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The effects of electroporation on viability and quality of *in vivo*-derived bovine blastocysts

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Abstract. The introduction of exogenous molecules into embryos is required for analyses of molecular dynamics and specific gene functions during early embryonic development. Electroporation is an effective method to transport exogenous molecules into cells, but is rarely used in bovine embryos. First, we evaluated the viability of *in vivo*-derived bovine blastocysts after electroporation with fluorescein (FAM) labeled-oligonucleotides with varying pulse numbers (3, 5, 7, and 10), while keeping the pulse duration at 1 msec and the electric field of 20 V/mm. Next, we examined the effects of zona pellucida status on blastocyst quality after electroporation, by comparing the average diameter of blastocysts before and after electroporation using blastocysts with intact zona pellucida and hatching/hatched blastocysts. Electroporation successfully introduced exogenous molecules into *in vivo*-derived bovine blastocysts without loss of viability. Moreover, the status of the zona pellucida may be associated with the quality of blastocysts after electroporation.
Key words: Bovine, Blastocyst, Electroporation, *In vivo*, Zona pellucida

Dramatic biological changes during embryonic development, such as gene expression with major embryonic genome activation [1] and epigenetic modifications with DNA methylation and histone modifications [2], have been analyzed in mammals, including cows. In bovine embryos, the methylation status and expression of specific genes in *in vitro*-developed blastocysts have been studied but suggested to be different from those in *in vivo*-derived blastocysts [3]. Observing *in vivo*-derived embryos is required to understand the precise mechanisms of biological changes during early embryonic development. Efficient introduction of exogenous molecules into embryos, such as small interference RNAs (siRNA) for RNA interference [4], morpholinos for disruption of targeted gene function [5], and molecular beacons for imaging specific endogenous RNAs in living cells [6], is required to investigate molecular dynamics and specific gene functions during early embryonic development.

Microinjection is a well-established technique for introducing exogenous molecules into embryos. However, microinjection requires expensive equipment and sophisticated techniques, and the direct insertion and retraction of the needle cause physical stress and mechanical damage to embryos [7–9]. Furthermore, the application of microinjection is generally limited to one- to two-cell stage zygotes/embryos. Electroporation is another effective and widely used method

for transporting exogenous molecules into cells [10, 11], and has the potential to be a core technique for efficient introduction of molecules into morula/blastocyst stage embryos, since this is difficult to achieve via microinjection. Recently, electroporation has been applied to introduce exogenous molecules into the zygotes/embryos of mice [12, 13], rats [14], and pigs [15], but it is still rarely used in cows. The effects of electroporation-induced damage on the viability of bovine embryos are also unknown.

In this study, we evaluated the viability of *in-vivo* derived bovine blastocysts after electroporation under different electrical conditions. We used fluorescein (FAM) labeled-oligonucleotides to confirm the successful introduction of molecules. Furthermore, we examined the effects of zona pellucida status on the quality of blastocysts after electroporation by comparing the diameters of blastocysts before and after electroporation.

First, *in vivo*-derived blastocysts were electroporated with varying numbers of unidirectional pulses (3, 5, 7, and 10), while maintaining the pulse duration at 1 msec and the electric field of 20 V/mm. A total of 10 blastocysts with intact zona pellucida were electroporated for each pulse number group. After culturing for 24 h, blastocyst viability was assessed microscopically by observation of blastocoel re-expansion. All blastocysts shrank immediately after electroporation in each experimental group but re-expanded after incubation for 24 h (Fig. 1). The fluorescence from the introduced FAM labeled-oligonucleotide was observed in each experimental group immediately after electroporation (Fig. 2). The blastocyst incubated with FAM labeled-oligonucleotide for 5 min without electroporation and the blastocyst electroporated without FAM labeled-oligonucleotide served as negative controls (Fig. 2).

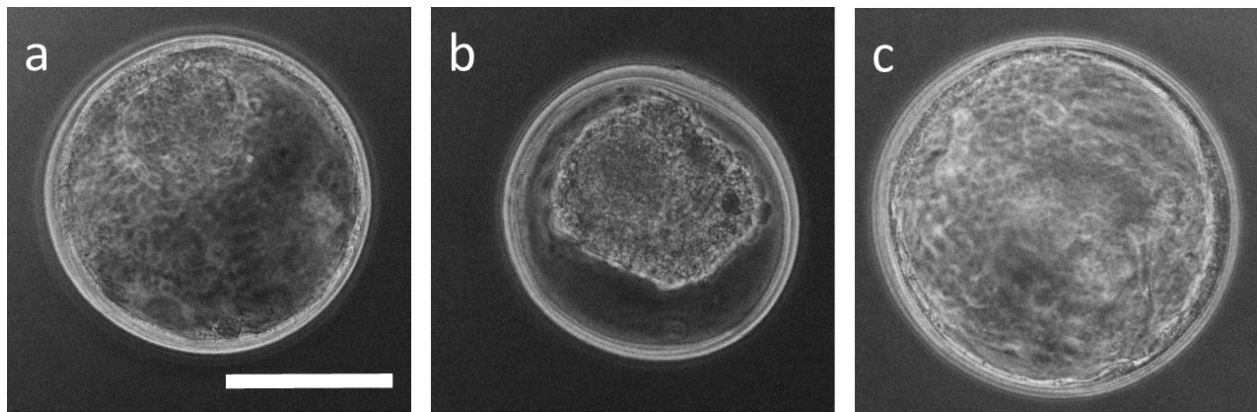Next, we performed electroporation (five 1-msec unidirectional

Fig. 1. Representative images of bovine blastocysts. (a) Expanded blastocyst before electroporation. (b) Shrunk blastocyst immediately after electroporation. (c) Re-expanded blastocyst 24 h after electroporation. Scale bar indicates 100 μm.
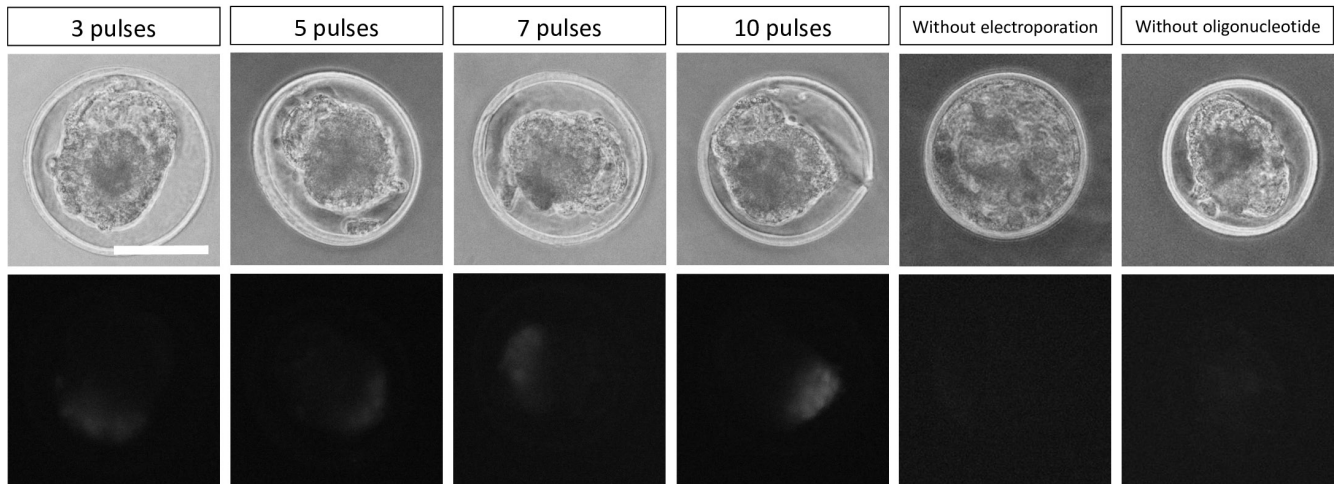

Fig. 2. Representative images of bovine blastocysts after introduction of FAM labeled-oligonucleotide by electroporation with three to ten pulses. Without electroporation: Blastocyst incubated with FAM labeled-oligonucleotide without electroporation as a negative control. Without oligonucleotide: Blastocyst after electroporation without FAM labeled-oligonucleotide as a negative control. Scale bar indicates 100 μm.

pulses at an electric field of 20 V/mm) to blastocysts with intact zona pellucida, and to blastocysts with partly cracked zona pellucida/without zona pellucida (hatching/hatched blastocysts). The diameters of blastocysts were measured using ImageJ software (version 1.52a; National Institutes of Health, Bethesda, MD, USA) for both groups before electroporation and 24 h after electroporation to evaluate the effects of zona pellucida status on the re-expansion of blastocysts. The diameter of each blastocyst was defined as the average of vertical diameter and horizontal diameter excluding the zona pellucida. The average diameter is expressed as mean ± SEM. The average diameter of blastocysts with intact zona pellucida was comparable before (170.8 ± 4.3 μm) and after electroporation (178.7 ± 7.3 μm). However, the average diameter of hatching/hatched blastocysts after electroporation (136.5 ± 9.2 μm) was significantly lower ($P < 0.01$) than that before electroporation (218.7 ± 10.5 μm) (Fig. 3).

We evaluated the viability of electroporated *in vivo*-derived bovine blastocysts by analyzing re-expansion of the blastocoel. In this study, the electroporation conditions were three to ten 1-msec pulses at an electric field of 20 V/mm. Regardless of the pulse number, all blastocysts with intact zona pellucida re-expanded after electroporation. In mouse *in vivo*-derived zygotes electroporated by the same electroporation system used in this study, three to seven 3-msec pulses at an electric field of 30 V/mm were the optimal conditions under which embryo viability was not significantly affected [16]. These results indicate that i*n vivo*-derived bovine blastocysts may be able to tolerate severe electroporation conditions comparable to the optimal electroporation conditions for mouse zygotes. However, we previously demonstrated that the resistance to electroporation of 1-cell *in vitro*-derived zygotes in pigs was lower than that in mice [15]. The resistance to electroporation is presumed to be different
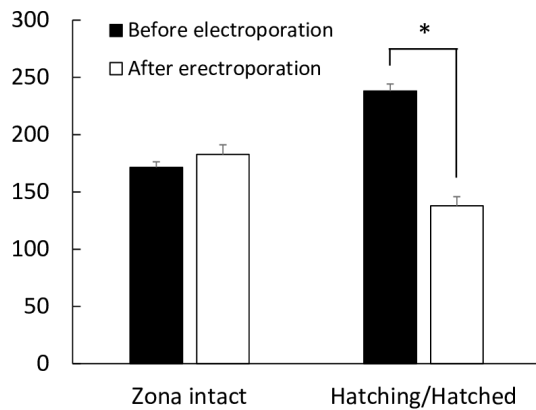
Fig. 3. The average diameter of blastocysts with intact zona pellucida (n = 12) and of hatching/hatched blastocysts (n = 9) before and 24 h after electroporation. * P < 0.01. Error bar, mean ± SEM.

among animal species. Moreover, *in vivo*-derived embryos have morphological differences with *in vitro*-produced embryos [17]. It has been reported that *in vitro*-produced embryos are more adversely affected by chilling than *in vivo*-derived embryos [17]. The sensitivity to cooling and freezing is presumed to be related to the relatively higher lipid content often observed in *in vitro*-produced embryos [17, 18]. These factors may affect the resistance to electroporation.

Electrofusion of two-cell stage embryos has been reported to be effective for the production of tetraploid blastocysts [19]. Electroporation of bovine embryos after the two-cell stage has the possibility to cause cell fusion. In this study, we did not evaluate cell fusion and polyploidy after electroporation of blastocysts. In general, electrofusion of blastomeres is performed by a single 30–250 μsec pulse at an electric field of 0.50–2.0 kV/cm [19–21]. In this study, our electroporation condition, 1-msec pulses at an electric field of 20 V/mm, was longer duration and lower voltage compared to general conditions of electrofusion. The effects of multiple pulses on electrofusion also have not been investigated well. The possibility of electrofusion of two-cell- to blastocyst stage-embryos producing zygotes/embryos during electroporation with multiple pulses with long duration and low voltage requires further investigation.

In this study, the introduction of FAM labeled-oligonucleotide into the blastocyst was achieved. However, a non-homogeneous fluorescence pattern was observed, indicating that some embryonic cells failed to take up the exogenous molecules by electroporation. This non-homogeneous fluorescence pattern is probably caused by the unidirectional electric pulse used in this study, since a bidirectional electric pulse for electroporation is presumed to improve the introduction of exogenous molecules. However, further studies are required because we previously found that the bipolar pulse affects blastocyst formation in porcine *in vitro*-fertilized zygotes [22]. Recently, the introduction of siRNA into 8–16-cell stage bovine embryos was achieved by the lipofection method after removal of the zone pellucida [23]. The combination of these technologies can be an effective strategy for improving introduction of exogenous molecules into blastocysts. Moreover, cell type is also a possible

factor affecting the fluorescence pattern introduced by electroporation. The optimal electroporation conditions for introducing exogenous molecules into cells are specific to cell type [24]. However, to our knowledge, optimal electroporation conditions for cells composing blastocysts, cells of inner cell mass and trophectoderm, have not been investigated. Therefore, further investigations are required.

Next, we demonstrated that the blastocysts with intact zona pellucida were expanded 24 h after electroporation, of which the average diameter was comparable to that before electroporation. On the other hand, the hatching/hatched blastocysts were shrunk by electroporation and failed to expand to the same degree with before electroporation. These results indicate that the cracking/ existence of the zona pellucida during electroporation affects the quality of blastocysts. In mice, the existence of zona pellucida is one of the factors affecting the viability of electroporated embryos [12]. Therefore, performing electroporation before hatching of zona pellucida can minimize electroporation-induced damages in bovine embryos. Other factors that may affect the viability of embryos after electroporation are the thickness of the zona pellucida and developmental stage of the embryos. The thickness of the zona pellucida is reduced by expansion of the blastocyst. A previous study in mouse embryos demonstrated that the thinner the zona pellucida, the more easily a macromolecule can be introduced into embryos under identical electric pulses, although embryo viability decreases after electroporation [13]. Moreover, our previous study using *in vitro*-derived porcine 1-cell zygotes showed that sensitivity to electroporation is affected by the zygote stage [15]. The developmental stage of bovine embryos could also affect their viability after electroporation. To our knowledge, the viability of embryos of various developmental stages after electroporation has not been investigated in cows. All these factors should be considered in order to maintain the viability of embryos after electroporation procedures.

In conclusion, electroporation successfully introduced exogenous molecules into *in vivo*-derived bovine blastocysts without loss of viability. Moreover, we demonstrated that the status of the zona pellucida may be associated with the quality of blastocysts after electroporation in *in vivo*-derived bovine blastocysts. To date, there are few studies of electroporation of blastocysts in domestic animals. Additional studies are warranted to identify the factors that affect the outcome of electroporation in various animal species including cows, to further improve this technique for efficient introduction of molecules into embryos.

Methods

Animals

All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Tokushima University. This study was approved by the Institutional Animal Care and Use Committee of Tokushima University (approval number: T2019-24).

Blastocyst collection

Holstein cows (n = 5), 4–8 years of age, were superovulated with follicle-stimulating hormone (FSH, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 30 A.U. decreasing over 5 days (6, 6/4, 4/3, 3/2, 2). A controlled intravaginal drug releasing device (CIDR; Eazibreed®;

Livestock Improvement Association of Japan, Tokyo, Japan) was inserted into the vagina of each cow with intramuscular injection of 2 mg estradiol-benzoate (Ovahormon®; ASUKA Animal Health, Tokyo, Japan) 96 h before the first FSH injection. Luteolysis was induced with one injection of 0.75 mg prostaglandin (PG) F2α analogue (Cloprostenol; Estrumate®, Intervet, Tokyo, Japan) given at the time of the seventh FSH injection. The CIDR was withdrawn at the eighth FSH injection and 200 μg gonadotropin-releasing hormone (GnRH) analogue (Fertirelin acetate; Spornen®, Kyoritsu Seiyaku) was injected 24 h after CIDR withdrawal. Cows were inseminated twice with 0.5 ml frozen-thawed semen (1×10^7 cells/ml), about 12 and 24 h after the Fertirelin acetate injection. Embryos were collected non-surgically 8 days after the first insemination, according to procedures described previously [25]. The embryos were examined microscopically for morphological quality and developmental stage as previously described [26]. The embryos were graded for quality: Grade A = excellent, Grade B = good, Grade C = fair, Grade D = poor, and Grade E = degenerate. Only Grade A embryos (n = 61) were used in this study. The embryos were placed in Dulbecco's phosphate-buffered saline (DPBS) with magnesium and calcium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), and were transported within 2 h to the laboratory at 37.5ºC.

Incubation of blastocysts

The blastocysts were washed with culture medium consisting of tissue culture medium 199 with Earle's salts (TCM 199; Gibco/Invitrogen) supplemented with 20% FBS (Thermo Fisher Scientific), 50 μM chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), 1 × insulin-transferrin-selenium supplement (ITS, Sigma-Aldrich) and 50 μg/ml gentamicin (Sigma-Aldrich). The blastocysts were subsequently incubated individually in 40 μl of culture medium under a layer of mineral oil in an ART Culture Dish 25 (NIPRO, Osaka, Japan) until electroporation and for 24 h after electroporation. The incubation of the blastocysts was conducted at 38.5ºC in a humidified incubator containing 5% CO_2 and 5% O_2

Electroporation

Electroporation was performed as described previously [15]. Briefly, the electrode (LF501PT1-20; BEX, Tokyo, Japan) was connected to a CUY21EDIT II electroporator (BEX) and set under a stereoscopic microscope. The blastocysts were washed with Opti-MEM I solution (Gibco/Invitrogen) and placed in a line in the electrode gap, in a chamber slide filled with 10 μl Opti-MEM I solution containing oligonucleotide labeled with FAM at the 5' and 3' ends, synthesized by Eurofins Genomics KK (Tokyo, Japan): 5'-[FAM]-ACGACGCGACTAGCGCACCGATACGTCGT-[FAM]-3'. The sequence of the oligonucleotide was confirmed by database analysis to have no homology with any bovine mRNA.

Statistical analysis

The diameters of blastocyst before and after electroporation were evaluated using a paired t-test for each group. The analysis was performed in StatView (Abacus Concepts, Berkeley, CA, USA). P < 0.05 was considered statistically significant.

Acknowledgments

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