韓國受精卵移植學會誌(2002) 第 17 卷 第 1 號
Korean J. Emb. Trans. (2002) Vol. 17, No. 1, pp. 13~21

Development of Bovine Embryos Produced by Intracytoplasmic Sperm Injection (ICSI)

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SUMMARY

Intracytoplasmic Sperm Injection (ICSI) has been widely used for both human infertility and basic research. However, the high incidence of chromosomal abnormality is severe problem in cattle. Various oocyte activation stimuli, therefore, were compared by assessment of developmental capacity and chromosome analysis. Motile sperm selected by Percoll-density gradient were treated with 5 mM dithiothreitol (DTT) and injected into an oocyte matured for 24 h. Eggs were then allocated into 5 treatment groups. Group 1 (control), sperm injection was performed without any further activation stimuli to the oocytes. Group 2 (handled control), sham injection was performed without sperm. In Group 3, oocytes exposed to 5 (M ionomycin for 5 min at 39(C. Group 4: ionomycine + 1.9 mM demethylaminopurine (DMAP, 3 h) and Group 5, ionomycine + 3 h culture in M199 + DMAP. Cleavage and the later development rate in Groups 1, 2 and 3 were significantly ($P<0.05$) lower than those in Groups 4 and 5. The incidence of chromosomal abnormality in the embryos treated directly with DMAP after ionomycine was relatively higher than in the embryo of Group 3 h, delayed DMAP treatment. From this results DMAP caused to be arrested the release of the 2nd polar body, resulting in changes of chromosomal pattern. Therefore, the time interval between ionomycin and DMAP is a crucial role in bovine ICSI.

(Key words: ploidy, activation, ICSI, bovine oocyte)

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has become a very widely applied means of overcoming infertility in humans, its clinical usefulness, and valuable research tool for studying fundamental aspects on how the two gametes interact during fertilization. Recently, the spontaneous ability of sperm cell to bind exogenous DNA molecules can be exploited by using spermatozoa as vectors for delivering foreign genetic information to eggs during fertilization and ICSI procedures.

The success of bovine ICSI depends not only on

Supported by Korean Research Foundation (KRF-99-041-G00150G7013) for Dr. Gyu-Jin Rho

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sperm treatment but also on activation of the oocyte (Chen and Seidel, 1997; Younis et al., 1989; Keefer et al., 1990). Although mechanical stimulation by the injection pipette can only occasionally bring about activation of the bovine oocyte, further stimuli of oocyte activation should be followed.

Activation of mammalian oocytes in several species has been attempted using various chemicals, physicals and enzymatic stimuli (Kaufman, 1983). Most of these stimuli, such as ethanol (Nagai, 1987; Kubiak, 1989; Minamihashi, 1993), calcium ionophore (Ware et al., 1989), electrical stimulation (Ware et al., 1989; Powell and Barnes, 1992; Campbell et al., 1993; Prochazka et al., 1993), temperature shock (Stice et al., 1994) can activate some oocytes, however, the efficiencies of early attempts at oocyte activation were low, often varied with the age of the oocytes (Stice and Robl, 1988; Ware et al., 1989; Yang et al, 1993; Takano et al., 1993). Activation procedures that mimic normal fertilization more closely have been somewhat more effective. These include the use of sperm factor (Stice and Robl, 1990) and of combination with an inhibitor of protein synthesis (protein phosphorylation or histon kinase) to prevent the re-accumulation of maturation promoting factor (MPF) (Fukui et al., 1992; Szollosi et al., 1993; Navara et al., 1994; Susko-Parrish et al., 1994; Yang et al., 1994). In addition, oocyte activation is always conducted in cattle to obtain better ICSI results (Qian et al., 1996; Chen and Seidel 1997; Rho et al., 1998b).

The principal objectives of this study were to develop a reliable and effective method on bovine ICSI. Based on that observation, it was hypothesized that time intervals between ionomycin and DMAP treatment is a crucial role for the extrusion of the second polar body. Once metaphase II oocyte is activated by ionomycin, the second polar body must undergo to be released to perivitelline space within 3～6 h. In the use of protein synthesis inhibitor directly prior to ionomycin treatment, the extrusion of the second polar body must inhibit and results in triploidy ICSI embryo. Therefore a 3-h interval between ionomycin treatment and DMAP was conducted to be tested the hypothesis in ICSI embryos whether the ploidy of the resulting embryos is 2n or others.

MATERIALS AND MATHODS

1. Chemicals and Media

Chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO). The medium (IVM-medium) used for maturation of cummulus-oocyte-complex (COCs) was M199 containing Earle's salts, 10% fetal calf serum (FCS), 10 μg/ml LH, 10 μg/ml FSH and 1 μg/ml estradiol -17 μ, 25 mM Hepes, 2.5 mM Na pyruvate, 1 mM L-glutamine, and 1.0% penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively; Pen-Strep; GIBCO). The medium (IVC-medium) used for culture of embryos was M199 containing Earle's salts, 10% FCS, 2.5 mM sodium pyruvate, 1 mM L-glutamine, and 0.5% Pen-Strep. M199 containing Earle's salts, 10% FCS, 5 mM sodium pyruvate, 1 mM L-glutamine, 1.0% Pen-Strep was used for the culture of bovine oviductal epithelial cells (BOEC). For IVM and IVC media, the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg. Tyrode's albumin lactate pyruvate medium containing 2% bovine serum albumin (BSA, Fraction V) either supplemented with 10 mM Hepes (HEPES-TALP) or without (IVF-TALP) was used for sperm preparation. The micromanipulation medium used for ICSI was performed in drops of Ham's F-10 containing 25 mM Hepes.

2. Oocytes Preparation

Cumulus-oocyte-complexes (COCs) collected from ovaries harvested at a local abattoir were matured in 50 μl droplets of IVM medium under paraffin oil (Yakuri, Japan) at 39℃ in a humidified atmosphere

of 5% CO_2 in air. After 24 h culture, the expanded cumulus cells were removed by vortexing for 2 min in 3% sodium citrate solution. Oocytes with the first polar body and dense cytoplasm were selected using an inverted microscope (× 400) for further experiments.

3. Sperm Preparation

Sperm preparation was conducted by a Percoll (Pharmacia, Uppsala, Sweden) density gradient (Rosenkrans et al., 1993). In brief, 100% Percoll solution was mixed with 10×salt solution (NaCl, 2.889 g; KCl, 0.238 g; KH_2PO_4, 0.116 g; $CaCl_2$, 0.112 g; Hepes, 0.163 g; 50 ml of mili-Q water) to from 90% Percoll solution and then formed 45% Percoll solution as addition of 1 (90% Percoll solution) to 1 (HEPES-TALP medium) (v/v). The gradient was formed by pipetting 1.5 ml of 90% Percoll solution into a 15 ml conical tube and then layered with 1.5 ml of 45% Percoll solution. Frozen-thawed bull semen were placed onto the top of 45% gradient and then centrifuged at 850×g for 15 min at room temperature. After removal of supernatant, the pellet was washed once with 10 ml of HEPES-TALP medium by centrifugation at 350 ×g for 10 min. The sperm pellet was then resuspended in IVF-TALP supplemented with 6 mg/ml BSA. In a 2 ml culture tube, a 0.1 ml sperm aliquot was layered under 0.8 ml IVF-TALP medium supplemented with of 5 mM dithiothreitol (DTT). After 30 min of incubation, the spermatozoa were washed by centrifugation at 350 (g for 10 min, in HEPES-TALP medium in order to minimize the effect of DTT. The sperm pellet was resuspended in 1ml HEPES-TALP medium and sperm cells were used for injection. Vital stain (*Fertil*Light^{tm}, Molecular Probes inc., Eugene, OR) was used to assess the integrity of sperm plasma membranes by the method of Rho et al (2001).

4. ICSI

In preparation for ICSI, 2 μl of the sperm suspension was transferred to 10 μl of Ham's F-10 medium containing with 10% polyvinylpyrrolidone (PVP; Mr. 360,000, v/v. Sigma) under paraffin oil in order to prevent spermatozoa from sticking to the inner surface of the micropipette and to reduce their motility. ICSI was performed at×200 magnification in 20 μl droplets of Ham's F-10 under mineral oil in 60 ml tissue culture dishes (Falcon; Fisher Scientific, Atlanta, GA) maintained at 37℃ on the heated stage of a Carl Zeiss Sedival inverted microscope with a Narishige micromanipulator (Narishige, Tokyo, Japan). The injection pipette with an inner diameter at the tip of 8 μm was connected to a pair of Narishige micromanipulator, and holding pipette was connected to a 1-ml tuberculin syringe.

Oocytes were held its position at 12 or 6 o'clock to the first polar body by the holding pipette. After injection pipette containing a spermatozoon was inserted into the ooplasm at 3 o'clock to the first polar body, a small volume of ooplasm was aspirated into the injection pipette in order to rupture the oolemma. Subsequently, the aspirated ooplasm and spermatozoon were expelled into the ooplasm with a minimum volume of medium. One hour after injection, the oocytes were re-examined, and any from which the spermatozoon could see to have been expelled into the perivitelline space were removed.

Sham injection was performed in a similar manner. The oolemma was ruptured; ooplasm was aspirated into the injection pipette and expelled back into the oocytes with a minimum volume of medium.

5. Activation Procedures and *In Vitro* Culture

Oocytes after injection were assigned to 5 experimental groups. Group 1: As control, sperm injection was performed without any further activation stimuli to the oocytes. Group 2: As handled con-

trol, sham injection was performed without sperm. Group 3: Oocytes exposed to 5 μM ionomycin for 5 min at 39℃, and then rinsed 2 times in HEPES -TALP containing 30 mg/ml BSA to stop activation. Group 4: Oocytes exposed to the ionomycin for 5 min, and than cultured in IVC-medium supplemented with 1.9 mM dimethylaminopurin (DMAP) for 3 h (Susko-Parrish et al., 1994). Group 5: Oocytes exposed for 5 min, cultured in IVC-medium to be allowed the extrusion of the second polar body, and than exposed to DMAP (Rho et al., 1998).

All eggs were then co-cultured with bovine oviductal epithelial cells (BOEC) in set of 15 in 50 μl drops of IVC-medium as described by Rieger et al. (1995). These co-cultures were maintained for 8 days. At Days 2 and 5 (Day 0 = day of injection), the culture were "fed" by adding 25 μl of IVC -medium to each drop. Embryonic development was assessed with an inverted microscope at 24 h intervals for up to 192 h after injection.

6. Cytological Procedures

At 16 h after ICSI, some oocytes were fixed overnight in methanol: acetic acid (3:1, v:v), stained with 1% aceto-orcein to reveal the presence of pronuclei (PN) formation and nuclear status at × 400 magnification. At 196 h post-activation, embryos developed to blastocyst stage were prepared and examined for their cytogenetic composition, as described by King et al. (1979). The stained chromosome spreads and nuclei were counted under a compound microscope at × 200 magnification. The chromosomes were evaluated at × 1,000 with oil-immersion optics. Embryos were classified as being haploid, diploid, polyploid or mixoploid.

7. Statistical Analysis

Differences were analyzed among treatments using one-way ANOVA after arc-sine transformation of the proportional data ($P<0.05$). Differences were considered Duncan's Multiple Range Test for variable.

RESULTS

1. Sperm Morphology by DTT Treatment

At 30 min of culture in 5 mM DTT solution, most sperm (~76%) still possessed an intact membrane as judged by vital staining, but displayed altered morphology in 45% of the sperm. At 2 h culture in a solution, ~38% of the sperm appeared as a dead and ~67% displayed altered morphology (data not shown), including head and tail. In a head of the sperm pronounced bending near the equatorial segment was exhibited. Tail

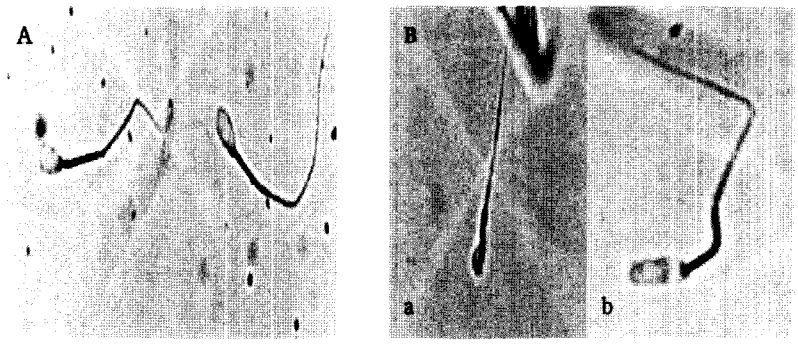

Fig. 1. Morphological changes of sperm cultured in IVF-TALP with DTT (B) and without (A) for 30 min (× 800). A) Normal appearance, B) Bending head (a) and swollen head (b).

appeared to be swollen and bent. From 30 min of culture, partially decondensation of sperm head observed as presented in Fig. 1. However, control sperm that cultured in DTT-free medium, did not appeared decondensation.

2. PN Formation of ICSI Eggs

PN formation of the eggs injected DTT-treated sperm was compared to those of DTT-untreated sperm. After injection of sperm treated with/without DTT, the eggs were activated with ionomycin and followed by culture in IVC-medium supplemented with 1.9 mM DMAP. At 16 h culture after activation, the eggs were fixed and evaluated their PN formation. Table 1 shows in 4 replicates that the proportion 2PN formation was higher significantly in the eggs injected with DTT-treated sperm than that of without DTT-treated sperm (50% vs. 32%, respectively, $P<0.05$). Although metaphase II oocytes contained intact sperm head in both groups, significantly higher ($P<0.05$) rates in DTT untreated group were revealed. In a case of male PN formation, some of eggs possessed unactivated metaphase Ⅱ plate (16%, untreated DTT; 17%, treated DTT, respectively).

3. Development of ICSI Eggs

As a consequence of the result shown in Table 1, resulting in higher PN formation, sperm that injected into the eggs in all subsequence of this experiment were treated with DTT. After injection of those sperm, eggs were then allocated into three activation groups, as described in a part of Materials and Methods, and compared their cleavage and developmental rates to control that carried sperm injection but not further activation, in 7 replicates. In both of groups 2 (handled control) and 1 (control, sperm injected but not activated), cleavage rates remained low (1~2%) and none of them developed to blastocyst stage embryo. However, activated groups (group 3, 4 and 5) developed into cleavage and later stage embryos significantly

Table 1. Pronuclear formation of the eggs injected with and without DTT-treated sperm

Sperm treatment	No. oocytes	Pronuclear formation (%)			
		2PN	MⅡ+1SP	MⅡ+1PN	3PN
No DTT	50	16 (32.0)[a]	22 (44.0)[a]	8 (16.0)	4 (8.0)
DTT	75	27 (50.0)[b]	11 (20.3)[b]	9 (16.7)	7 (13.0)

* Percentages with different superscripts within a column differ significantly, $P<0.05$.

2PN, male+female PN; MⅡ+1SP, MⅡ oocyt+intact sperm; MⅡ+1PN, MⅡ oocyte + male PN; 3PN, more than 3PN.

Table 2. Developmental capacity of ICSI eggs

Group (treatment)	No. oocytes	Development (%)	
		Cleavage	Blastocyst
1 (control)	98	2 (2.0)[a]	0
2 (handled control)	101	1 (1.0)[a]	0
3 (Ionomycin)	159	27 (17.0)[b]	1 (0.6)[a]
4 (Ionomycin+DMAP)	116	61 (52.6)[c]	18 (15.5)[b]
5 (Ionomycin+3h+DMAP)	128	57 (44.5)[c]	15 (11.7)[b]

* Percentages with different superscripts within a column differ significantly. $P<0.05$.

Table 3. Ploidy of embryos produced by sperm injection

Group (treatment)	No. blastocysts	Ploidy (%)		
		Diploid	Mixoploid	Polyploid
4 (Ionomycin+DMAP)	18	6 (33.3)	10 (55.5)	2 (11.1)
5 (Ionomycin+3h+DMAP)	15	8 (53.3)	6 (40.0)	1 (6.7)

higher ($P<0.05$) than those of groups 1 and 2 (Table 2). Although 17% of the eggs in group 3 activated with only ionomycin was cleaved but, one out of cleaved (1/27, 4%) developed to later stage embryos. However, in groups 4 and 5, the rates of cleavage and development differ significantly ($P<0.05$) than those of other 3 treated groups (53 and 16%, and 45 and 12%, respectively). Between two treated groups, there did not differ the rates of cleavage and development.

4. Ploidy of ICSI Embryos

From all 33 blastocysts, 259 spread chromosome sets were analyzed as being diploid (2n), mixoploid (blastomeres having each different chromosomal sets) and polyploid ($\geq$ 3n). Embryos developed to blastocysts in groups 4 and 5 were determined of their ploidy (Table 3). Although there was no significant difference in the frequency of chromosomal abnormalities in the embryos between two groups (56% and 11% mixoploid and polyploid , respectively, in group 4; and 40% and 7%, respectively, in group 5), group 5 tended to a few higher rate of diploid compared to group 4 (53% vs. 33%, respectively).

DISCUSSION

This study has demonstrated that the efficiency of ICSI by pre-treatment of sperm with 5 mM DTT for 30 min, and followed by activated with the combination of ionomycin and DMAP, was improved on male PN formation, and reducing abnormal ploidy of the resulting embryos in cattle.

Treating the sperm with 5 mM DTT compare to untreated sperm group before using them for ICSI clearly increased the rate at which they gave rise male pronuclei in injected ooytes. This result corroborates a previous report (Perreault et al., 1988) that DTT-treated cattle sperm nuclei were able to participate more fully in DNA synthesis in the hamster oocyte due to more rapid decondensation and PN formation. This improvement may be caused by the reduction of stabilizing disulfide bonds of sperm nucleus. Disulfide-reducing agents as DTT, anionic detergent, proteases, and high or low concentrations of salts promote the decondensation of nuclear chromatin in mammalian sperm coincide with parallel researches (Zirkin et al., 1980; Perreault et al., 1982; Rho et al., 1998). In DTT-treated sperm group, the observation of an intact sperm cell residing to injected oocyte has also been made by Rho et al. (1998) in the bovine and by Gomez et al. (1998) in the sheep. Cattle sperm have very tightly packaged nuclei since they contain Type 1 protamine, which is maximally cross-linked (Perreault et al., 1988). In the present study on sperm morphological changes by use of DTT clearly shows that its head altered by decondensation and/or by pronounced bending near the equatorial segment, suggesting that it contains protamine close to the equatorial segment of sperm head. Similarly the perforatorium of rat sperm also contains DTT receptor site, resulting in decondensation of sperm head by treatment with DTT (Calvin et al., 1971). In addition, tail also appeared to be swollen and bent from 2 h of culture. However, control sperm that cultured in DTT-free

medium, no such changes were observed. In human (Van Steirteghem et al., 1993), mice (Kimura et al., 1995) and rabbit (Perreault et al., 1988), injection procedure itself is sufficient to be activated the oocytes, as both of nuclei of sperm and oocyte can undergo their decondensation and PN formation. However, the present study clearly shows that injection procedure rarely leads to sperm decondensation and PN formation. When the eggs injected with DTT-treatment sperm did not activated with any of chemicals, 1~2% of the eggs cleaved and none of them developed to later stage embryo. Similarly Rho et al. (1998) also opined that bovine eggs which were injected with sperm rarely lead to be activated.

Calcium plays an important part in the intracellular signaling responsible for the initiation and propagation of oocyte activation in all mammalian species. However, the present study has demonstrated that oocytes activation procedure using the combination of ionomycin and DMAP is more efficient than ionomycin alone, suggesting that being arrested the MPF level of the activated oocytes with calcium to be minimal amount plays more important. A previous other report (Rho et al., 1998) suggested that the combination of ionomycin and DMAP greatly increases the incidence of chromosomal abnormality in bovine parthenotes, causing to failure of the second polar body by DMAP treatment. In mammals, the extrusion time of the second polar body required about more than 2 h after fertilization with sperm or stimulation by activators (Tombes et al., 1992; Jones et al., 1998). And hence a 3-h interval between ionomycin and DMAP treatment has suggested in the present study in order to allow the extrusion of the second polar body. After sperm injection into oocytes, cleavage and development into blastocysts did not differ in the eggs activated by 3-h delayed treatment compare to ionomycin + DMAP. When compared the ploidy of the resulting embryos, a 3-h delayed DMAP treatment tended to a few higher rate of diploid than ionomycin + DMAP treatment. Between the treatments, chromosomal abnormality as assessed by mixploidy and polyploidy in without delayed DMAP treatment was appeared by 67% of the resulting embryos. The occurrence of chromosomal abnormality may be due to inhibition of the extrusion of the second polar body, with some nuclei presumably re-entering S-phase of the cell cycle without having passed through metaphase. In contrast to this result, a study of parthenote reported by Rho et al. (1998) concluded that a 3-h delayed DMAP treatment increased significantly higher haploid rate than ionomycin + DMAP treatment, as measured by normal ploidy in the parthenotes. However, contrasts markedly with the 17% of chromosomal abnormalities estimated to occur in bovine embryos resulting from conventional *in vitro* fertilization (De la Fuente and King, 1998).

In conclusion, the results indicate that the pretreatment of sperm with 5 mM DTT and the activation of oocyte with ionomycine and a 3-h delayed DMAP treatment are effective for facilitating sperm decondensation and subsequent development with normal chromosome after being injected into the oocytes. Further study remains in vivo study by produced ICSI embryos and adopting transgenic field by sperm-mediated gene transfer.

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(접수일: 2002. 1. 7/ 채택일: 2002. 3. 14)