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Effect of anisomycin, a protein synthesis inhibitor, on the *in vitro* developmental potential, ploidy and embryo quality of bovine ICSI embryos

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Summary

Increasing the efficiency of intracytoplasmic sperm injection (ICSI) in domestic animals has been attempted by many researchers, however embryonic development to the blastocyst stage remains low compared with that of *in vitro* fertilization (IVF) embryos. One of the main problems observed in cattle is inadequate oocyte activation after ICSI. The present study compared the effect of cycloheximide (CHX), 6-dimethylaminopurine (DMAP), and anisomycin (ANY) on the fertilization rate, development, ploidy and quality of bovine embryos generated by ICSI. Although no differences were observed between treatments in terms of cleavage, higher blastocyst rates were observed for ANY (37.3%) compared with CHX (21.8%, $P < 0.05$) and DMAP (28.6%, $P > 0.05$) treatments. No differences were observed in the quality of embryos as assessed by the total number of cells, their distribution to the different embryo compartments [inner cell mass (ICM) and trophectoderm (TE)], the proportion of ICM cells to the total cell numbers and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive cells. Similarly, no differences were observed in the normal ploidy of embryos (56, 67, and 55%) for ANY, CHX and DMAP, respectively. However, higher fertilization rates were observed for ANY (75%) and CHX (87%) treatments compared with DMAP (35%). In conclusion, ANY showed a superior developmental rate compared with CHX treatment. Although no significant differences were observed compared with an improved protocol of DMAP (2Io-DMAP), the lower fertilization rate recorded with DMAP strongly suggests that ANY could be a better alternative for oocyte activation than traditional chemical compounds used currently in ICSI.

Keywords: Activation, Anisomycin, Cycloheximide, Dimethylaminopurine, ICSI

Introduction

Intracytoplasmic sperm injection (ICSI) is an assisted reproductive technique that consists of the injection of a spermatozoon into a metaphase II (MII) oocyte. ICSI has been widely used in humans to solve male fertility problems (Palermo *et al.*, 1996) but also constitutes a valuable tool for endangered species preservation and for the production of desired sex domestic cattle, for transgenic animals production, and for studying biological aspects related to the interaction between gametes (Hamano *et al.*, 1999; Perry *et al.*, 1999; Probst & Rath, 2003; Rodger *et al.*, 2009; Garcia-Vazquez *et al.*, 2010).

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In domestic animal species, *in vitro* embryo development after ICSI is lower than that observed for conventional *in vitro* fertilization (IVF) (Catt *et al.*, 1996; Li *et al.*, 1999; Arias *et al.*, 2015). One of the main problems observed with this technique is inadequate oocyte activation. Consequently, activation through physical (Galli *et al.*, 1999) or chemical stimuli (Goto *et al.*, 1990; Rho *et al.*, 1998a; Chung *et al.*, 2000; Suttner *et al.*, 2000; Horiuch *et al.*, 2002; Galli *et al.*, 2003; Abdalla *et al.*, 2009) is essential for the success of this technique (Yanagimachi, 1994; Abdalla *et al.*, 2009). However, published data on the activation of oocytes after ICSI are controversial in bovine species; some authors have reported that oocyte activation is triggered by sperm components in the absence of exogenous stimulus (Galli *et al.*, 2003; Wei and Fukui, 2002). In contrast, others have observed no calcium oscillations after ICSI or only a single calcium peak 1 h after sperm injection of oocytes, which is insufficient to inactivate the maturation-promoting factor (MPF) (Malcuit *et al.*, 2006).

Several studies have attempted to solve this problem using different activation treatments (Rho *et al.*, 1998b; Suttner *et al.*, 2000; Oikawa *et al.*, 2005; Abdalla *et al.*, 2009). The most frequent protocols used for the activation of bovine oocytes are those described by Rho *et al.* (1998a) and Suttner *et al.* (2000), who employed ionomycin followed by the treatment with 6-dimethylaminopurine (DMAP) or cycloheximide (CHX), respectively. To date, only a few improvements have been incorporated into these protocols, including the combination of single, double or triple compounds (Lazzari *et al.*, 2002; Lagutina *et al.*, 2007). New chemical activation compounds capable of generating substantially better embryonic development than those reported with DMAP and CHX have not been reported so far.

Anisomycin (ANY), a protein synthesis inhibitor that acts specifically in the translational stage (Joiakim *et al.*, 2004), has been recently used in bovine species by our research group, demonstrating high embryo development and normal ploidy of parthenotes and reconstructed somatic cell nuclear transfer (SCNT) embryos (Felmer & Arias, 2015). Here, we report the effect of activating bovine sperm-injected oocytes with ANY on the fertilization rate, preimplantation embryo development, ploidy and quality of the generated embryos, compared with the traditional protocols of CHX and DMAP.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Collection of ovaries, selection of oocytes and IVM

Ovaries were collected from a local slaughterhouse (Frigorifico Temuco, Temuco, Chile). Cumulus–oocyte complexes (COCs) were aspirated from 2–7 mm follicles. Good quality oocytes having a corona of cells of at least six layers and a uniformly granulated cytoplasm were matured in TCM-199 medium, supplemented with 10% inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA) and 6 μg/ml luteinising hormone (LH) hormone (Sioux Biochemical, Inc. Sioux City, IA, USA), 6 μg/ml follicle stimulating hormone (FSH) hormone (Bioniche Life Science Inc. Belleville, Ontario, Canada) and 1 μg/ml estradiol, and then incubated for 18–19 h at 38.5°C, in 5% CO_2 in air and saturation humidity.

Intracytoplasmic sperm injection (ICSI)

Before ICSI, 2 μl of commercial sperm suspension (Alta Genetics Inc., Alberta, Canada) was mixed with 8 μl of phosphate-buffered saline (PBS) containing 10% polyvinylpyrrolidone (PVP; Irvine Scientific, Santa Ana, CA, USA). After 18 h post maturation (hpm), oocytes were denuded of granulosa cells by vortexing in the presence of 1 mg/ml hyaluronidase and selected for the presence of the first polar body. ICSI and parthenotes were performed as described previously by our group (Arias *et al.*, 2014).

Oocyte activation and embryo culture

Injected oocytes were randomly assigned to the three activation treatments. Activation of oocytes was carried out with 5 μM ionomycin (Calbiochem, San Diego, CA), for 5 min followed by incubation in potassium simplex optimized medium (KSOM; EmbryoMax®, Millipore Corp, Billerica, MA, USA) containing either 1 μg/ml anisomycin for 5 h (ANY treatment), 10 μg/ml cycloheximide for 5 h (CHX treatment), or in KSOM medium for 3 h followed by a new incubation in 5 μM ionomycin for 5 min and medium containing 1.9 mM 6-dimethylaminopurine for 4 h (DMAP treatment) (Bevacqua *et al.*, 2010). Anisomycin concentration and exposure time were previously established in parthenotes and SCNT embryos (Felmer and Arias, 2015). After activation, oocytes were allocated to 50 μl culture drops consisting of KSOM medium supplemented with 1% (v/v) BME – essential amino acids, 1% (v/v) MEM – non-essential amino acids and 4 mg/ml fatty acid free bovine serum albumin (BSA). Culture was carried out at 38.5°C with a gas mixture of 5% CO_2, 5% O_2, 90% N_2 and saturated humidity (Felmer *et al.*, 2011). Cleavage rate was recorded on day 3 of culture (ICSI = day 0), at which time embryos were supplemented

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with 5% fetal bovine serum (FBS). Embryos were further cultured until day 8 to register the blastocyst rate.

Fertilization assessment

At 17 h post activation (hpa), some presumptive zygotes were fixed in 4% w/v paraformaldehyde in PBS containing 10 μg/ml Hoechst stain for 10 min. Fertilization was recorded according to the presence of a second polar body, one male pronucleus (MPN) or a decondensed sperm head (DSH) and one female pronucleus (FPN). The zygotes with abnormal nuclear configurations (1PN/1PB/1 condensed sperm head (CSH) or 1PN/2PB/1 (CSH)) were recorded in the analysis as "others."

Total number, cell allocation and TUNEL staining

A double-staining procedure combined with TUNEL was used to assess the total number of cells and their allocation to the trophectoderm (TE) and inner cell mass (ICM) cells and DNA integrity in day 8 morphologically similar expanded blastocysts generated by each method (Fouladi-Nashta *et al.*, 2005; Arias *et al.*, 2015). Embryos were permeabilized with 0.2% Triton X-100 for 20 s, washed twice with PBS/BSA and incubated with 10 μg/ml propidium iodide for 5 min at 38°C. Then, embryos were washed twice with PBS/BSA again and incubated for 30 min at room temperature in 4% paraformaldehyde containing 10 μg/ml of Hoechst stain.

For TUNEL assay, embryos were additionally permeabilized for 5 min with 0.1% sodium citrate containing 0.1% Triton X-100, washed twice with PBS/BSA and incubated with labelling reagent according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science, Indianapolis, IN, USA). Finally, embryos were mounted onto a glass slide on drops of 10 μl of anti-fade and examined under an epifluorescence microscope (Nikon Eclipse TS100, Tokyo, Japan) coupled with UV-2E/C DAPI and/or enhanced green fluorescent protein (EGFP) filters. A positive control for TUNEL was carried out by treating embryos with 75.4 U DNase I for 15 min at 38°C before the TUNEL assay, and a negative control by incubating embryos with the fluorescent labelling reagent in the absence of the terminal transferase dUTP enzyme.

Karyotype analysis

At 96 h after activation embryos were cultured in KSOM/0.4% BSA plus 5% FBS medium containing 0.05 μg/ml Colcemid (KaryoMax®; Life Technologies, Carlsbad, CA, USA) for 18 h. Then, embryos were exposed to a hypotonic 0.75 M KCl solution for 3–5 min to induce nuclear swelling. Subsequently, embryos were placed on a clean glass slide in a small volume of medium. A methanol–acetic acid solution (1:1; v/v) was dropped on the embryos while gently blowing with the slides placed under the stereoscope and allowed to dry for at least 24 h at room temperature. After drying, slides were stained with 5% Giemsa solution (Invitrogen, Carlsbad, CA, USA) for 10 min. Chromosome spreads were evaluated at ×1000 magnification with oil immersion optics (Nikon Instruments Inc., New York, NY, USA). Embryos were classified as being haploid, diploid, triploid, tetraploid, mixoploid or others.

Experimental design

We compared the effect of anisomycin to protocols using CHX and DMAP in bovine ICSI. Initially we analyzed the *in vitro* development and quality of sperm-injected oocytes activated by the different treatments. Matured oocytes were randomly allocated to each activation treatment and the experiments were replicated 7 times, except for CHX and DMAP that were replicated 6 times. The same comparison was conducted to assess the developmental potential of parthenotes used as controls. The number of cells in embryos was assessed in day-8 morphologically similar expanded blastocysts. In total, 10 blastocysts were analyzed for each treatment. The fertilization rate of presumptive zygotes activated by ANY ($n = 16$), CHX ($n = 16$), or DMAP ($n = 20$) were assessed 17 h after activation. The final experiment investigated the effects of the different activation treatments on the ploidy of sperm-injected oocytes 96 h after activation.

Statistical analysis

A chi-squared test with Bonferroni's correction was used to compare the proportions of cleaved embryos, total blastocysts (early, expanded and hatched), chromosomal composition and pronuclear formation with the Statgraphics Centurion XV software version 15.1.02 (StatPoint Technologies, Inc., Virginia, USA). In cases in which the expected cell frequencies were < 5, a Fisher exact test was employed. The mean numbers of ICM cells, TE cells, total cells and TUNEL-positive cells were compared using one-way analysis of variance (ANOVA) with Scheffe post-hoc test. An error probability of $P < 0.05$ was considered significant for all analyses.

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Table 1 *In vitro* development of sperm-injected bovine oocytes after different chemical activation treatments

Treatments	*n*	Cleavage (%)	Blastocysts				
			n	Percentage per cultured embryo	Early blastocysts (%)*	Expanded blastocysts (%)*	Hatched blastocysts (%)*
ICSI-ANY	193	161 (83.4)^{a}	72	37.3^{a}	33 (45.8)^{a}	31 (43.0)^{a,b}	8 (11.1)^{a,b}
ICSI-CHX	165	123 (74.5)^{a,b}	36	21.8^{b}	18 (50.0)^{a}	16 (44.4)^{a,b}	2 (5.6)^{b}
ICSI-DMAP	154	125 (81.2)^{a}	44	28.6^{a,b}	20 (45.5)^{a}	11 (25.0)^{a}	13 (29.5)^{a}
Parthenotes-ANY	113	89 (78.8)^{a,b}	24	21.2^{b}	8 (33.3)^{a}	16 (66.7)^{b}	0^{b}
Parthenotes-CHX	105	55 (52.3)^{c}	21	20.0^{b}	11 (52.3)^{a}	8 (38.1)^{a,b}	2 (9.5)^{a,b}
Parthenotes-DMAP	99	64 (64.6)^{b,c}	22	22.2^{b}	7 (31.8)^{a}	10 (45.5)^{a,b}	5 (22.7)^{a}

Cleavage was recorded at 72 h of culture and blastocyst rates were recorded at 192 h, respectively (six replicates/CHX and DMAP groups, and seven replicates/ANY group). *Percentages are based on the total number of blastocysts. ^{a,b,c}Data followed by different letters in the same column are significantly different ($P < 0.05$).

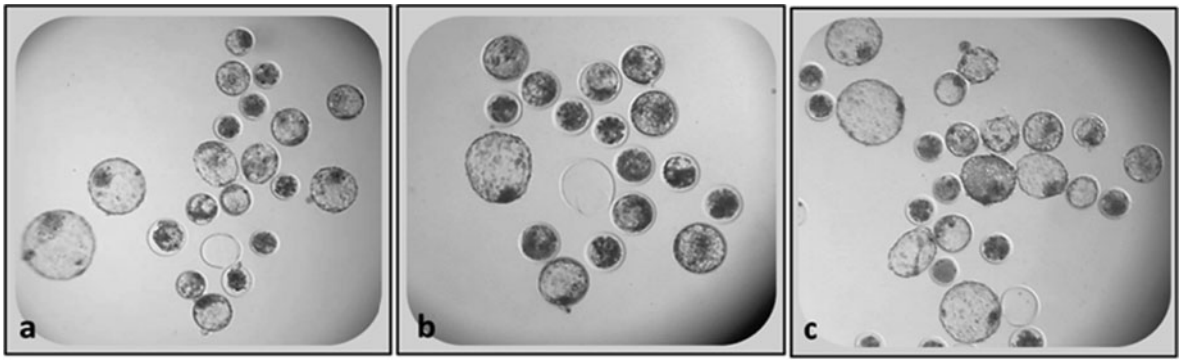

Figure 1 Bovine blastocysts generated by ICSI (192 h) activated with ANY (a), CHX (b), or DMAP (c). Magnification: ×100.

Results

In vitro development of sperm-injected oocytes activated by different chemical treatments

The results of 512 sperm-injected oocytes activated with ionomycin in combination with ANY ($n = 193$), CHX ($n = 165$) and DMAP ($n = 154$) showed no differences in terms of cleavage rate at 72 h of embryo culture (83.4, 74.5 and 81.2%, respectively). However, higher percentages of blastocysts per cultured embryo were observed with ANY (37.3%, $P < 0.05$) and DMAP (28.6%, $P > 0.05$) treatments compared with CHX (21.8%) (Table 1 and Fig. 1).

Although differences were observed in the cleavage rate of parthenotes activated by the same treatments no significant differences were observed in the percentage of blastocysts per cultured embryo (Table 1). The morphological appearance of early blastocysts was not different between sperm-injected oocytes and parthenogenetically activated groups. However, differences were observed in the rate of expanded and hatched blastocysts between treatments (Table 1). Accordingly, the majority of blastocysts generated by ICSI and parthenogenesis were early and expanded blastocysts (Table 1).

Effect of the activation treatment on the embryo cell number and ploidy of sperm-injected oocytes

The quality of embryos generated by sperm-injected oocytes activated by the different treatments showed no differences in the total number of cells, ICM cells, TE cells and in the proportion of ICM: total cell number between ANY, CHX, and DMAP, respectively (Table 2).

The TUNEL assay also showed no differences between the different oocyte activation treatments in the proportion of TUNEL-positive cells: total cells, TUNEL-positive cells: ICM cells, and TUNEL-positive cells: TE cells (Table 2).

The chromosome analysis in embryos generated by sperm-injected oocytes subjected to different activation treatments showed no differences in ploidy (2n) between ANY (56%), CHX (67%) and DMAP (55%) treatments ($P > 0.05$; Table 3 and Fig. 2).

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Table 2 Effect of the oocyte activation method on the total number of cells (Total), inner cell mass (ICM) cells, trophectoderm (TE) cells and TUNEL-positive cells of bovine blastocysts generated by ICSI

	Mean cell number (±SD)			TUNEL staining			
Treatment	Total	TE	ICM	ICM: total (%)	TUNEL-positive cells: total cells (%)	TUNEL-positive cells: ICM cells (%)	TUNEL-positive cells: TE cells (%)
ANY	141.7 ± 23.2[a]	87 ± 26.9[a]	54.7 ± 5.7[a]	39.5 ± 8.5[a]	1.9 ± 1.0[a]	1.8 ± 1.7[a]	2.1 ± 1.2[a]
CHX	157.2 ± 51.4[a]	93.3 ± 43.8[a]	63.9 ± 16.4[a]	42.7 ± 10.5[a]	2.7 ± 1.5[a]	3.7 ± 3.3[a]	2.6 ± 2.1[a]
DMAP	143.5 ± 24.6[a]	97.6 ± 31.3[a]	53.0 ± 14.9[a]	36.0 ± 10.5[a]	2.5 ± 1.6[a]	3.4 ± 2.6[a]	3.1 ± 2.1[a]

Cells were counted in good quality expanded blastocysts at 192 hpa ($n = 10$ per treatment).

Table 3 Chromosomal composition of 8-cell embryos generated by sperm-injected oocytes activated by different chemical treatments

Treatment	Informative embryos	2n	3n	4n	Mixoploid (2n/4n)	Other	Total abnormal
ANY	9	5 (56%)	0	0	3 (33%)	1 (11%)	4 (44%)[a]
CHX	15	10 (67%)	0	0	2 (13%)	3 (20%)	5 (33%)[a]
DMAP	11	6 (55%)	0	1 (9%)	0	4 (36%)	5 (45%)[a]

In total, 86 metaphases were evaluated (2.5 per informative embryo).

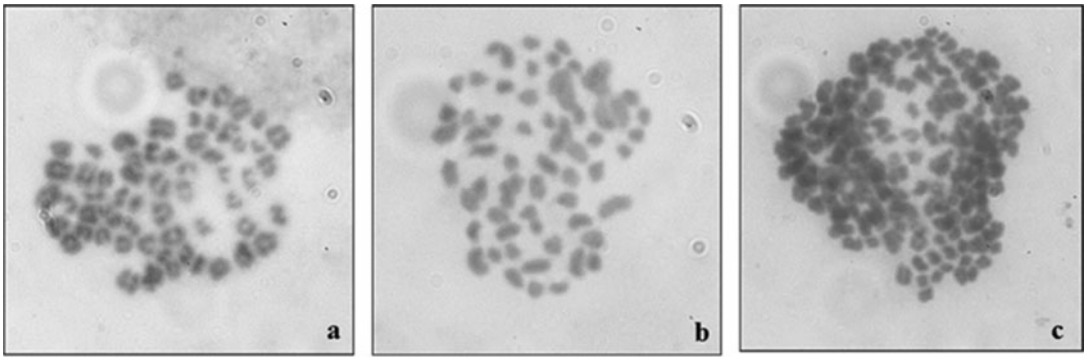

Figure 2 Representative chromosomal spreads from 8-cell stage bovine embryos. (a, b) Normal ploidy; and (c) abnormal ploidy. Magnification: ×1000.

Pronuclear formation of sperm-injected oocytes activated with different chemical treatments

The fertilization rate was established by assessing the pronuclear formation. This analysis showed a higher ($P < 0.05$) fertilization rate in oocytes activated with ANY (75%) and CHX (87%) compared with DMAP (35%) treatment (Table 4).

Discussion

In some species, including mice and humans, the puncture of the oolemma by the injection pipette and aspiration of cytoplasm during the ICSI procedure is sufficient to trigger oocyte activation (Kimura & Yanagimachi, 1995; Tesarik & Sousa, 1995). However, in bovine species, external stimuli are required for meiosis resumption, extrusion of the cortical granules, formation of the MPN and the start of the embryonic development (Malcuit *et al.*, 2006). Different chemical treatments to induce calcium oscillations in combination with protein synthesis/phosphorylation inhibitors have been used to increase cytosolic calcium and decrease the MPF activity, respectively (Rho *et al.*, 1998b). However, there are few studies in the literature that sufficiently assess the effect of these chemicals on the activation, embryonic development, quality and ploidy of sperm-injected oocytes.

Although no differences were found in the cleavage rates among the different activation treatments, ANY showed a significantly higher blastocyst rate compared with CHX treatment. However, blastocyst rates did not differ compared with DMAP treatment. It is

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Table 4 Pronuclear formation rate of sperm-injected oocytes activated with different chemical treatments

Treatment	*n*	Pronuclear formation	
		1MPN (or DSH) + 1FPN + 2 PB	Others
ANY	16	12 (75%)[a]	4 (25%)[a]
CHX	16	14 (87%)[a]	2 (13%)[a]
DMAP	20	7 (35%)[b]	13 (65%)[b]

MPN: male pronucleus, FPN: female pronucleus, DSH: decondensed sperm head, PB: polar body, Others: 1PN/1PB/1 sperm head condensed and/or 1PN/2PB/1 sperm head condensed. [a,b]Data followed by different letters in the same column are significantly different ($P < 0.05$).

necessary to note that the DMAP treatment used in this study was an improved protocol of double ionomycin treatment (2Io-DMAP) that has previously shown to improve the blastocyst rate of bovine ICSI-sperm-mediated gene transfer (Bevacqua et al., 2010). Therefore, the ANY treatment used in the present study achieved one of the highest blastocyst rates recorded so far in bovine sperm-injected oocytes, which is comparable with the rates achieved in conventional IVF.

No differences were observed in the blastocyst formation rates between DMAP and CHX treatments, which is consistent with previous reports in bovine sperm-injected oocytes (Abdalla *et al.*, 2009) and in bovine reconstructed SCNT embryos (Bhak *et al.*, 2006; Ross *et al.*, 2009). However, these results are in contrast with a previous study by Suttner *et al.* (2000) in bovine ICSI. These authors also found no differences in the cleavage rate between these two activation treatments but they described a higher blastocyst formation rate with DMAP compared with CHX treatment (Suttner *et al.*, 2000). Activation of buffalo sperm-injected oocytes also revealed a higher blastocyst formation rate with DMAP compared with CHX treatment (Liang *et al.*, 2011).

The blastocyst formation rates of parthenotes activated with ANY was lower compared with its respective activated sperm-injected oocyte group, while no differences were observed between parthenotes and sperm-injected oocytes activated with CHX and DMAP. This is an important issue for embryos generated by ICSI because external activation can induce parthenogenetic embryonic development as it will be discussed below. The results of parthenotes activated with DMAP were slightly higher than those reported by Liang *et al.* (2011) in buffalo and Rho *et al.* (Rho *et al.*, 1998b) in bovine. However, these data differ markedly with those reported by Abdalla *et al.* (2009) who observed a very low cleavage (~20%) and blastocyst formation (~2%) rates with CHX and DMAP. This divergence could be attributed to differences in the protocols used in both laboratories (e.g. the use of stored ovaries, a piezo drill or a different culture medium used in the Abdalla's study).

The quality of the embryos produced *in vitro* could be related to their capacity for post-implantation development once they are transferred into synchronized recipient females. The assessment of the total number of cells, their allocation to the ICM and TE compartments and the proportion of ICM cells to the total cell number are therefore frequently used to evaluate the quality of bovine blastocysts produced *in vitro* (van Soom *et al.*, 1997). In the present study, the quality of ICSI-generated embryos assessed by the total number of cells and their distribution between the TE and ICM compartments showed no differences between the activation treatments, which suggests that the activation did not affect the cell differentiation of these embryos. This result is in agreement with the previous study of Abdalla *et al.* (2009), who also found no differences between DMAP and CHX treatments. Apoptosis is also an important marker of embryo quality because the post-implantation developmental potential is affected by the incidence of apoptosis at the preimplantation stage (Kumar *et al.*, 2007). The TUNEL assay carried out in our ICSI embryos did not show differences in the degree of TUNEL-positive cells between the activation treatments. In fact, the number of TUNEL-positive cells was very similar to that reported in embryos generated by IVF (Wang *et al.*, 2008; Arias *et al.*, 2015) and much lower than that observed in bovine diploid parthenogenetic blastocysts activated by CHX and DMAP (Wang *et al.*, 2008).

Despite some controversy, it is widely accepted that bovine ICSI requires external stimulus for the activation of oocytes and meiosis resumption (Chung *et al.*, 2000; Li *et al.*, 1999; Rho *et al.*, 1998b). We have also demonstrated previously that activation of bovine oocytes after ICSI in the absence of chemical stimulus has a poor cleavage rate (<7%) and no development to the blastocyst stage (Arias *et al.*, 2014). However, chemical activation increases the possibility of parthenogenetic development. Activation protocols

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capable of inducing both high fertilization rates and embryonic development to the blastocyst stage should therefore be preferred for ICSI procedures (Nasr-Esfahani *et al.*, 2010; Montag *et al.*, 2012). To confirm successful fertilization after ICSI, the assessment of pronuclear formation is of crucial importance. The results of the present study confirmed that most of the oocytes subjected to ICSI and activated with ANY and CHX treatments were fertilized compared with the smaller proportion of the sperm-injected oocytes activated with DMAP. This highlights the fact that a greater proportion of ICSI embryos activated by DMAP are the result of parthenogenetic development.

Activation protocols for bovine oocytes frequently combine the use of calcium ionophore followed by a treatment with DMAP or CHX (Wells *et al.*, 1999; Bhak *et al.*, 2006; Salamone *et al.*, 2006; Arias *et al.*, 2014). However, abnormalities during fertilization have been described following some of these treatments (Szollosi *et al.*, 1993), including chromosomal aberrations that could be responsible for the low pregnancy rates observed after SCNT (De La Fuente & King, 1998; Bhak *et al.*, 2006). In the present study, the ploidy of sperm-injected oocytes was similar between ANY, CHX and DMAP treatments. This result is consistent with previous data reported with DMAP by Rho *et al.* (1998b) and Ock *et al.* (2003). In contrast, Suttner *et al.* (2000) reported a very low incidence of chromosomal abnormalities (10%) in sperm-injected oocytes activated with CHX.

Abnormal ploidy normally observed in sperm-injected oocytes activated by these chemicals could be associated with a failure in the release of the second polar body or to parthenogenetic development. In the case of ANY and CHX treatments, it is more likely to be due to a failure in the release of the second polar body because the MPN formation was high (75 and 87%, respectively) in both treatments. Meanwhile, the abnormal ploidy observed with DMAP could be the result of parthenogenetic development, because the MPN formation recorded with this treatment was much lower (35%). Although a much higher correlation between developmental rates and the ploidy of ICSI embryos activated with DMAP would have been expected, this was not observed probably due to a certain bias with the ploidy analysis in which truly fertilized embryos and those that result from parthenogenetic development (ICSI oocytes without male pronuclear formation) are chosen at random for this analysis.

In conclusion, we report here for the first time the use of ionomycin followed by ANY on the activation of bovine sperm-injected oocytes. ANY improved the *in vitro* developmental potential of ICSI-bovine embryos compared with CHX treatment. Furthermore, ANY generated embryos with similar quality and ploidy than the traditional activation protocols employing DMAP and CHX. Although the improved DMAP treatment showed a similar developmental rate compared with ANY, the lower fertilization rate recorded with DMAP strongly suggests that ANY might be a better alternative for the activation of sperm-injected oocytes and may have important implications in the activation of oocytes in other animal species. Future studies are needed to determine the effect of this activation treatment on the gene expression pattern and the post-implantation developmental potential of embryos generated by ICSI.

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Conflict of interest

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

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