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Chemical Activation with a Combination of Ionomycin and Dehydroleucodine for Production of Parthenogenetic, ICSI and Cloned Bovine Embryos

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Contents

The aim of this study was to evaluate the potential of dehydroleucodine (DhL), a new drug isolated from a medicinal herb used in Argentina, for activation of bovine oocyte. Several DhL concentrations and exposure times after ionomycin (Io) treatment were tested. The optimal DhL treatment, found for parthenogenetic development, was employed to produce bovine embryos by intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). The best parthenogenic embryo developments were observed with 5 μM Io for 4 min followed by 5 μM DhL concentration and after 3-h exposure time (52.3% cleavage; 17.4% morulae; 7.3% blastocyst; n = 109). This treatment generated no significant differences with standard Io plus 6-dimethylaminopurine (DMAP) treatment in preimplantation embryo development. In our conditions, the embryo development reached after ICSI and SCNT assisted by the DhL treatment did not differ in terms of cleavage and blastocyst development from activation with standard Io plus DMAP treatment ($p > 0.05$). In conclusion, DhL utilization to activate oocytes and induce development of parthenogenotes, ICSI-embryos or SCNT-embryos is reported here for first time.

Introduction

Artificial activation stimuli are essential in some reproductive technologies, such as intracytoplasmic sperm injection (ICSI) (Rho et al. 1998) or somatic cell nuclear transfer (SCNT) in ruminant species (Wilmut et al. 1997; Wells et al. 1999). In humans, ICSI is widely applied to overcome several types of male infertility, like oligozoospermia and fertilization failure without any type of artificial activation treatment (Palermo et al. 1996; Nagy et al. 1998). By contrast, low activation rate and poor *in vitro* development is achieved after sperm injection in livestock animals. An additional artificial activation treatment, before or after ICSI, improved embryo development (Rho et al. 1998; Hamano et al. 1999; Chung et al. 2000; Hwang et al. 2000; Suttner et al. 2000; Ock et al. 2003), although few ICSI domestic animal births have been reported (Goto et al. 1990; Hamano et al. 1999; Horiuchi et al. 2002; Wei and Fukui 2002; Galli et al. 2003; Oikawa et al. 2005). This difficulty could be attributed to deficient level of sperm decondensation and pronuclear formation even after chemical or physical ICSI assistance (Perreault et al. 1988; Catt and Rhodes 1995; Malcuit et al. 2006). On the contrary, in cloning by SCNT, artificial activation is always required to obtain embryonic development and live offspring (Wilmut et al. 1997; Cibelli et al. 1998; Wells et al. 1999; Salamone et al. 2006).

Mammalian oocytes arrested at metaphase II stage of meiosis could be activated by spermatozoa (fertilization) or by an artificial stimulus (parthenogenetic activation). Oocyte activation depends on maturation promoting factor (MPF) inhibition. Maturation promoting factor is a heterodimer of cyclin dependent kinase p34/cdc2 and cyclin B, and it displays a cyclic activity with a peak at metaphase (Gautier et al. 1990; Kubiak et al. 2008). In immature oocytes, MPF is an inactive complex, where cdc2 is phosphorylated on both Thr-161 and Thr-14/Tyr-15 residues. This phosphorylation is regulated by specific kinases like CAK, Myt-1 and Wee-1 (Nurse 1990; Wohlbold et al. 2006). Dephosphorylation of Thr-14/Tyr-15 is necessary to start MPF activation, which is induced by Cdc 25 phosphatase activation (Nishijima et al. 1997; Liu et al. 1998).

In natural fertilization, when a spermatozoon activates an oocyte, periodic oscillations of intracellular calcium are promoted (Ducibella et al. 2002; Ducibella and Fissore 2008). These oscillations are responsible for events like destruction of cyclin B, MPF inactivation, reinitiation of meiosis, extrusion of the second polar body (2PB), pronuclei formation, recruitment of maternal mRNAs and cell cycle resumption (Schultz and Kopf 1995; Ducibella et al. 2002; Hyslop et al. 2004; Ducibella and Fissore 2008). Before mitotic entry, accumulation of cdc25 is important for dephosphorylation of cdc2 (Liu and Yang 1999).

On the other hand, a wide variety of artificial chemical or physical stimuli can induce the oocyte activation in several mammal species, even without sperm intervention, such as electrical stimulation (Collas et al. 1993), ethanol (Loi et al. 1998; Yi and Park 2005), thimerosal/DTT (Machaty et al. 1997), strontium (Méo et al. 2007), calcium ionophore A23187 (Liu et al. 2002) or Io (Rho et al. 1998; Bhak et al. 2006). However, in bovine, most authors use an Io treatment, to generate a transient inactivation of MPF by a single Ca^{2+} increase, with a persistent inhibition of MPF, induced by non-specific kinase inhibitors as DMAP (Susko-Parrish et al. 1994). This treatment has been shown to be particularly effective in inducing bovine oocyte activation (Susko-Parrish et al. 1994; Wells et al. 1999). In full parthenogenetic activation (pronuclear development), both cyclin B destruction and phosphorylation of cdc2, dephosphorylation of ERK2, and later cdc25 accumulation occurred (Liu and Yang 1999).

Dehydroleucodine (DhL, formula in Fig. 1) is a sesquiterpene lactone of the guaianolide type, isolated

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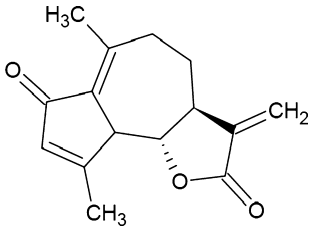

Fig. 1. Molecular structure of dehydroleucodine (DhL), a sesquiterpene lactone of the guaianolide type

from aerial parts of the *Artemisia douglasiana* Besser, a medicinal herb used in Argentina (Giordano et al. 1990). It has previously been shown that DhL selectively induces a transient arrest in G2 of both meristematic cells and vascular smooth muscle cells (Lopez et al. 2002; Cruzado et al. 2005). It has been suggested that DhL did not directly inhibit MPF and early stages of the Cdc 25 activation cascade were proposed as DhL targets in bufo arenarum oocytes (Sánchez Toranzo et al. 2007). Aiming to induce activation and embryo development, we evaluated the effect of DhL in bovine oocyte activation, testing several DhL concentrations and exposure times after Io treatment. The optimal DhL treatment achieved for parthenogenetic embryo development was employed to produce bovine embryos by ICSI and SCNT.

Materials and Methods

Except where 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 in Dulbecco phosphate buffer saline (DPBS, 14287-072; Gibco BRL, Grand Island, NY, USA) containing 10% foetal bovine serum (FBS, 10499-044; Gibco BRL) and 2% antibiotic-antimycotic (ATB; 15240-096; Gibco BRL). Follicular oocytes covered by at least three layers of granulose cells and with an evenly granulated cytoplasm were selected for *in vitro* maturation. The maturation medium was bicarbonate-buffered TCM-199 (31100-035; Gibco BRL), containing 2 mM glutamine (G-8540), 10% FBS, 2 mg/ml follicle stimulating hormone (NIH-FSH-P1, Folltropin®; Bioniche, Belleville, Ontario, Canada), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% ATB. The oocytes were incubated in 500 μl of medium in four-well dishes (Nunclon; Nunc, Naperville, IL, USA) covered with mineral oil (M8410). *In vitro* maturation conditions were 6.5% CO_2 in humidified air at 39°C for 24 h. After maturation, cumulus cells were removed from all oocytes by vortexing for 2 min in hyaluronidase (H-4272) (1 mg/ml DPBS) and washed three times in HEPES-buffered TCM-199 (H4034).

Parthenogenetic activation

To obtain haploid parthenogenetic embryos, *in vitro* matured oocytes were activated with 5 μM Io (I24222; Invitrogen, Carlsbad, CA, USA) for 4 min, cultured in TCM-199 for 3 h to permit extrusion of their 2PB, and treated by 1.9 mM DMAP (D2629) for 3 h (Io + 3h + DMAP) or by 5 μM DhL for 3 h (Io + 3h + DhL). To obtain diploid parthenogenetic embryos, *in vitro* matured oocytes were activated with 5 μM Io for 4 min followed by 1.9 mM DMAP treatment for 3 h (Io + DMAP) or followed by different incubation periods in different concentrations of DhL (Io + DhL) depending on the experiment.

Intracytoplasmic sperm injection (ICSI)

Sperm microinjection

Intracytoplasmic sperm injection was performed in microdroplets of 10 μl of Tyrode's albumin lactate pyruvate buffered with HEPES (TALP-H; Bavister and Yanagimachi 1977) under mineral oil (M8410) in 100 × 20 mm tissue culture dishes (Corning, 430167). Intracytoplasmic sperm injection was performed using a Narishige hydraulic micromanipulators (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA). The injection pipettes used had an inner diameter of 8 μm. Frozen semen was thawed in a 37°C water bath for 30 s. Each spermatozoon for injection was selected from a 4 μl droplet of 10% polyvinylpyrrolidone (PVP, 99219; Irvine Scientific, Irvine, CA, USA). A motile spermatozoon was immobilized by breaking its tail, with the tip of an injection needle, against the bottom of the dish. The selected spermatozoon was aspirated tail-first into the injection pipette and moved to the drop containing the oocytes. Metaphase II oocytes were held under negative pressure on the holding pipette, with the polar body at the 6 or 12 o'clock position. The microinjection pipette was pushed through the zona pellucida and into the cytoplasm of the oocyte at the 3 o'clock position, where aspiration was used to break the oolemma. The spermatozoon and aspirated ooplasm were then expelled into the oocyte with a minimal volume of PVP.

Injected oocyte activation

Injected oocytes were immediately activated in TALP-H with 5 μM Io for 4 min and placed in TCM-199 for 3 h to permit extrusion of their 2PB. Subsequently, they were transferred to a drop of 1.9 mM DMAP (Io + 3h + DMAP) or 5 μM DhL (Io + 3h + DhL) in TCM-199 for 3 h.

Somatic cell nuclear transfer (SCNT)

Preparation of recipient cytoplasts

In vitro matured oocytes were denuded of cumulus cells and mechanically enucleated using the same micromanipulation system utilized for ICSI procedure. Enucleation was performed using 20 μm internal diameter pipettes. Metaphase chromosomes were visualized under

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ultraviolet (UV) light (< 10 s) after staining with 1 μg/ml bisbenzimide (Hoechst 33342) for 10 min. The integrity of the ooplast was assessed by evaluation of stained M-II chromosomes inside the pipette, confirming successful enucleation.

Preparation of donor karyoplasts

Foetal fibroblasts were obtained from a 75-day-old Jersey foetus and used as nuclear donors. Isolation, freezing and culture of cells was performed by standard procedures. Cell synchronization was performed by serum starvation (0.5% FBS) for 72 h before being used as donor nuclei. A single cell was inserted into the perivitelline space of the enucleated oocyte by using a 20 μm internal diameter pipette. Subsequently, they were fused, and cultured as described below.

SCNT-embryos fusion

Fusion of somatic cells was carried out by manually aligning cells in a fusion chamber (BTX Instrument Division; Harvard Apparatus, Holliston, MA, USA) so that the membranes to be fused were parallel to the electrodes. Fusion was performed by administering one electrical DC pulse of 2.4 Kv/cm for 30 μs (Electroporator ECM 830; BTX Instrument Division; Harvard Apparatus).

SCNT-embryo activation

Two hours after fusion, oocytes were activated by incubation in 5 μM Io for 4 min and then placed in 1.9 mM 6-DMAP (Io + DMAP) or 5 μM DhL (Io + DhL) in TCM-199 for 3 h. The inhibitor was then removed by washing three times in TALP-H and embryos were cultured as described below.

In-vitro embryo culture and evaluation

Activated, ICSI and SCNT-embryos were returned to the original maturation medium after replacing 50% of the medium with fresh medium and were cocultured with cumulus cells. Cleavage, morulae and blastocyst formation rates were evaluated at 48 h, 5 and 7 days after activation respectively. Blastocyst stage embryo nuclei were stained on slides in TCM-199 with 1 μg/ml of bisbenzimide. A drop of staining solution containing one embryo was placed in the centre of a slide and a cover slip was placed over the drop and the edges sealed. Nuclei were visualized and counted using an UV microscope.

Experimental design

Initially, we evaluated the effects of different DhL concentrations and exposure times on parthenogenic activation of bovine oocytes. In a second experiment, development of parthenogenetic embryos under the optimal conditions of the first experiment was tested on three replicates. Finally, we activated ICSI and SCNT embryos with the most favourable DhL treatment and their development was compared at least three replicates for each.

Statistical analysis

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

Results

Experiment 1. Effects of different DhL concentrations and exposure times on parthenogenic activation of bovine oocytes

The cleavage rates of *in vitro* matured oocytes after exposure to 5 μM Io for 4 min and different concentrations of DhL for 1, 3 or 5 h were evaluated (Fig. 2).

The highest oocyte cleavage rates were observed with 1 μM and 5 μM DhL concentrations. At those concentrations, significantly more oocytes were cleaved after 3 h and 5 h of exposure compared with 1 h of exposure. At 30, 60 or 100 μM concentrations, cleavage was not observed. After this first approach, we selected four DhL activation conditions, with two DhL concentrations (1 μM and 5 μM) and two exposure times (3 h and 5 h).

Experiment 2. Effect of DhL activation on parthenogenic development of bovine embryos

Although no differences were found for cleavage rate in three of four treatments, some differences were observed in parthenogenetic bovine embryo development (Table 1). One DhL treatment (Io + 5 μM, 3 h) generated non-significant differences (52.3% cleavage; 17.4% morulae; 7.3% blastocyst; n = 109) with standard Io + DMAP oocyte activation treatment on preimplantation embryo development (56.6% cleavage; 21.7% morulae; 9.6% blastocyst; n = 83). This activation procedure allowed the 2PB extrusion in 60% of the

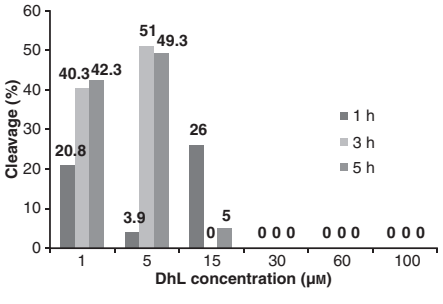

Fig. 2. Effects of different dehydroleucodine (DhL) concentrations and exposure times on parthenogenic activation of bovine oocytes. The cleavage rates of *in vitro* matured oocytes after exposure to 5 μM Io for 4 min and different concentrations of DhL (1, 5, 15, 30, 60 and 100 μM) for 1, 3 or 5 h were evaluated in three replicates (n = 629). The highest oocyte cleavage rates were observed with 1 μM and 5 μM DhL concentrations. At those concentrations, significantly more oocytes were cleaved after 3 h and 5 h exposure times ($p < 0.05$, Fisher's test). DhL, Dehydroleucodine; h, hours

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Table 1. *In-vitro* development of dehydroleucodine (DhL) activated bovine oocytes

Treatment	DhL time incubation (h)	n	Cleavage n (%)	Morulae n (%)	Blastocyst n (%)
Io + 1 μM DhL	3	105	37 (35.2)^{a}	2 (1.9)^{ab}	0 (0)^{a}
Io + 1 μM DhL	5	103	43 (41.7)^{ab}	4 (3.9)^{ab}	0 (0)^{a}
Io + 5 μM DhL	3	109	57 (52.3)^{b}	19 (17.4)^{c}	8 (7.3)^{bc}
Io + 5 μM DhL	5	103	49 (47.6)^{b}	7 (6.8)^{b}	2 (1.9)^{ab}
Io + DMAP	NA	83	47 (56.6)^{b}	18 (21.7)^{c}	8 (9.6)^{c}
Io	NA	60	5 (8.3)^{c}	0 (0)^{a}	0 (0)^{ab}

Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's test). Three independent repetitions were performed for each treatment and no differences were observed between repetitions.
Io, ionomycin; DMAP, 6-dimethylaminopurine; DhL, dehydroleucodine; NA, not applicable.

oocyte treated. These DhL conditions were therefore chosen for successive experiments.

Experiment 3. Effect of DhL activation after ICSI on subsequent embryo development

The Io + 3h + DhL treatment was assayed as assistance for ICSI (Table 2). Several parallel controls were added, remarking an ICSI assisted or not by the standard activation treatment (Io + 3h + DMAP), and haploid parthenogenetic activation treatment.

The embryo development reached after ICSI assisted by Io + 3h + DhL treatment did not differ in terms of cleavage and blastocyst rates (56.1% and 1.8% respectively) from ICSI assisted by Io + 3h + DMAP treatment (51.2% and 5.8% respectively). In our conditions, low cleavage rate (12.7%, n = 102) and no morulae were obtained by ICSI without chemical assistance, confirming the incomplete activation for injection mechanical stimulus in bovine. In our conditions, no blastocyst was obtained by haploid parthenogenote activation with Io + 3h + DhL.

Experiment 4. Effect of DhL activation after SCNT on subsequent embryo development

In Table 3, results of *in vitro* SCNT embryo development after different oocyte activation procedures were shown. Activation of SCNT-embryos by Io + DhL did not differ in terms of cleavage and blastocyst rates (42.6% and 4.3% respectively) from activation by Io + DMAP (57.9% and 7.9% respectively). The blastocyst development rate in activated parthenogenotes controls with Io + DhL (10.3%) is not significantly different from those activated by Io + DMAP (12.1%). However, cleavage rates in embryos activated with Io + DMAP (76.9%) were greater than in SCNT groups. Blastocyst cell number was not significantly different in all treatments.

Discussion

In this study, we introduced DhL, a new drug to induce activation of bovine oocytes. Dehydroleucodine, a lactone purified from an Argentinean medicinal herb, which transiently arrests several kinds of cultured cells at G2 (Lopez et al. 2002; Cruzado et al. 2005). Although DhL molecular targets are still unknown, its utilization to activate and induce development of parthenogenotes, ICSI-embryos or SCNT-embryos was reported here for the first time.

In experiments 1 and 2, we evaluated the effect of DhL in bovine oocyte activation, testing several DhL concentrations and exposure times. No information on this subject was found in the literature. The highest parthenogenic embryo development rates were observed with Io + 5 μM DhL for 3 h. This treatment generated

Table 2. *In-vitro* development of intracytoplasmic sperm injection (ICSI) bovine embryos

Method	Activation treatment	n	Cleavage n (%)	Morulae n (%)	Blastocysts n (%)	Cell number (SD)
ICSI	Io + 3h + DhL	114	64 (56.1)^{ab}	11 (9.6)^{a}	2 (1.8)^{ab}	76 (±54)
ICSI	Io + 3h + DMAP	86	44 (51.2)^{ab}	12 (14.0)^{a}	5 (5.8)^{a}	53 (±32)
ICSI	None	102	13 (12.7)^{c}	0 (0)^{b}	0 (0)^{b}	0 (0)
Parthenogenetic control	Io + 3h + DhL	113	53 (46.9)^{a}	14 (12.4)^{a}	0 (0)^{b}	0 (0)
Parthenogenetic control	Io + 3h + DMAP	116	76 (65.5)^{b}	18 (15.5)^{a}	4 (3.4)^{ab}	71 (±32)

Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's test). Three independent repetitions were performed for each treatment, and no differences were observed between repetitions.
DMAP, 6-dimethylaminopurine; DhL, dehydroleucodine; ICSI, intracytoplasmic sperm injection; Io, ionomycin.

Table 3. *In-vitro* development of somatic cell nuclear transfer (SCNT) embryos activated with dehydroleucodine (DhL)

Method	Activation treatment	n	Cleavage n (%)	Morulae n (%)	Blastocysts n (%)	Cell number (SD)
SCNT	Io + DhL	47	20 (42.6)^{a}	7 (14.9)	2 (4.3)	86 (±14)
SCNT	Io + DMAP	38	22 (57.9)^{a}	9 (23.7)	3 (7.9)	79 (±22)
Parthenogenetic control	Io + DhL	116	79 (68.1)^{ab}	21 (18.1)	12 (10.3)	96 (±19)
Parthenogenetic control	Io + DMAP	91	70 (76.9)^{b}	21 (23.1)	11 (12.1)	111 (±7)

Values with different superscripts in a column are significantly different ($p < 0.05$, Fisher's test). Three independent repetitions were performed for each treatment and no differences were observed between repetitions ($p > 0.05$, Fisher's test).
DMAP, 6-dimethylaminopurine; DhL, dehydroleucodine; Io, ionomycin; SCNT, somatic cell nuclear transfer.

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no significant differences in pre-implantation development with standard Io + DMAP diploid treatment. Our study has also demonstrated that oocyte activation procedure employing Io + DhL is more efficient than Io alone. This result suggested that activation of oocytes by Io was incomplete, it being necessary to apply an additional chemical agent such as DhL or DMAP, as shown by other authors (Méo et al. 2007; Ock and Rho 2008).

6-dimethylaminopurine inhibits 2PB body extrusion and parthenogenote embryos showed a low haploid rate (4%; Bhak et al. 2006). Activation treatments with or without incubation window between Io and DMAP are already described (Susko-Parrish et al. 1994; Rho et al. 1998). The 'window' procedure consists of oocyte activation initiation by a brief exposure to Io, followed by a 3 h culture period to allow the extrusion of 2PB, and finally incubation for 3 h in DMAP to complete the activation process. If incubation time window between Io and DMAP treatments is absent, 2PB is not extruded in most activated oocytes, resulting in the formation of diploid parthenotes, which have better development than haploid ones (Szöllösi et al. 1993; Lagutina et al. 2004). So, activated oocytes after ICSI are cultured for 3 h before DMAP treatment to allow for the extrusion of the 2PB (Rho et al. 1998). The main conclusion of these experiments is that Io + 5 μM DhL for 3 h is the most effective DhL treatment of parthenogenetic activation, in terms of the embryo cleavage and blastocyst production.

Concerning the ICSI experiment, our results showed, in agreement with other authors, that the ICSI technique by itself is not enough to induce bovine embryo development (Keefer et al. 1990; Chung et al. 2000). Low cleavage rate and no morulae stage embryos were obtained by ICSI without chemical assistance. Mechanical sperm injection and presence of sperm in the ooplasm are enough to activate mouse and human oocytes (Palermo et al. 1992; Kimura and Yanagimachi 1995), but an additional activation stimulus after ICSI is needed for successful embryo development in cattle (Rho et al. 1998; Hamano et al. 1999; Chung et al. 2000 Hwang et al. 2000; Suttner et al. 2000; Ock et al. 2003; Oikawa et al. 2005). Several activation procedures have been applied after bovine ICSI including ethanol, electrical stimulation and Io plus DMAP or cycloheximide (CHX) (Rho et al. 1998; Hamano et al. 1999; Chung et al. 2000 Hwang et al. 2000; Suttner et al. 2000; Ock et al. 2003; García-Roselló et al. 2009). Moreover, although development to blastocyst is reached employing piezo-electric ICSI without artificial activation in cattle, blastocyst rates improved significantly after chemical activation (Oikawa et al. 2005). The results of our experiment showed that development of ICSI-embryos assisted by Io + 3h + DhL were similar to the development of ICSI-embryos assisted by standard Io + 3h + DMAP treatment. The results of experiment 2 suggest that in DhL treatments the time incubation window might not be necessary to allow the 2PB extrusion. In further experiments, DhL could be used without 3 h incubation window for haploid ICSI activation assistance.

In bovine, SCNT activation treatments usually employ calcium ionophore, followed by a treatment with CHX or DMAP (Wells et al. 1999; Bhak et al. 2006; Salamone et al. 2006). However, these activation treatments induce abnormal embryo ploidy in many cases, and could be responsible for many embryonic losses and the low pregnancies rates obtained after SCNT (De la Fuente and King 1998; Bhak et al. 2006). These facts encouraged us to evaluate this new activator drug to assist SCNT. Our results demonstrated that development of bovine SCNT-embryos assisted by Io + DhL was similar to the development of SCNT-embryos assisted by standard Io + DMAP treatment. Based on the results of experiment 2, to improve developmental rates of SCNT-embryos and reduce rates of haploid embryo development, DhL must be tested in further experiments in combination with cytochalasin B, to inhibit 2PB extrusion (Liu et al. 1998).

To our knowledge, this is the first report in which DhL was used to activate oocytes and induce development of parthenogenotes, ICSI-embryos or SCNT-embryos in bovine. Chromosomal abnormalities of embryos following DhL activation must be investigated in detail, and *in vivo* experiments with receptor cattle must be performed to evaluate foetal development rate and calf production.

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Author contributions

G. Vichera, J. Alfonso: designed study, acquisition and analysis data, drafted paper, C.C. Duque, M.A. Silvestre: acquisition data, F. Pereyra-Bonnet: designed study and acquisition the data, R. Fernández-Martín: analysis data, designed study, drafted paper, D. Salamone: designed study, acquisition and analysed data, drafted paper, revising it critically.

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