). Addition of DMAP or DhL for 6 h (from 15 to 21 h post IVF) was evaluated. Cleavage rates were >70% after all treatments. Although blastocyst rates were not significantly different among treatments, both *ceDNA* alone and *leDNA-v* + DMAP differed significantly from the IVF control group. The *egfp* expression rates were higher when linear pCX-EGFP was used ($P < 0.05$), independent of treatment. In addition, expression was detected earlier ($P < 0.05$) when linear transgene was employed. However, when *ceDNA* was employed, *egfp* blastocyst rates were not significantly different between *ceDNA*-v and *ceDNA* groups. Conversely, blastocysts expressing *egfp* were fewer for *leDNA* alone compared with *leDNA-v* (Table 1). Addition of 6-DMAP or DhL for 6 h did not increase total transgene-expressing embryos, although there was a tendency to increase *egfp*-expressing blastocysts in DMAP groups.

3.3. Experiment 3: FISH analysis of bovine embryos produced by vesicle and by transgene alone-mediated transgenesis

Blastocyts expressing *egfp* produced by *leDNA-v* and by *leDNA* injection were analyzed by FISH. Positive signals were detected in one blastocyst produced after each treatment (1/2 embryos from *leDNA-v*; 1/1 embryo from *leDNA* alone). The signals were observed in pairs which could be associated with each sister chromatid in interphase cells (Fig. 1).

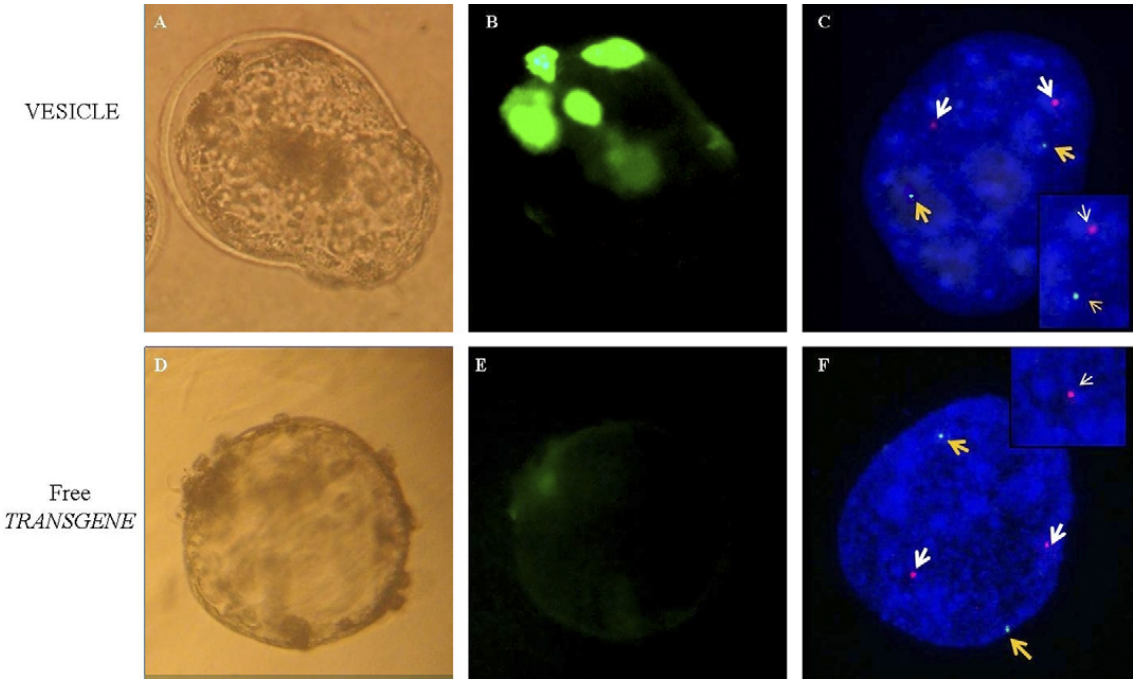

Fig. 1. Bovine blastocysts produced by linear exogenous DNA coincubated with vesicles (*leDNA-v*) (A, B, C) and by linear exogenous DNA alone (*leDNA*) (D, E, F) cytoplasmic injection mediated transgenesis. (A, D) Under bright light; (B, E) under blue light (488 nm); (C) fluorescent in situ hybridization (FISH) analysis of an *leDNA-v* blastocyst; and (F) FISH analysis of an *leDNA* blastocyst. White arrows indicate pCX- enhanced green fluorescent protein (EGFP) Rhodamine labeled probe (red). Yellow arrows indicate bovine chromosome 5 pericentromeric probe, FITC labeled (green).

3.4. Experiment 4: effect of transgene and cell cycle inhibitors on DNA damage measured by γH2AX in the pronuclei of presumptive transgene expressing zygotes

The presence of DNA double-stranded breaks in the pronuclei of presumptive zygotes produced after various treatments was measured by immunocytochemistry against γH2AX (Table 2, Figs. 2 and 3). All presumptive zygotes were evaluated at the one-cell stage. In some cases, chromosomes were totally condensed, indicating that these zygotes had already reached M phase (N = 5 for IVF control group; N = 1 for sham injection; N = 2 for sham vesicles; N = 1 for *leDNA-v*; and N = 6 for *leDNA*; these data were excluded from Table 2). The γH2AX foci area was higher for the group incubated in DMAP than for *leDNA* alone, sham injection, and IVF control groups ($P < 0.05$).

Table 2
Detection of the phosphorylation of histone H2AX (γH2AX) foci number and γH2AX foci area on bovine presumptive zygotes as an indicator of DNA damage induced by the transgene and by cell cycle inhibitors.

Treatment	Analyzed embryos		Total embryos (1 PN/embryo)*		2 PN embryos	
	Total N	2 PN	Mean ± SD foci	Mean ± SD foci area ($\mu m^2 \times 10^3$)	Mean ± SD foci	Mean ± SD foci area ($\mu m^2 \times 10^3$)
leDNA-v + DMAP	9	5	355.7 ± 291.2	245.5 ± 225.2[a]	275.8 ± 194.2	349.0 ± 253.11[a]
leDNA-v + DhL	11	8	216.4 ± 176.2	110.2 ± 169.2[a,b]	173.0 ± 122.9	115.9 ± 194.6[a,b]
leDNA-v	8	6	195.4 ± 98.3	93.0 ± 78.1[a,b]	200.5 ± 113.5	112.5 ± 81.2[a,b]
leDNA	9	7	209.8 ± 142.1	33.9 ± 39.0[c]	213.6 ± 154.7	37.2 ± 43.1[c]
Sham vesicles	8	4	155.6 ± 104.4	161.1 ± 150.3[a,b]	101.5 ± 60.2	131.1 ± 194.3[a,b]
Sham injection	7	4	137.9 ± 81.3	47.9 ± 25.8[c]	100.8 ± 49.1	36.5 ± 26.8[c]
IVF	8	7	126.9 ± 128.8	27.8 ± 43.3[c]	134.7 ± 137.0	31.0 ± 45.7[c]

Within a column, percentages without a common superscript differed ($P < 0.05$).

leDNA, linear exogenous DNA alone injection; *leDNA-v*, linear exogenous DNA coincubated with vesicles; *leDNA-v* + DhL, linear exogenous DNA coincubated with vesicles followed by dehydroleukodine (DhL) incubation; *leDNA-v* + DMAP, linear exogenous DNA coincubated with vesicles followed by dimethylaminopurine (DMAP) incubation; PN, pronuclei; sham injection, injection of medium alone; sham vesicles, ooplasmic vesicles injected (not coincubated with transgene).

* Only one PN was measured per embryo (the PN with largest foci area/embryo; all analyzed embryos were included).

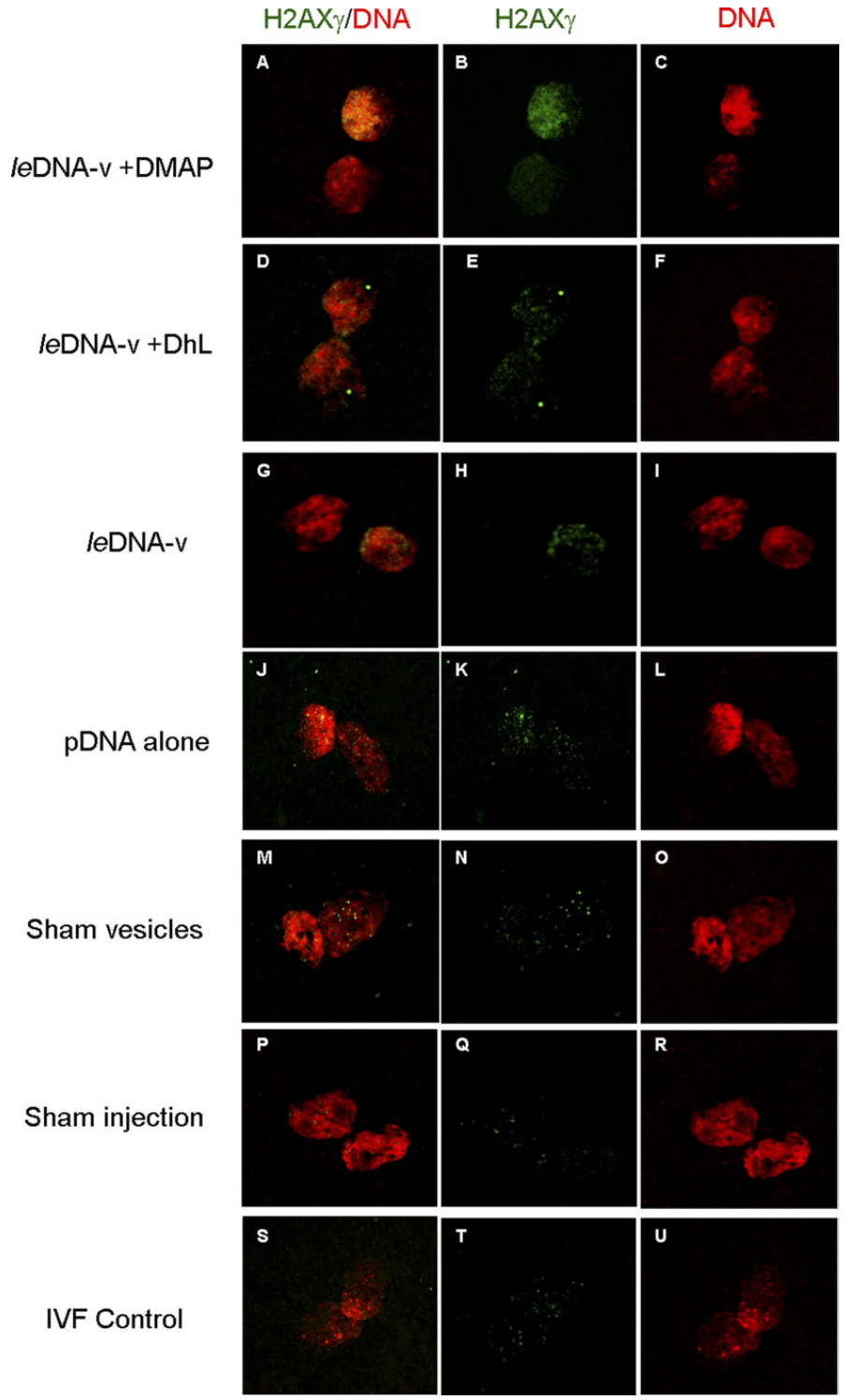

Fig. 2. The phosphorylation of histone H2AX (γH2AX) in bovine zygotes at 21 h of various transgenesis strategies: linear exogenous DNA coincubated with vesicles (*leDNA-v*) + 6-dimethylaminopurine (DMAP) (A, B, C); *leDNA-v* + dehydroleukodine (DhL) (D, E, F); *leDNA-v* (G, H, I); and *leDNA* alone (J, K, L). Controls consisting of: sham vesicles (M, N, O), sham injection (P, Q, R), and IVF (S, T, U) were also included. Embryos were labeled by immunofluorescence using an antibody to γH2AX (green); DNA was counterstained with propidium iodide (PI) (red). (A, D, G, J, M, P, S) Juxtaposition of red and green channels. (B, E, H, K, N, Q, T) Green channel. (C, F, I, L, O, R, U) Red channel. *leDNA-v* + DhL, linear exogenous DNA coincubated with vesicles followed by DhL incubation; *leDNA-v* + DMAP, linear exogenous DNA coincubated with vesicles followed by DMAP incubation; sham injection, injection of medium alone; sham vesicles, ooplasmic vesicles injected (not coincubated with transgene).

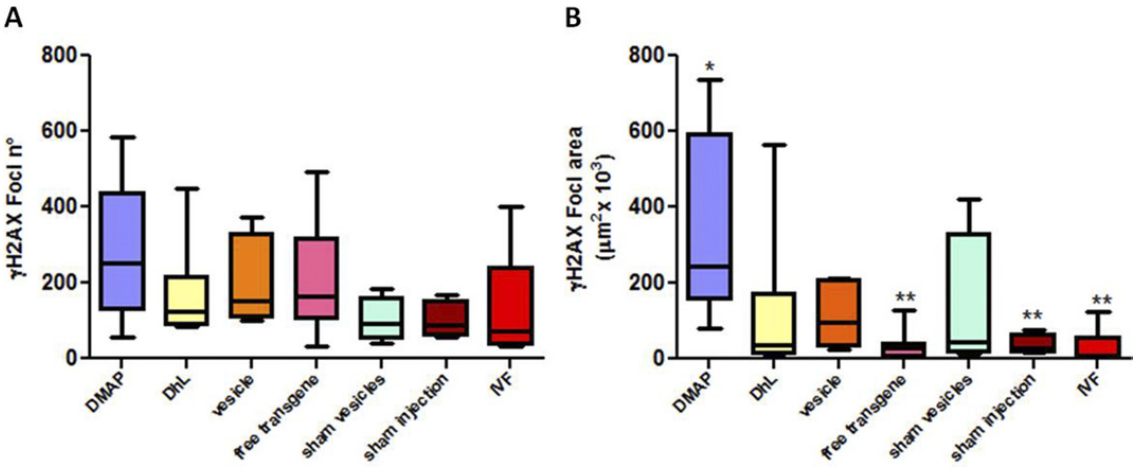

Fig. 3. Quantitative data on the phosphorylation of histone H2AX (γH2AX) in bovine zygotes at 21 h of different transgenesis strategies: linear exogenous DNA coincubated with vesicles (*leDNA-v*) + 6-dimethylaminopurine (DMAP); *leDNA-v* + dehydroleukodine (DhL); *leDNA-v*; and *leDNA* alone. Controls consisting of sham vesicles, sham injection and IVF were also included. (A) Box and whiskers plot of γH2AX foci number in 2 pronuclei (PN) embryos subjected to the different treatments. (B) Box and whiskers plot of γH2AX foci area in 2 pronuclei embryos subjected to the different treatments. *, ** Statistical differences. Statistical analysis was done using one-way ANOVA and Tukey's post test. Horizontal bar indicates mean. Numbers for (A) and (B) are listed in Table 2. *leDNA-v* + DhL, linear exogenous DNA coincubated with vesicles followed by DhL incubation; *leDNA-v* + DMAP, linear exogenous DNA coincubated with vesicles followed by DMAP incubation; sham injection, injection of medium alone; sham vesicles, ooplasmic vesicles injected (not coincubated with transgene).

3.5. *Experiment 5: cloning of* egfp*-expressing blastomeres produced by various treatments*

Transgene-expressing embryos were produced by the treatments tested in this study, but many of them had *egfp* mosaicism. Homogeneous expression rates were higher ($P < 0.05$) for *leDNA-v* + DMAP compared with *leDNA* injection (21.6% and 11.6% respectively, Table 3). Day 3 *egfp*-expressing blastomeres produced by these treatments were used as donor nuclei for cloning. Blastocyst development after cloning was not significantly different among the three treatments, although blastocyst rates were lower ($P < 0.05$) than in the parthenogenetic control group (Table 3, Fig. 4). Transgene expression at Day 4 after cloning was higher ($P < 0.05$) for *leDNA-v* + DMAP. Also, homogeneous expression of clones was higher ($P < 0.05$) for *leDNA-v* + DMAP and for *leDNA-v* compared with *leDNA* donor blastomeres.

4. Discussion

This work describes, apparently for the first time, treatment of presumptive zygotes with cell cycle inhibitors to increase the efficiency of transgenesis. Both expression mosaicism and double-stranded breaks, determined by quantification of γH2AX foci, were measured. Incubation with DMAP increased γH2AX foci

Table 3
Cloning of *egfp* blastomeres produced by *leDNA-v*, *leDNA-v* + DMAP, and by *leDNA* alone injection mediated transgenesis.

egfp blastomeres production	N	Donor embryos		Cloned blastomeres, N	Cleavage (%)	Blastocysts (%)	*egfp* expression (%)*	Homogenous expression (%)†	*egfp* blastocysts (%)‡
		egfp expression (%)	Homogeneous expression						
leDNA-v + DMAP	102	65 (63.7)^{a}	22 (21.6)^{a}	106	87 (82.1)^{a,b}	8 (7.5)^{a}	84 (96.5)^{a}	84 (100)^{a}	8 (100)
leDNA-v	100	47 (47)^{b}	18 (18)^{a,b}	81	56 (69.1)^{a}	6 (7.4)^{a}	53 (65.4)^{b}	53 (100)^{a}	5 (83.3)
leDNA	129	53 (41.1)^{b}	15 (11.6)^{b}	99	82 (82.8)^{b}	8 (8.1)^{a}	65 (65.6)^{b}	58 (89.2)^{b}	7 (87.5)
Control	214	—	—	—	188 (87.9)^{b}	63 (29.4)^{b}	—	—	—

Within a column, percentages without a common superscript differed ($P < 0.05$). The control was parthenogenetic activation in the same conditions as in the cloning groups.
leDNA, linear exogenous DNA alone injection; *leDNA-v*, linear exogenous DNA coincubated with vesicles; *leDNA-v* + DMAP, linear exogenous DNA coincubated with vesicles followed by dimethylaminopurine (DMAP) incubation.
* At Day 4.
† Calculated over total *egfp*-expressing cloned embryos at Day 4.
‡ Calculated over total blastocysts.

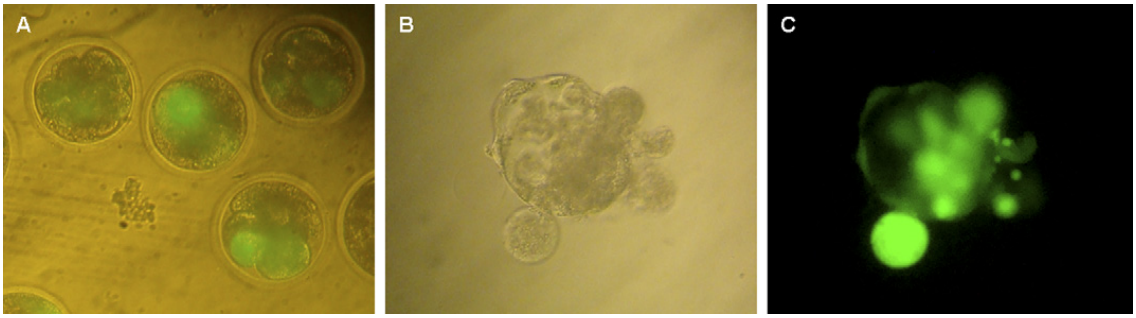

Fig. 4. (A) Bovine embryos produced by vesicle mediated transgenesis plus 6-dimethylaminopurine (DMAP), with mosaic expression patterns. (B, C) Bovine *egfp*-expressing blastocysts produced by SCNT in the Well of the Well (WOW) system employing as donor cells blastomeres derived from linear exogenous DNA coincubated with vesicles (*leDNA-v*) + DMAP (linear exogenous DNA incubated with vesicles followed by DMAP). (B) Under bright light; (C) under blue light (488 nm).

area and improved *egfp* expression rates after cloning. As well, it tended to increase rates of transgenesis and homogenous expression.

In the first experiment, treatment with cell cycle inhibitors was tested to prolong first cellular division. Both DMAP and DhL induced reversible blockage of the first embryonic cleavage. The potential of DMAP to inhibit embryo division has been previously shown for eight-cell stage bovine embryos [27], consistent with the present study. To avoid detrimental effects on development, incubation with the agents was confined to a 6-h interval (from 15 to 21 h post IVF). The commencement of DMAP and DhL treatment (15 h post IVF) was determined according to the onset of the first S phase in bovine embryo development after IVF [28,29].

In the second experiment, development of embryos injected with transgene and subsequently incubated with cell cycle inhibitors was evaluated. Green blastocysts over total blastocyst number tended to increase with DMAP incubation. On the contrary, the rate of green blastocysts production was not improved by DhL incubation. As well, this experiment confirmed that linear pCX-EGFP improved total *egfp* expression rates, both after injection of transgene alone, or coincubated with vesicles. Another interesting observation was that incubation of transgene with oolema vesicles improved rates of *egfp*-expressing blastocyst only when the linear transgene was employed, with no significant differences in *egfp* blastocysts rates for groups injected with circular DNA.

In this respect, Iqbal et al. [30] reported that injection of free circular plasmids into the cytoplasm of fertilized bovine and murine zygotes was efficient for expression of exogenous DNA in embryos. Nevertheless, in our case, the highest transgenesis rates were obtained after injection of *leDNA* coincubated with vesicles. Page [31] demonstrated the efficient production of transgenic mice by cytoplasmic injection of a polylysine/DNA mixture into pronuclear stage embryos, being unable to reproduce these results when the exogenous DNA was injected alone. For our technique, we hypothesized that vesicles acted as a protective barrier against nuclease activities present in the ooplasm. The presence of a DNase I-like activity in the cytoplasm has been demonstrated in pigs [32], and could be responsible for free linear DNA degradation before its arrival to the nucleus, as previously suggested for cattle [33].

To determine if efficiency of transgenesis mediated by injection of vesicles and treatment with DMAP was related to the genomic damage present in the nuclei, γH2AX (marker of DNA DSBs) was measured in Experiment 4. Previous reports demonstrated that foci number linearly correlated to the number of DNA double stranded breaks [34], being estimated that approximately 2000 H2AX molecules were phosphorylated per double stranded break [35]. To our knowledge, this is the first report measuring γH2AX in transgene expressing embryos. In this work, we evaluated DMAP or DhL incubation with the objective of lengthening the first pronuclear phase and of inducing more ruptures to consequently increase transgenesis. Incubation in DMAP increased γH2AX focal area. Previous reports in somatic cells indicated that increased focal area is a consequence of γH2AX spreading over a large chromatin domain, providing additional binding sites for DNA damage response proteins [36]. This amplified presence of DNA ruptures in zygotes produced by *leDNA-v* + DMAP might be responsible for an increased integration frequency after DMAP treatment, and could also be the reason for the increased proportion of transgene-expressing blastocysts and clones observed for this treatment. In our conditions, development to blastocysts was not compromised, in contrast to

previous reports on fertilization with highly irradiated spermatozoa, which resulted in substantial DNA damage and failure of embryos to reach the blastocyst stage [37]. Perhaps DMAP incubation under the conditions tested in this work allowed induction of DNA breaks and the related increase in *egfp*-expressing blastocysts, but apparently did not compromise blastocyst development. Conversely, DhL incubation did not increase DNA ruptures or result in higher rates of transgene expression.

Interestingly, γH2AX foci number and foci area did not significantly increase after cytoplasmic injection with vesicles coincubated with transgene, or after the microinjection procedure in the absence of transgene (sham-v and sham-injection groups). These results differed from observations for pronuclear microinjection and for ICSI-mediated transgenesis. In that regard, pronuclear microinjection, comet assay and karyotype analysis revealed that the micromanipulation procedure per se was responsible for high DNA damage rates, independent of the presence of the transgene [38]. Conversely, for ICSI-mediated transgenesis, sperm injection did not induce chromosomal breaks, but injection of sperm coincubated with transgene-induced DNA damage [38].

In this work, positive FISH signals were obtained for groups cytoplasmically injected with linear transgene coincubated with vesicles and with linear transgene alone.

Unlike polymerase chain reaction (PCR), the FISH technique is an excellent predictor of stable integration in the guest genome. However, as the lowest resolution of FISH is approximately 100 kb, and the plasmid used in this work was 5.5 kb, only tandem associations could be detected. The possibility of tandem association of the genetic constructs has been reported [4,7], allowing the use of this technique in the present conditions. The positive signals detected allowed us to confirm that integration occurred after generation of transgenic embryos by cytoplasmic injection of linear transgene alone or incubated with vesicles.

In the last experiment, our objective was to evaluate continuity of transgene expression after additional rounds of cellular division, as well as to determine mosaic expression patterns. Previous to cloning, the group injected with linear transgene coincubated with vesicles followed by DMAP had higher homogeneous expression rates than the one injected with linear transgene alone (Table 3). However, expression mosaicism rates were high in all cases. These observations might indicate transgene incorporation after the first S phase [4]. Subsequent to cloning of *egfp*-expressing blastomeres, *egfp* expression rates were higher for *leDNA-v* + DMAP than for the other groups. As well, there was an increase in homogeneous expression that was statistically higher after linear transgene coincubated with vesicles injection, followed or not by DMAP, compared with the injection of linear transgene alone. This could also be considered an indicator of higher transgene incorporation after injection of vesicles or vesicles plus DMAP than after injection of DNA alone.

In conclusion, incubation in DMAP of presumptive zygotes injected with transgene increased γH2AX foci area and resulted in higher transgene expression after cloning. As well, it tended to increase *egfp* blastocyst rates, and also homogeneous *egfp* rates. Our results also supported the possibility of producing transgene-expressing embryos by cytoplasmic injection of the transgene. This technique is promising for domestic animal transgenesis, for which pronuclear microinjection has many technical difficulties.

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