Piezo-assisted Intracytoplasmic Sperm Injection in Cattle

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ABSTRACT

Intracytoplasmic sperm injection (ICSI) is one of the artificial fertilization methods when only a few sperm are available for insemination, and an important tool for the preservation of genetic materials of endangered animal species, especially the male is infertile. Different from other species such as mice and pigs, the conventional ICSI method which uses spiked pipette for injection (Spike-ICSI) is exhibited low success rates in cattle because the bovine sperm head membrane is hard to break during injection procedure. We chose piezo-assisted ICSI (Piezo-ICSI) for the improvement of the injection procedure including sperm head membrane rupture and efficient puncture of the plasma membrane of the oocytes. In this experiment, we compared the efficacy of the bovine ICSI embryo production between the Piezo-ICSI and Spike-ICSI. The second polar body extrusion, pronuclear formation, cleavage and blastocyst formation were evaluated after implementation of two different ICSI techniques. The Piezo-ICSI tended to show comparably higher rates of the second polar body extrusion (41.7%), the pronuclei formation (42.9%) and the two-cell cleavage (41.4%) than Spike-ICSI does (33.3%, 28.6% and 23.5%, respectively) although there is no statistic significance between two groups. In addition, the blastocysts were only obtained from the Piezo-ICSI group (10.3%). Our finding shows that the Piezo-ICSI may be used as an artificial fertilization method in cattle when *in vitro* fertilization is not applicable.

(Key words : intracytoplasmic sperm injection (ICSI), bovine, piezo-driven micromanipulation)

INTRODUCTION

The technique of intracytoplasmic sperm injection (ICSI), which consists of the direct microinjection of a single sperm into an oocyte in metaphase II, has expanded the possibilities of assisted fertilization techniques in Human (Palermo *et al.*, 1993). In domestic animal species, ICSI could be applied as an artificial reproduction option to solve various problems in reproduction. Different from human, however, it is less focused on low male fertility. The technique of ICSI in livestock can be employed as assisted reproduction for conservation of the endangered animal species and transgenic animal production.

In cattle, the first calf by ICSI was derived from the injection of the head of a sperm frozen without cryoprotectant (Goto *et al.*, 1990). Hamano *et al.* (1999) also produced healthy calves by ICSI although the rate of *in vitro* production of ICSI derived blastocysts was quite low. The main cause of the failure of ICSI in cattle seems to be related to the failures in pronuclear formation and ovum activation (Chen and Seidel, 1997). In general, introducing the sperm is sufficient for oocyte activation, induction of sperm head decondensation, female and male pronuclear formation and initiating subsequent embryonic development. In the bovine and porcine species, however, the conventional ICSI using the spiked injection pipette (Spike-ICSI) is not adequate for such events (Rho *et al.*, 1998; Tian *et al.*, 2006, respectively). In the ICSI procedure, the retention of acrosome or subacrosomal perinuclear theca seems to prevent decondensation of sperm chromatin in ooplasm (Sutovsky *et al.*, 1997; Katayama *et al.*, 2002). To support for the sperm chromatin decondensation, physical removal of the acrosomal membranes (Goto, 1993) or damaging the sperm membranes by freezing and thawing (Keefer *et al.*, 1990) methods are suggested. Permeabilization or removal of the sperm membranes may have a role in facilitating sperm chromatin decondensation, and it results in pronuclear formation after ICSI.

The use of an electric piezo-actuated micromanipulator for sperm injection (Piezo-ICSI) has resulted in higher rates of activation, fertilization and embryonic development after ICSI in horses (Choi *et al.*, 2002) and cattle (Galli *et al.*, 2003). This device causes vibration in the injection micropipette, fa-

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cilitating the penetration into the zona pellucida and facilitating the junction of the sperm and oocyte membranes. In horses, the application of the Piezo-ICSI increases the activation and the cleavage rates when *in vitro* matured oocytes are used (Choi *et al.*, 2002). However, the number of researches on Piezo-ICSI in cattle is still not very successful.

In the present study, two different ICSI techniques (Piezo-ICSI versus Spike-ICSI) are compared for the artificial fertilization in cattle.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were purchased from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated otherwise.

2. Oocyte Recovery and *In Vitro* Maturation (IVM)

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 2~3 h in saline at 25~35℃. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3- to 8-mm follicles using an 18-gauge hypodermic needle attached to a 5-ml disposable syringe. After washing three times in IVM medium, COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. Selected COCs were cultured in 4-well culture dishes (Nunc, Denmark) containing 500 μl of IVM medium under warmed and gas-equilibrated mineral oil for 20~22 h at 38.5℃, 5% CO_2, 5% O_2 and 90% N_2. The IVM medium for oocytes is composed of tissue culture medium 199 with Earle's salts and L-glutamine (TCM 199, Invitrogen, USA) supplemented with 10% FBS (Invitrogen), 10 μg/ml FSH-P (Folltropin-V, Veterpharm, UK), 0.2 mM sodium pyruvate, 1 μg/ml estradiol-17β and 10 ng/ml EGF.

3. Intracytoplasmic Sperm Injection (ICSI)

After maturation, the oocytes were denuded of cumulus cells by vortexing in the presence of 0.1 % hyaluronidase for 4 min and then were returned to maturation medium. The oocytes having a visible first polar body were selected for ICSI. Frozen semen of a Holstein was thawed in 35℃ water for 20 s and harvested sperm pellet using swim-up method followed by centrifugation of supernatants. The sperm pellet was re-suspended in 5 ml phosphate buffer saline (PBS, Invitrogen) supplemented with 0.1% polyvinylalcohol (PVA). The 1 μl of sperm suspension was diluted in a drop containing 10% polyvinylpyrrolidone (PVP), just before sperm microinjection. The oocytes was prepared in a separated drop containing 5 μg/ml cytochalasin B. For injection, a sperm was aspirated tail-first and injected into the ooplasm through the zona pellucida using a injection pipette of 5- to 8-μm diameter. In the drop containing PVP and sperm, the sperm were selected according to their progressive movement, the tail was cut with a circular movement of the injection pipette, and the immobilized sperm was sucked into the injection pipette. With the holding pipette, the oocyte was placed with its polar body to 6 or 12 O'clock position before sperm injection. When the Piezo-ICSI was performed, piezo pulses of speed 6 and intensity 3 of piezo-actuated micromanipulator (Primetech, Japan) was applied to the tip of the injection pipette for drilling of zona pellucida and pulses speed 1 and intensity 1 were applied for puncturing the plasma membrane of the oocyte. The image of injection pipettes for Spike-ICSI and Piezo-ICSI are shown in Fig. 1.

4. Oocyte Activation and *In Vitro* Culture (IVC)

Oocyte activation was performed after ICSI. The oocytes were incubated in CR2 medium (CR1aa; Rosenkrans and First, 1994) supplemented with 0.1% PVA and 10 μM Ca-ionophore for 5 min. Then, the oocytes were washed and transferred to CR2 medium with 0.3% BSA for 3 h to check second polar body extrusion. The oocytes having second polar body were selected and transferred to CR2 medium supplemented with 0.3% BSA and 2.5 mM 6-dimethylaminopurine (6-DMAP) for 3 h. After the treatment, the fertilized oocytes were transferred to IVC medium. To check male and female pronuclear formation, presumptive zygotes were stained by Hochest33342 after 3 h of culture. The rest of the zygotes were cultured in 20 μl drops of CR2 medium with 0.3% BSA for 3 days and

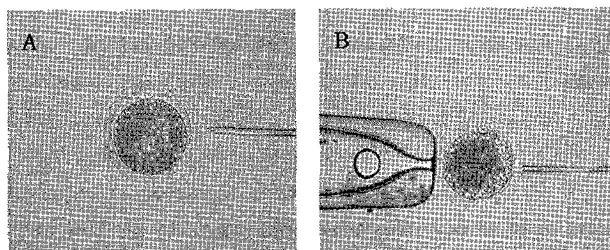

Fig. 1. Two type of ICSI methods. A. Spiked injection pipette for conventional ICSI (Spike-ICSI), B. Blunt injection pipette for ICSI using piezo-actuated micromanipulator (Piezo-ICSI).

then transferred to CR2 medium with 0.15 % BSA and 0.15 % FBS for 5 days at 39℃ in 5% CO_2, 5% O_2 and 90% N_2. The culture drops were covered by mineral oil and 10 to 15 embryos were placed in each drop.

The assessment of the outcome of Spike- and Piezo-ICSI was performed by the observation of second polar body extrusion and pronuclear formation after activation, cleavage rates on Day 3 and the formation of blastocysts on Day 8 of culture.

5. Statistical Analysis

Experiments were repeated three times, and the proportion of second polar body extrusion, pronuclear formation, cleavage and the blastocyst formation were evaluated by Student's *t*-test. Difference at $p<0.05$ was considered significant.

RESULTS

The Piezo-ICSI tended to show higher rates of the second polar body extrusion (41.7%), the pronuclear formation (42.9%) and the two-cell cleavage (41.4%) than the Spike-ICSI does (33.3%, 28.6% and 23.5%, respectively; Table 1 and 2) although there is no statistic significance between two groups due to small sample sizes. In addition, the blastocysts were only obtained from the Piezo-ICSI group (10.3%; Table 2 and Fig. 2).

DISCUSSION

The results of this study show that Piezo-ICSI in cattle is comparably efficient for the production of bovine ICSI blastocysts *in vitro*. The rate of blastocyst formation in our Piezo-ICSI experiment is also higher than the rates reported by Goto *et al.* (1990; 1.8%) and Hamano *et al.* (1999; 6.9%) by Spike-ICSI. Using Piezo-actuated micromanipulator may be one of the reasons for comparably higher blastocyst rate in our present report. This result is in agreement with the reports of Oikawa *et al.* (2005), using a piezo-electric actuator, the combination of immobilizing the motile spermatozoa just before injection was useful for the production of blastocysts following Piezo-ICSI. Katayose *et al.* (1999) also reported that the use of an electric piezo drill supports the fertilization success after ICSI remarkably when compared to the use of conventional Spike-ICSI in cattle.

Table 1. Comparison of the second polar body extrusion and pronuclear formation on Spike- and Piezo-ICSI

Group	No. of oocytes	No. (%) of 2nd polar body extrusion	Percentage (rate) of pronuclear formation
Spike-ICSI	24	8 (33.3)	28.6 (2/7)
Piezo-ICSI	36	15 (41.7)	42.9 (3/7)

Three replicates.

Table 2. Comparison of the cleavage rates and embryo development on Spike-and Piezo-ICSI

Group	No. of presumptive zygotes*	No. of cleavage (%)	No. of blastocysts (%)
Spike-ICSI	17	4 (23.5)	–
Piezo-ICSI	29	12 (41.4)	3 (10.3)

Three replicates.
*Only the oocytes having 2nd polar body after activation were strictly selected for the culture.

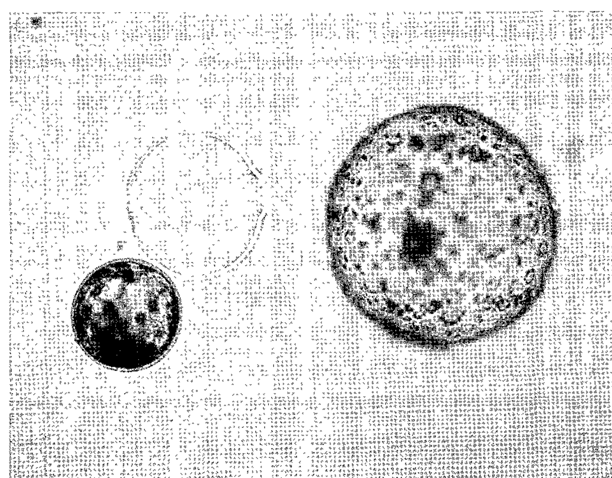

Fig. 2. Expanded and hatched blastocysts obtained from Piezo-ICSI.

The plasma membrane of the bovine oocytes is flexible and resistant to the spiked pipette and this makes difficult for the deposition of sperm inside the oocyte. During ICSI process, part of the oolemma is normally sucked into the micropipette because it is not easy to confirm the breaking of the plasma membrane. However, this procedure can cause lysis of the cells and subsequent degeneration of the oocytes, damaging the embryo (Keskintepe and Brackett, 2000). The use of the piezo-actuator drill facilitates small puncture on the plasma mem-

brane of the oocytes, leading to less damage during sperm injection. Different from *in vitro* fertilization (IVF), rupturing the sperm membranes before injection seems to be important in ICSI because this ensures the release of sperm cytosolic factors which is important in oocyte activation (Hinrichs *et al.*, 2005). In normal fertilization process including (IVF), acrosomal membranes are ruptured before the penetration and sperm plasma membrane is fused with the plasma membrane of the oocyte during the penetration process. In ICSI, however, sperm passed through all membranes of the oocyte aided by micropipette and is placed in the ooplasm. Hence mechanical removal of sperm membranes and inducing disclosure of cytosolic factors is important for the success of ICSI program. Sperm treatments prior to ICSI that may result damage to the sperm plasma membrane have been shown to affect the timing of onset of calcium oscillations, oocyte activation and pronuclear formation after ICSI (Yanagida *et al.*, 1997; Kasai *et al.*, 1999). Piezo pulse during injection process may affect not only puncturing the plasma membrane of the oocyte but also disrupting the membranes of the sperm in our experiment.

In conclusion, our data shows that the Piezo-ICSI in association with oocyte activation supports the blastocyst formation in cattle. The Piezo-ICSI can be used as an alternative assisted reproductive technology program when IVF is not applicable and/or not proper in cattle.

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