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Effects of Activation Conditions on the Production of Bovine Intracytoplasmic Sperm Injection Embryos Using *in vitro*-Matured Oocytes

Hitoshi Ushijima[1*], Tetsukazu Kosaki[2], Yukihiro Ishimura[3], Katsuya Sakamoto[1] Takayuki Ishikawa[4] and Hiroshi Nagashima[5]

[1]Chiba Prefectural Livestock Experimental Center, Ichihara, Chiba, 290-0513
[2]NOSAI Chiba, Katori 289-0407
[3]Sanmu Agricultural High School, Oami-shirasato 299-3251
[4]Nippon Institute for Biological Science, Kobuchizawa, Yamanashi, 408-0041
[5]Laboratory of Reproduction Engineering, Department of Animal Production, Meiji University, Kawasaki 214-8571, Japan

ABSTRACT

This study examines the effects of activation conditions on the production of bovine blastocysts using bovine intracytoplasmic sperm injection (ICSI) on *in vitro*-matured oocytes. Frozen-thawed sperm was inserted deep into the oocyte cytoplasm by the Honolulu method. The rates of normal pronuclear formation and *in vitro* development of ICSI embryos were increased when ICSI embryos were artificially activated with mobile sperm (73% vs. 54%, $P< 0.05$). ICSI embryos activated with ionomysin and then dimethylaminopurine developed to blastocysts at a high rate (34%). However, parthenogenetic embryos were found amongst these resultant ICSI embryos, it is necessary to improve the activation protocol used in bovine ICSI. In addition, a development rate of 30% was obtained for ICSI embryos activated with 2–3 pl of 200 mM inositol 1,4,5-trisphosphate, being a significantly higher rate than that using conventional IVF (15%, $P< 0.01$). These results demonstrate that ICSI can improve the success of *in vitro* procedures for bovine embryos.

Key words: intra-cytoplasmic injection, Honolulu method, *in vitro*-produced bovine embryo, parthenogenetic activation

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*Correspondence: Dr. H. Ushijima

INTRODUCTION

The *in vitro* embryo production (IVP) system is used to obtain embryos from the ovaries of slaughtered cows, providing a vital means for increasing meat production [1]. In fact, a new system is now developing in which the IVP system is combined with reproduction engineering techniques and the collection of oocytes from donor cattle using an ultrasound-guided trans-vaginal ovum pickup method [2]. However, as embryo production based on conventional *in vitro* fertilization (IVF) systems [3] is known to be heavily influenced by individual differences in bull semen [4], this restricts the amount of semen that can provide suitable results using conventional IVF methods [5]. i.e., some of the valuable semen had an insufficient sperm concentration [6] making it unsuitable for crossing conventional IVF conditions [5] or embryo collection from donor cattle treated for super-ovulation [7]. Accordingly, IVF is the bottleneck for producing embryos using IVP systems in practical field applications. We also know that intracytoplasmic sperm injection (ICSI) bypasses critical *in vitro* fertilization events such that it very well could overcome problems due to bull variations in bovine IVF, e.g., sperm motility, sperm concentration, and sensitivity for capacitation agents.

Following the establishment of the Honolulu method [8, 9], ICSI has been shown to be practical in several animal species [10]. Injection of spermatozoon results in embryo activation and subsequent development at a high ratio. However, bovine *in vitro*-matured (IVM) oocytes are not completely activated by the ICSI procedure [11-13]. Productivity of normal ICSI embryos might be improved by overcoming bovine singular problems. Here, we therefore compare artificial activation conditions which induce normal pronuclear formation of ICSI embryos using IVM oocytes. Utility of ICSI for use in the livestock industry is also examined by *in vitro* developmental competence of ICSI embryos in comparison with that of IVF embryos.

MATERIALS AND METHODS

Collection of in vitro matured oocytes

The viability of IVP bovine embryos is known to be affected by the storage conditions of cumulus oocyte complexes (COCs), i.e., conditions established by law to prevent the transmission of bovine spongiform encephalopathy (BSE) [14]. COCs were collected from ovaries of slaughtered cattle and cultured in 25 mM Hepes buffered TCM-199 (culture medium; Gibco Laboratories, Grand Island, NY, USA) supplemented with 5% (v:v) FCS (Gibco), 50 μg/ml streptomycin (Meiji Conf. Co., Tokyo,

Japan), and 100 unit/ml penicillin-G (Meiji). COCs were cultured in a single drop of culture medium (500 μl) covered with mineral oil while still in the slaughterhouse, and after inspection confirmed no possibility of BSE, samples were transported to the laboratory. After 22–24 h of *in vitro* maturation (IVM) culture, cumulus cells were removed by repeated pipetting through a fine-bore pipette in PBS (Gibco) containing 1 mg/ml hyaluronidase (Sigma Co., St. Louis, MO, USA). Oocytes with a homogeneous cytoplast having a first polar body were selected for ICSI [14].

Selection of Semen

IVF and ICSI were performed using sperm suspensions from frozen semen collected from super-sires with second-crop daughter progeny-test information on hand to confirm high repeatability. The frozen semen was immersed in a 37 °C water bath for 30 s. Thawed semen was washed with BO and were centrifuged at 500 × g for 5 min to remove cryoprotectants. Pellet spermatozoa were resuspended with BO containing 5 mM caffeine (Sigma), 5 μg/ml heparin (Novo-heparin, Kodama Ind., Japan), and 0.1% BSA (Fraction five, Sigma) and centrifuged [3]. The final concentration of spermatozoa in the IVF medium was 5–10 × 10^6/ml. The sperm suspension was used for IVF by culturing with IVM oocytes for 6–8 h. The remaining suspension was used for ICSI. The spermatozoa were centrifuged with PBS containing 10% polyvinylpyrrolidone (PVP medium, Sigma) at 500 × g for 5 min. A 5-μl sperm suspension was kept under mineral oil in a 90 × 5 mm plastic dish (Lux) until ICSI.

Micromanipulation Instrument

We used a previously described set of micromanipulator instruments (Narishige, Tokyo, Japan) attached to an inverted microscope equipped with Hoffman modulation optics (Hoffman Modulation Contrast, Model EP: Nikon) [9]. Manipulation procedures were performed in PBS containing 20% FCS (PB1) at 22 C. Prior to ICSI, the insert position of the zona pellucida (ZP) was cut by a microneedle to shorten the procedure time for ICSI [15]. ICSI was carried out as previously described using the so-called Honolulu method [8,9]. Briefly, the inner surface of injection pipette was coated by washing several times with PVP medium, then by rubbing the neck at the tip of the needle against the bottom of the dish, a motile sperm was immobilized and aspirated in the needle at the position with tail first. Insert position of the ZP was at 2-3 o'clock position when the 1st polar body of oocyte was at the 12 o'clock position. The injection pipette penetrated deep into the cytoplasm and sucked into the small amounts of

cytoplast through the slit of the ZP. A single piezo pulse (int-2, speed-2) was then applied to break the oolemma, after which an immobilized spermatozoon was expelled into the cytoplasm [16]. Obtained ICSI embryos were held for 20 min at 22 C in PB1 to slowly heal the broken membrane [8].

Activation of IVM Oocytes

An extended time after *in vitro* oocyte maturation adversely affects pronuclear formation and subsequent embryo developmental competence of IVF and ICSI embryos [17, 18]. The optimum activation period for non-aged oocytes is considered to be 24–27 h after maturation culture. To induce activation of IVM bovine oocytes, researchers have used calcium ionophore A 23187 [11, 19], ethanol [10, 20, 21], electric stimuli [22], ionomycin (IA) [23-26], and inositol 1,4,5-trisphosphate (IP3, potassium salt, Sigma) [16, 27]. Actually, a combined multiple activation method is needed for inducing the oocytes 24–27 h after maturation culture [28, 29]. One activation treatment combines IA and dimethylaminopurine (DMAP) which increases the cleavage and blastocyst rates of ICSI-treated bovine oocytes [24-26, 30]. Oocyte activation was accordingly performed using one of seven methods. After activation the ICSI embryos were cultured at 38.5 C in humidified air containing 3% CO_2, and the activated embryos were used for either nuclei observation or further culture testing.

Karyomorphism Examination at Pronuclear Stage of ICSI Embryos

Embryos were mounted on slides and fixed in 25% acetic alcohol using the hole mount method 18 h after ICSI. Karyomorphism was then determined at least 30 h after fixation by staining with 2% aceto-orcein (Wako) and examining with a phase contrast microscope at 200 and 400 × magnification. Because incompletely decondensed sperm and asynchronized pronuclei are observed in ICSI embryos [20, 26], the oocyte karyomorphism were classified into three groups [12]: (1) a normal pronucleus (NP), i.e., oocytes with 1st and 2nd polar bodies and one male and one female pronuclei; (2) an abnormal pronucleus (AbP) without NP, including for example oocytes without 2nd polar body extrusion, oocytes with a condensed sperm head, and multiple female pronuclei; and (3) non-activated nuclei (NA) without any female pronuclei in the oocytes.

In vitro Culture of Bovine ICSI Embryos

Embryos were cultured for 5 d in pre-equilibrated drops of culture medium (0.5 ml) under the same maturation culture conditions (Day 0 = ICSI). After 5 d the embryos were transferred into

microdrops of culture medium containing 100 mM -merchaptoethanol (Sigma). Finally the developmental rate to blastocyst stage was determined at day 8. Embryos without any degenerated blastomeres were judged as transferable quality embryos.

Experimental Designs

Selection of Injection Sperm

In experiment 1, we investigated the effect of sperm mobility and the inducement of artificial activation on *in vitro* development of ICSI embryos.

Adequate Activation Conditions for ICSI

To confirm an adequate activation method for ICSI, we compared the normal pronucleus formation of activated embryos by seven different methods. Control activation consisted of (1) Two 75-V/mm 50-μs electric pulses given to oocytes in 0.3 M sucrose containing 0.05 mM $CaCl_2$ and 0.1mM $MgSO_4$ [29], or (2) embryos were exposed to 7% ethanol medium for 5 min [31]. The cell membrane of oocytes undergo stress due to micropores occurring as a result of electrostimulation treatment or osmolality shock by ethanol activation, and hence another activation method might be suitable for ICSI embryos whose ooeloma are injured during puncturing by ICI [32]. The third group (3) of embryos were therefore exposed for 4 min to ionomycin (5 μM: Cal Biochem, La Jolla, CA) in Ca-free PBS supplemented with 0.1 mg/ml PVA (Sigma) [23-25].

IP3 is considered to be important for activation of mammalian oocytes at the time of fertilization, and it could act as an artificial activator of oocytes [33]. Since IP3 injected into bovine IVM oocytes [27] also produces dynamic changes in intercellular calcium release, we investigated using IP3 as a new activation method for bovine embryos, i.e., group (4) in which a few pl of 200 mM IP3 diluted by 0.9% NaCl was microinjected into oocytes [16]. In group (5) combined activations were performed using IP3 and in group (6) using IA followed by culturing with 1.9 mM DMAP in culture medium for 3.5 h [24, 27].

Normal nuclei formation of a single female pronuclei and second polar body extrusion was recently observed in activated oocytes after injection of sperm factor into the cytosonic fraction [34]. i.e., the sperm solution (SS) was extracted from a sperm suspension. Briefly, collected fresh bovine semen was washed in PBS (-) and centrifuged 2 times at 500 ×g for 20 min. Gathered spermatozoa were sonicated at 7.5 MHz for 10 min. These suspensions, i.e., group (7) were filtered at 0.22 μm and 1–2 pl of SS was injected into the ooplasm to induce oocyte activation.

In vitro development of ICSI Embryos with Artificial Activation

Pronucleus formation and subsequent embryo developmental competence of ICSI embryos subjected to artificial activation were compared to conventional IVF embryos and ICSI embryos with no activation.

RESULTS

Nearly 50% of ICSI embryos injected with immobilized sperm formed pronuclei (Fig. 1).

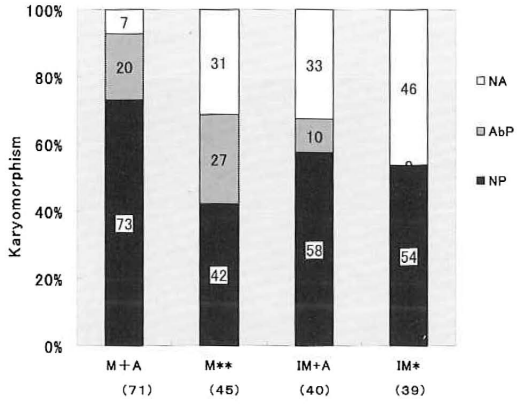

Fig. 1. Karyomorphism of bovine oocytes injected with mobile (M) or immobilie (IM) sperm and with (+A) and without activation 18 h after ICSI treatment.

Values in parentheses indicate the number of embryos examined. Values inside the bar graphs indicate the percentage of the number of embryos used. Closed, gray, and open bars respectively indicate the frequency of normal pronucleus (NP), abnormal pronucleus (AbP), and not-activated nuclei (NA). The NP frequency of ICSI embryos with M+A is significantly different from that of IM and M groups (*: P < 0.05; **: P < 0.01).

However, the frequency rate of NP formation of the ICSI embryos was significantly (P< 0.05) lower than that of ICSI embryos injected with mobile sperm followed by additional artificial activation. We also found that *in vitro* developmental competence of ICSI embryos injected with mobile sperm followed by activation was higher (P< 0.01) than those groups of embryos injected with immobilized sperm (Table 1).

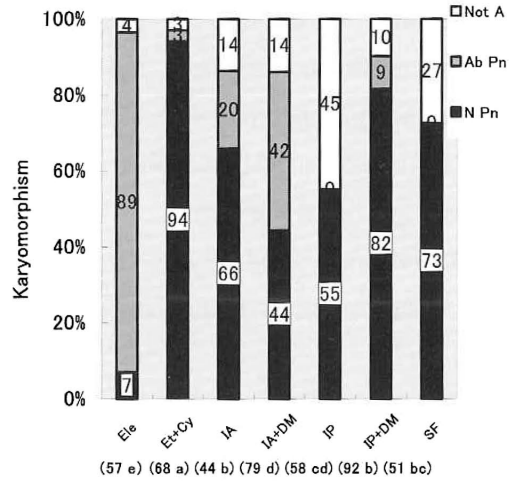

Fig. 2. Karyomorphism of bovine parthenogenetic oocytes activated with various method. Values within columns with different superscripts significantly differ, a, b, c P< 0.05; d, e P< 0.001.

Figure 2 shows karyomorphology of parthenogenetic embryos 18 h after activation. Activation by ethanol (94%), IP3 (55%), or SS (73%) leads to haploid female pronuclei formation with 2nd polar body extrusion resulting in NP. On the

Table 1. *In vitro* development of bovine embryos injected with mobile or immobile sperm and with or without activation treatment.

Sperm mobility	Activation	No. of embryos				
		Cultured	Cleaved (%)		Blastocyst (%)	Transferable (%)
−	−	54	18 (33)		0 (0) [b]	0 (0)
−	+	65	18 (28)	[b]	3 (6)	0 (0)
+	−	54	27 (50)	[a]	3 (6)	0 (0)
+	+	34	19 (56)	[a]	3 (9) [a]	0 (0)

Differences within the experiment groups were verified using Chi-squares test. $P< 0.05$ was considered significant. Values within columns with different superscripts significantly differ, [a, b] $P< 0.01$.

Table 2. *In vitro* development of bovine ICSI embryos activated with different methods.

Activation	DMAP	No. of embryos							
		Cultured	Cleaved		Blastocyst (%)		Transferable (%)		
-	-	52	24 (46)	[a]	6 (12)	[a]	0 (0)	[a]	
IA	-	70	52 (74)	[b]	14 (20)		5 (7)		
IA	+	65	53 (82)	[b]	22 (34)	[b]	13 (20)	[b]	
IP	-	53	32 (60)		16 (30)	[c]	10 (19)	[b]	
IP	+	30	21 (70)	[c]	8 (27)		2 (7)		
IVF	−	235	107 (46)	[a]	36 (15)	[a]	11 (5)	[a]	

IA, ionomycin; IP, inositol 1,4,5-triphosphate; IVF, conventional IVF; and DMAP, dimethylaminopurine. Values within columns with different superscripts significantly differ, [a, b] $P< 0.01$; a, c $P< 0.05$.

other hand, activation by IA (20%) or electrical stimuli (89%) tends to inhibit the release of the 2nd polar body. Of particular interest is that the use of DMAP culture leads to a higher rate ($P< 0.05$) of activated oocytes without 2nd polar body extrusion in IA + DMAP (42%) and IP + DMAP (9%).

Table 2 shows *in vitro* development of ICSI embryos. Note that the rate of *in vitro* development to blastocyst stage in IVF embryos is 15%, whereas the rates

for ICSI embryos activated with IP3 is 30 %, being a significantly higher rate than for IVF embryos and non-activated ICSI embryos ($P< 0.05$). For ICSI embryos activated with IA and then DMAP, *in vitro* development into a 2-cell stage, blastocyst stage, and blastocyst with transferable quality was also higher ($P< 0.01$) than that of the control IVF embryos.

Note that ICSI embryos activated with IP3 and cultured for 7 d led to the birth of a normal offspring after transfer to a surrogate recipient (Fig. 3).

Fig. 3. A normal offspring obtained from an ICSI embryo activated with IP3.

DISCUSSION

Following the production of the first ICSI-derived calves by Goto *et al.* [11], use of immobilized sperm for ICSI has been reported, e.g., sexed sperm heads [21], dead sperm [11], and freeze dried sperm [30]. These embryos were found to have a high frequency of male pronuclear formation reaching blastocysts [23, 35]. In humans and mice, however, sperm immotility and time-dependence after sperm immobilization treatment leads to an increase in DNA fragmentation of the sperm head [36], lower chromosomal normality, and decreased developmental competence in ICSI embryos [37, 38]. In this experiment, developmental competence of ICSI bovine embryos using mobile sperm is higher than that of dead sperm. Additional artificial activation also increased developmental competence of ICSI embryos producing similar results obtained by others [11-13]. A protocol involving motile sperm injection followed by oocyte activation should therefore improve the productivity of bovine ICSI.

Our data showed that karyomorphology of the bovine oocytes after activation is dependent on the type of activation treatment. ICSI embryos activated with IA treatment contained parthenogenetic embryos such as one or two female pronuclei without 2^{nd} polar body extrusion or multiple nuclei with a male pronuclei. The frequency of parthenogenetic embryos for the resultant ICSI embryos was increased by activation followed by DMAP treatment. Parthenogenetic embryos developed to blastocysts at a high rate, yet these embryos could not reach full term

development. Oikawa *et al.* [39] of blastocysts obtained from IA + DMAP-treated ICSI embryos was extremely low, suggesting that a high proportion of the blastocysts produced using this method are parthenogenetic embryos. This phenomenon led to adverse effects on offspring production when resultant blastocysts obtained from ICSI were used to produce calves. It is accordingly essential to establish ICSI conditions that form male/female haploid pronuclei at a high frequency.

Unlike DMAP, cycloheximide (CH) does not block both the extrusion of the 2nd polar body and male pronuclear formation [25, 40]. CH treatment may therefore be more effective [23], although further investigation is required to confirm the significance of these findings. Here, injection of SS leads to oocyte activation and NP formation at a high frequency, so further use as an activation agent for ICSI-embryos should be investigated. On the other hand, Horiuchi and coworkers [10,39] found that about 60% of ICSI embryos injected with motile sperm extruded a 2nd polar body 4 h after ICSI. When such embryos were selected and then activated artificially, they not only developed *in vitro* into blastocysts at a high frequency, but offspring were produced at high pregnancy rate [39]. This method is considered to be feasible to gather normally fertilized embryos with high demonstrated that full-term development viability from ICSI embryos at an early stage of fertilization.

Insufficient IVM conditions leads to a decrease in IP3 stored via cumulus cells such that a possibility exists that this adversely affects egg activation and male pronuclei formation [41]. In fact, failure of pronuclear formation commonly found in the bovine is not specific to ICSI, as it also occurs IVF oocytes [41, 42]. We found that ICSI embryos activated with IP3 had a frequency of NP formation and developed into blastocysts, being a significantly higher rate than that of IVF embryos or non-activated ICSI embryos. A normal offspring obtained from the resultant blastocyst was born after transferring to a surrogate recipient; this being the first known report in which ICSI bovine embryos activated with IP3 reached full term. IP3 injection into the ooplasm is therefore considered to be an effective parthenogenetic activation for IVM oocytes.

Although ICSI expands the possibilities using reproductive technologies in the cattle industry, *in vitro* development to the blastocyst stage of ICSI embryos (20–30%) is still lower compared to typical results from IVF [23]. Here, when valuable frozen semen was used for embryo production, ICSI increased the rate of normal fertilization by avoiding the drawback of non-fertilization and polyspermy in the

conventional IVF system. Additionally, subsequent *in vitro* development of ICSI embryos was higher than that using conventional IVF, indicating that ICSI can improve *in vitro* productivity of bovine embryos in field trials.

CONCLUSIONS

ICSI can clearly improve the success of *in vitro* procedures used on bovine embryos. Use of artificial activation, however, which is useful for effectively producing ICSI embryos, also has a drawback of producing parthenogenetic embryos; thus, this requires the establishment of ICSI conditions that form male/female haploid pronuclei at a high frequency.

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