IJAS Iranian Journal of Applied Animal Science

Effects of Various Activators on Bovine Embryonic Development Following Intracytoplasmic Sperm Injection

Research Article

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Received on: 19 Mar 2011
Revised on: 10 Jun 2011
Accepted on: 17 Jun 2011
Online Published on: Jun 2012

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Online version is available on: www.ijas.ir

ABSTRACT

The objective of this study was to compare the effectiveness of various combination activators of bovine oocyte following intracytoplasmic sperm injection (ICSI) in terms of cleavage and embryo development. Bovine ICSI oocytes were treated with a variation of treatments of combined or single activators as follows; 30 V for 15 x 2 µsec of electrical pulse +10 µg/mL cycloheximide (CHX), 7% ethanol + CHX, 5 µM Calcium ionophore A23187 (Ca-I) + CHX, Ca-I + 1.9 mM 6-dimethylaminopurine (6-DMAP), CHX alone, Ca-I alone. The highest cleavage (73.5%-79.7%), and blastocyst rates (28.0%-35.3%) were obtained in Ca-I + 6-DMAP, ethanol + CHX and electrical stimuli + CHX groups. The treatment with CHX only or Ca-I only gave a significantly lower ($P<0.05$) yield of cleavage rate and blastocyst than those treatments with Ca-I + 6-DMAP, ethanol + CHX, electrical stimulation and Ca-I + CHX. In conclusion, these results show that post activation of ICSI oocytes with Ca-I + 6-DMAP, ethanol + CHX, electrical pulse + CHX and Ca-I + CHX are more effective treatments for increasing blastocyst development than those CHX only or Ca-I only, however, activation with ethanol + CHX is recommended.

KEY WORDS activators, bovine embryonic development, intracytoplasmic sperm injection.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a microfertilization technique by which a spermatozoon is directly injected into an oocyte. ICSI bypasses sperm competition occurring within the reproductive tract and also the sperm-oocyte interaction at the levels of zona pellucida and oolema (Fujinami *et al*. 2004a; Fatehi *et al*. 2006; Ajduk *et al*. 2006). This technique has proven its efficacy in producing live offspring in animals and humans especially when sperm number and quality are insufficient for conventional *in vitro* fertilization (Galli *et al*. 2003). ICSI has also been demonstrated as a useful way of delivering the foreign DNA into the oocyte for agriculturally genetic manipulation (Niemann and Kues, 2000; Houdebine, 2002; Moisyadi *et al*. 2009). In addition, this technique also allows for an understanding of the chronology consequences during sperm and oocyte activation during the first cell cycle of embryo development (Galli *et al*. 2003). Although the fertilization of bovine oocytes by the injection of immobilized spermatozoa and normal calves have been reported (Goto *et al*. 1990; Galli *et al*. 2003), successful ICSI in bovine species has however been markedly restricted by the poor rates of sperm head decondensation following ICSI (Chung *et al*. 2000). To improve its efficacy, appropriate sperm and oocyte activation prior to or after ICSI is necessary to achieve normal fertilization/early embryo development in several species, including cow (Horiuchi *et al*. 2002; Wei and Fukui, 2002; Oikawa *et al*. 2005), pig (Tian *et al*. 2006; Garcia-Rosello *et al*. 2006), sheep (Shirazi *et al*. 2009) and

buffalo (Liang *et al*. 20091; Lu *et al*. 2006). Cycloheximide (CHX) inhibits the synthesis of a group of highly labile proteins such as cytostatic factor (CSF), which maintains a high level of maturation promoting factor (MPF) activity within the mature, unfertilized oocyte (Suttner *et al*. 2000). Cycloheximide alone induces oocyte activation without eliciting a Ca^{2+} transient and in combination with Calcium ionophore A23187 (Ca-I) drove the oocyte to resume meiosis (Atabay *et al*. 2000) and support subsequent development of activated eggs (Suttner *et al*. 2000). Electrical pulses caused a Ca^{2+} transient wave and oocyte activation, subsequent treatment with CHX prevented de novo synthesis of cyclin B and CSF, thus enhancing activation (Tien *et al*. 2006). 6-dimethylaminopurine (6-DMAP) can inhibit the extrusion of the second polar body, due to inhibition of phosphorylation necessary for the spindle apparatus (Tian *et al*. 2006). The ethanol treatment can cause a surge of intracellular free calcium which inactivates the existing liable proteins, and the protein synthesis inhibitor, cycloheximide, stops new synthesis of nascent proteins, degradation of cyclin B and thus inactivation of MPF occur (Yang *et al*. 1994). Ethanol is effective for the activation of *in vitro* matured bovine oocyte and has been used to activate buffalo oocytes (Liang *et al*. 2011). It is possible that the ethanol treatment may have already "overloaded" the calcium elevation response and therefore the electric pulse imposed afterwards results in no free calcium response by the oocyte (Yang *et al*. 1994). Therefore, the combined treatment synergistically drives the oocytes out of the metaphase stage and thus induces activation of the oocytes (Yang *et al*. 1994) and could enhance bovine embryonic development (Tian *et al*. 2006).

Activation of oocytes with additional stimuli during ICSI procedure increases free cytosolic calcium which causes the destruction of the cytostatic factor and degradation of the maturation-promoting factor (MPF) essentially for reinitiation of the second meiosis and formation of the male and female pronucleus (Suttner *et al*. 2000; Oikawa *et al*. 2005). Oocyte activation can be induced by a variety of physical and chemical agents, including an electrical pulse (Kubota *et al*. 2000; Tien *et al*. 2006), ionomycin, calcium ionophore (Rho *et al*. 1998; Suttner *et al*. 2000; Chung *et al*. 2000; Galli *et al*. 2003; Oikawa *et al*. 2005), 6-dimethylaminopurine (Tian *et al*. 2006; Chung *et al*. 2000; Rho *et al*. 1998; Suttner *et al*. 2000; Oikawa *et al*. 2005), cycloheximide (Yang *et al*. 1994; Suttner *et al*. 2000; Tian *et al*. 2006) and ethanol (Yang *et al*. 1994; Oikawa *et al*. 2005). The results obtained from these activators have been inconsistent among species studied (Keefer *et al*. 1990; Rho *et al*. 1998; Chung *et al*. 2000; Galli *et al*. 2003).

This study aimed to examine the effects of activators, 6-dimethylaminopurine, ethanol, electrical stimuli, calcium ionophore and cycloheximide ,on the developmental competence of ICSI-oocytes.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Oocyte collection and *in vitro* maturation

Bovine ovaries were obtained from an abattoir and transported to the laboratory in 0.9% (W/V) saline solution plus antibiotics. Within 3 h, 3-6 mm follicles were aspirated via a 18-gauge needle attached to a 10-mL disposable syringe, the cumulus oocyte complexes (COCs) were then morphologically classified at a magnification of x 40 using a stereomicroscope (Nikon SMZ-10 binocular, Japan). Only intact COCs with a compact and multilayered cumulus investment were used in this study. A group of 10 COCs were matured for 20-21 h at 38.5 ˚C under a humidified atmosphere of 5% CO_2 in a 50 µL droplet of maturation medium under mineral oil.

The maturation medium consisted of tissue culture medium 199 (TCM199) supplemented with 10% (v/v) bovine follicular fluid, 50 IU/mL human chorionic gonadotropin (chorulon®; Intervet-International BV., Boxmeer, Netherlands), 0.02 AU/mL follicle stimulating hormone and 1 µg/mL estradiol-17ß. After maturation, the oocytes with expanded cumulus cells were freed from the cumulus using 0.2% (W/V) hyaluronidase supplemented with 1 mg/mL polyvinylpyrrolidone (PVP, average molecular weight 40000) with gentle pipetting. The denuded oocytes were washed in TCM199 supplemented with 10% (v/v) fetal bovine serum (FBS) and 12.5 mM HEPES.

Sperm preparation and intracytoplasmic sperm injection

Frozen-thawed sperm from a proven-fertile bovine was used in this study. Motile sperm was selected by 'swim-up' for 30 min at 37 ˚C in Tyrode-albumin-lactate-pyruvate (TALP) supplemented with 5 mM caffeine. The recovered sperm was subsequently mixed with 10% PVP prior to ICSI. The ICSI procedure was performed using an inverted microscope (Carl Zeiss/Axiovert 135, Oberkochen, Germany) and the ICSI was driven by a micromanipulator (eppendorf, Cell Tram®, vario Microinjector, America). To perform ICSI, the motile sperm was immobilized by scoring the sperm tail with the tip of the injection needle against the bottom of the petri-dish. The oocytes were positioned on the holding pipette (inner diameter 20-30 µm) in such a way that the first polar body was oriented at 90 degree (12 o'clock position). The ICSI needle (inner diameter 9-11 µm) containing the sperm was then advanced through the

zona pellucida and oolemma at 180 degree (3 o'clock position) and the sperm was released into the ooplasm (Chung *et al*. 2000; Horiuchi *et al*. 2002). Following ICSI, the ICSI-oocytes were washed four times in TCM 199 supplemented with 10% (v/v) FBS and 12 mM HEPES and held at room temperature (approximately 26 ˚C) before oocyte activation. The oocytes after sperm injection are shown in Figure 1.

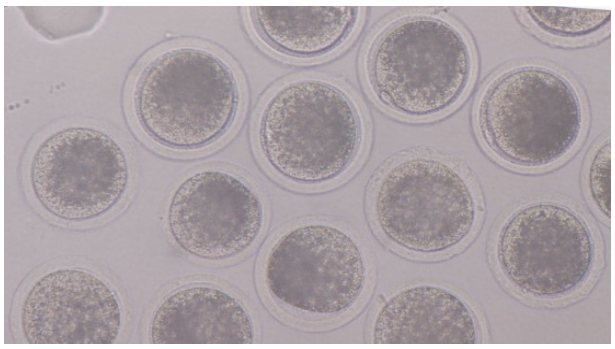

Figure 1 Bovine oocytes after sperm injection

Oocyte activation and embryo culture

After ICSI, the oocytes were activated according to the following treatment protocols:

1. Calcium ionophore followed by 6-dimethylaminopurine: the injected oocytes were exposed to 5 uM calcium ionophore in TCM199 supplemented with 10% FBS and 12 mM HEPES for 5 min at 38.5 ˚C under a humidified atmosphere of 5% CO_2. After four times washing, they were transferred to culture in SOFaa supplemented with 10% FBS and 1.9 mM 6-DMAP in SOFaa supplemented with 10% FBS for 3 h.

2. Ethanol followed by cycloheximide: the injected oocytes were treated with 7% ethanol for 5 min in TCM199 supplemented with 10% FBS and 12 mM HEPES. After four times washing, they were transferred to culture in SOFaa supplemented with 10% FBS and 10 µg/mL cycloheximide at 38.5 ˚C under a humidified atmosphere of 5% CO_2 for 5 h.

3. Electrical stimulation followed by cycloheximide: the injected oocytes were placed individually between electrodes and were induced with the pulse of direct current of 30 V for 15 x 2 µsec (Tasripoo *et al*. 2007) by electro cell fusion (BTX Electro manipulator Cell 200 San Diego, CA) in Zimmerman fusion medium (Zimmermann and Vienken, 1982). The electrical pulse also simultaneously induced initial oocyte activation (Kubota *et al.* 2000). After four times washing, they were transferred to culture in SOFaa supplemented with 10% FBS and 10 ug/mL cycloheximide at 38.5 ˚C under a humidified atmosphere of 5% CO_2 for 5 h.

4. Calcium ionophore followed by cycloheximide: the injected oocytes were exposed to 5 uM calcium ionophore in TCM199 supplemented with 10% FBS and 12 mM HEPES for 5 min at 38.5 ^{o}C under a humidified atmosphere of 5% CO_2. After four times washing, they were transferred to culture in SOFaa supplemented with 10% FBS and 10 ug/mL cycloheximide at 38.5 ˚C under a humidified atmosphere of 5% CO_2 for 5 h.

5. Cycloheximide only: the injected oocytes were transferred to culture in SOFaa supplemented with 10% FBS and 10 ug/mL cycloheximide at 38.5 ˚C under a humidified atmosphere of 5% CO_2 for 5 h.

6. Calcium ionophore only: the injected oocytes were exposed to 5 µM calcium ionophore in TCM199 supplemented with 10% FBS and 12 mM HEPES for 5 min at 38.5 ˚C under a humidified atmosphere of 5% CO_2.

7. Sham injection: the injected oocytes were injected without sperm and performed in the same manner as sperm injection group. They were subsequently treated with Ca-I followed by CHX or CHX only. Calcium ionophore plus CHX and CHX only were used in the sham injection groups.

Following activation treatments, presumptive embryos were subsequently cultured *in vitro* at 38.5 ˚C in SOFaa supplemented with 1% (v/v) FBS under a humidified condition of 5% CO_2, 5% O_2, and 90% N_2 for 48 h. The cleaved embryos were then co-cultured with bovine oviductal cells in SOFaa supplemented with 5% (v/v) FBS in a humidified atmosphere of 5% CO_2 for further 4-5 days. The culture medium was changed every 2 days, and embryo development continued till hatched blastocyst was recorded. (Day 0=day of *in vitro* fertilization).

Statistical analysis

The cleavage rates, 8-cell stage rates, morula and blastocyst rates from each treatment are presented as a percentage. Comparisons between treatments groups were analyzed by Chi square tests. Significant differences were defined when $P<0.05$.

RESULTS AND DISCUSSION

Sperm injection group

Effects of Ca-I followed by 6-DMAP, ethanol followed by CHX, electrical stimuli followed by CHX and Ca-I followed by CHX after ICSI on embryonic development *in vitro* are shown in Table 1.

A similar significant ($P<0.05$) cleavage rate (78.4%, 79.7% and 73.5%, respectively), 8-cell embryo (62.7%, 59.4% and 58.3%, respectively), morula (47.7%, 44.4% and 43.1%, respectively), blastocyst rate (34.6%, 35.3% and 28.0%, respectively) and hatched blastocyst rate (13.0%,

15.0% and 10.6%, respectively), were obtained from ICSI oocytes after treatment with Ca-I followed by 6-DMAP, ethanol followed by CHX, electrical stimuli followed by CHX. The cleavage, 8-cell embryos, morula and blastocyst production of ICSI oocytes in the Ca-I + 6-DMAP, ethanol + CHX, electrical stimuli + CHX and Ca-I + CHX group were higher significantly (P<0.05) than those in the Ca-I only and CHX only treatment group. The cleavage rate in the Ca-I + CHX group was not significantly (P>0.05) different from CHX group. The lowest blastocyst rate (1.9%) was from treatment with the Ca-I only treatment. The cleavage rate and blastocyst rate of ICSI oocytes in the ethanol+CHX group were higher significantly (P<0.05) than those in the Ca-I + CHX group. But the 8-cell embryos, morula and hatched blastocyst rate were not significantly (P>0.05) different between these two groups. The cleavage rate, 8-cell embryos, morula, blastocyst and hatched blastocyst rate of ICSI oocytes in electrical stimulation + CHX group were not significantly (P>0.05) different from the Ca-I + CHX group. The cleavage rate and later development till hatched blastocyst rate of ICSI oocytes in the Ca-I + 6-DMAP group were higher significantly (P<0.05) than those in the Ca-I + CHX group.

Effects of Ca-I followed by CHX, CHX only and Ca-I only after ICSI on embryonic development *in vitro* are shown in Table 2.

The cleavage, 8-cell embryos, morula, blastocyst and hatched blastocyst yield in the Ca-I + CHX group were significantly higher (P<0.05) than those in the CaI only treatment group.

The cleavage rate in the Ca-I + CHX group was not significantly (P>0.05) different from those in CHX only treatment group. But the later development till hatched blastocyst in the Ca-I + CHX group were significantly higher (P<0.05) than those in the CHX only treatment group. The cleavage and morula rate in the Ca-I group was significantly lower (P<0.05) from those in the CHX only treatment group. But 8-cell embryos, blastocyst and hatched blastocyst rate in the CaI group was not significantly (P>0.05) different from those in the CHX only treatment group. It is noted that there was no hatched blastocyst yield in both group.

Sham injection group: (Table 2)

Effects of Ca-I followed by CHX and CHX only after ICSI on embryonic development *in vitro* are shown in Table 2.

A significantly (P<0.05) higher cleavage rate (78.8% and 28.4%, respectively), 8-cell stage yield (48.3% and 10.4%, respectively), morula rate (31.8% and 3.0%, respectively), blastocyst rate (23.2% and 2.2%, respectively) and hatched blastocyst rate (7.3% and 0.8%, respectively), respectively, were obtained after treatment oocytes with Ca-I followed by CHX than those with CHX only, respectively.

Sperm injection versus sham injection: (Table 2)

Effects of Ca-I + CHX on embryonic development *in vitro* are shown in Table 2.

The cleavage rate (78.8%), in sham injection was significantly (P<0.05) higher than those of sperm injection group (63.8%).

Table 1 *In vitro* development of buffalo embryos derived from intracytoplasmic sperm injection and followed by activation with CaI followed by 6-DMAP, ethanol followed by CHX, electrical stimuli followed by CHX, Ca-I followed by CHX, Ca-I only and CHX only

Treatment	Total number of oocytes injected	No. of cleavage (%)	No. of 8-cell (%)	No. of Morula (%)	No. of blastocyst (%)	No. of hatched blastocyst (%)
Ca-I + 6-DMAP	153	120 (78.4)^{a}	96 (62.7)^{a}	68 (47.7)^{a}	53 (34.6)^{a}	20 (13.0)^{a}
Ethanol + CHX	153	106 (79.7)^{a}	77 (59.4)^{ac}	59 (44.4)^{ac}	47 (35.3)^{a}	20 (15.0)^{a}
Electric stimulation + CHX	132	97 (73.5)^{ac}	77 (58.3)^{ac}	57 (43.1)^{ac}	37 (28.0)^{ac}	14 (10.6)^{a}
Ca-I + CHX	141	90 (63.8)^{bc}	68 (48.2)^{bc}	49 (34.7)^{bc}	34 (24.1)^{bc}	13 (9.2)^{a}
CHX	147	81 (55.1)^{b}	39 (26.5)^{d}	22 (15.0)^{d}	9 (6.1)^{d}	0 (0)^{b}
Ca-I	107	35 (32.7)^{d}	18 (16.8)^{d}	5 (4.7)^{e}	2 (1.9)^{d}	0 (0)^{b}

The means within the same column with at least one common letter, do not have significant difference (P>0.01).

Table 2 *In vitro* development of buffalo embryos derived from intracytoplasmic sperm injection and followed by activation with Ca-I followed by CHX and CHX only in the sperm injection group and sham injection group

Treatment	Total no. of oocytes injected	No. of cleavage (%)	No. of 8-cell (%)	No. of Morula (%)	No. of blastocyst (%)	No. of hatched blastocyst (%)
CaI + CHX	141	90 (63.8)^{a}	68 (48.2)^{a}	49 (34.7)^{a}	34 (24.1)^{a}	13 (9.2)^{a}
Sham injection Ca-I + CHX	151	119 (78.8)^{b}	73 (48.3)^{a}	48 (31.8)^{a}	35 (23.2)^{a}	11 (7.3)^{a}
CHX	147	81 (55.1)^{a}	39 (26.5)^{ac}	22 (15.0)^{b}	9 (6.1)^{b}	0 (0)^{b}
Sham injection + CHX	134	38 (28.4)^{c}	14 (10.4)^{c}	4 (3.0)^{c}	3 (2.2)^{b}	1 (0.8)^{b}

The means within the same column with at least one common letter, do not have significant difference (P>0.01).

But, 8-cell stage embryo (48.2% and 48.3%, respectively), morula (34.7% and 31.8%, respectively), blastocyst rate (24.1% and 23.2%, respectively) and hatched blastocyst rate (9.2% and 7.3%, respectively), were not significantly (P>0.05) different between those of sperm injection and sham injection group.

Effects of CHX only after ICSI on embryonic development *in vitro* are shown in Table 2.

The cleavage rate (55.1% and 28.4%, respectively), 8-cell stage (26.5% and 10.4%, respectively) and morula rate (15.0% and 3.0%, respectively), respectively, of the sperm injection group were significantly (P<0.05) higher than those in sham injection group. But, blastocyst yields (6.1% and 2.2%, respectively) and hatched blastocyst (0% and 0.8%, respectively), was significantly not different between sperm injection and sham injection group. The representative of embryonic development at blastocyst and hatched blastocyst stages are shown in Figure 2 and Figure 3.

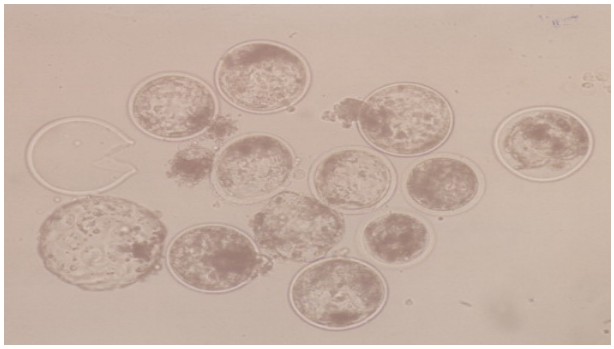

Figure 2 Representative of blastocyst and hatched blastocyst development of bovine embryos

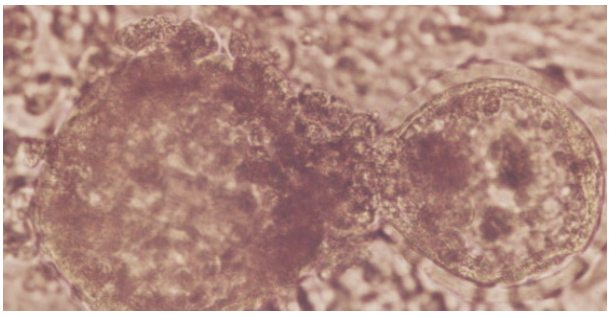

Figure 3 Representative of hatched blastocyst stage of bovine embryo development co-cultured with oviductal cell monolayer

Our results demonstrated that activation protocol after ICSI affected the developmental competence of bovine ICSI oocytes. Among the activators in our study, using Ca-I + 6-DMAP, ethanol + CHX, electrical stimuli + CHX and Ca-I + CHX showed significantly higher efficiency in increasing cleavage and embryonic development than CHX or Ca-I only. Previously, the use of ethanol plus CHX (Yang *et al*. 1994), Ca-I + 6-DMAP, Ionomycin + 6-DMAP, (Rho *et al*. 1998; Suttner *et al*. 2000; Chung *et al*. 2000; Oikawa *et al*. 2005) and DC pulse + DMAP (Tian *et al*. 2006) successfully increased the ICSI oocyte development capacity. There were few reports of the comparison treatment of ICSI-oocytes with varieties of chemical activators. It has been reported that Io + 6-DMAP or ethanol + CHX supported the highest second polar body extrusion and resulted in the highest blastocyst rates and cell numbers per embryo in buffalo (Liang *et al*. 2011). It has been reported that post-ICSI oocyte activation with ethanol is more effective than activation with ionomycin alone or with ionomycin + DMAP for the production of viable blastocyst of calves (Oikawa *et al*. 2005).

Our results also corresponded to the report in buffalo by Liang *et al*. (2011) that treatment with Ionomycin + 6-DMAP and ethanol + CHX gave highest blastocyst formation (24%-29%). The cleavage and blastocyst rate using Ca-I plus CHX were similar to the report by Galli *et al*. (2003), using Ionomycin + CHX (59.7% and 16.7%, respectively). The Ca-I + 6DMAP treatment group showed a similar cleavage and blastocyst yield (83.9% and 40.1%, respectively) reported by Oikawa *et al*. (2005). The treatment of ICSI - oocytes with ethanol + CHX showed similar percentages of 8-cell (59.4%) with those (45%) previously reported by Yang *et al*. (1994). It is noted that our results (using ethanol+CHX) were similar to those previously reported by Horiuchi *et al*. (2002), who used only 7% ethanol for post activation, at 8-cell stage (42.3%), morula rate (33.9%) and blastocyst rate (20.3%). Our study gave a higher cleavage rate, 8-cell stage, morula and blastocyst rate in the Ca-I + 6-DMAP treatment than those reported by Chung *et al*. 2000. The blastocyst yield from the Ionomycin + 6-DMAP treatment group in the present study was lower than that (23.9%) reported by Rho *et al*. (1998), but, our cleavage and morula were higher than those (61.2% and 37.2%, repectively) reported by Rho *et al*. (1998).

There was no hatched blastocyst stage or very few obtained when using CHX or Ca-I only in the sperm injected group and also in sham injected group. This may clearly indicate the beneficial effects of combined chemical activation. It is noted that the study of embryonic development until hatched blastocyst stage showed clearly higher development capacity of ICSI ooctye from each treatment especially with the 2-combination activators than those single activator (CHX only and Ca-I only). In the present study cleavage and blastocyst rate using Ca-I plus CHX were similar to the report by Galli *et al*. (2003), using Ionomycin + CHX (24%). Furthermore, blastocyst rate (20.3%), using ethanol + CHX treatment, was similar to those previously reported by Horiuchi *et al*. (2002) who used only 7% ethanol for post activation of ICSI oocytes.

Furthermore, it was reported that using repetitive treatment or increasing the concentration of Ionomycin could induce a higher yield of oocyte with 2 PN than a single stimulation (Chung *et al*. 2000). Additional stimulation may have allowed the oocytes to continue the cascade of activation events (Lee *et al*. 2003).

It may be inferred that, *in vivo*, cleavage and subsequently development of the inseminated oocyte need a great number of inducing agent. However, the low rate of 2 pronuclear formations remained low even using the repetitive treatment with ionomycin (Chung *et al*. 2000).

In the sham injected group when treated with Ca-I + CHX, also showed evidence that 2 combination activator gave higher efficiency development than using one activator (CHX only or Ca-I only). This also agrees with the previous report of Oikawa *et al*. 2005 (using Ionomycin+DMAP) and Tian *et al*. 2006 (using DC pulse plus DMAP). It is noted that ICSI - oocyte treated with Ca-I followed by CHX and CHX only showed a non-significant difference of cleavage rate and further embryonic development corresponded to both in sperm injected group and sham injected group. Thus, our results might be different from others in that, sperm did not clearly play an important role in the development of injected oocyte when treated with Ca-I + CHX and CHX only. This result contradicts the reports by Chung *et al*. (2000) that significantly higher activation rates were observed in sperm injected, activated ,than in sham injected, activated with ionomycin + DMAP. These demonstrated that spermatozoa contributed to the activation process as in *in vitro* fertilization (IVF. This also suggests further investigation with ethanol + CHX, electrical stimuli + CHX and Ca-I + 6-DMAP and also single stimulation in the sham injection treatment. This study also showed that, without sperm (sham-injection) plus post-activation, the injected oocyte could develop past the 16-cell stage and further develop until reaching the same rate of hatched blastocyst stage as those of sperm injected group.

However, our study in bovine did not show the evidence of the presence of pronuclear formation. Our data clearly showed that significantly higher blastocyst yields, were from these 2-combination activators; Ca-I + 6-DMAP, ethanol + CHX, electrical stimulation + CHX, and Ca-I + CHX than single activator, CHX and Ca-I only. Our results demonstrated the correspondence with other reports that using 2 combinations showing higher yield of cleavage and later embryonic development than those with a single activator. It is noted that these combined activators have showed the most consistent efficiency in ICSI bovine. Among the four combined activators used in this study, we recommend using 7% ethanol followed with 10 µg/mL of CHX, because it is simpler, more convenient and low cost.

In conclusion, these results showed that the two combination methods, Ca-I + 6-DMAP,ethanol + CHX, electrical pulse + CHX and Ca-I + CHX, used in this trial have been shown to be effective for the activation of bovine oocytes for increasing blastocyst development, however, activation with ethanol + CHX is recommended.

ACKNOWLEDGEMENT

The study was financially supported by a Thai government budget under the A Scientific Thai–Netherlands Co-operative Project (Project Code TH/NL 02/51), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, THAILAND.

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