FULL PAPER

Biochemistry

Introduction of a plasmid and a protein into bovine and swine cells by water-in-oil droplet electroporation

**Takeshi ISHINO[1), Hirofumi KURITA[2), Rikio KIRISAWA[3), Yoshinori SHIMAMOTO[4),
Rika NUMANO[2) and Hiroshi KITAMURA[1)***

[1)Laboratory of Veterinary Physiology, Departments of Veterinary Medicine, School of Veterinary Medicine,
Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[2)Department of Applied Chemistry and Life Sciences, Graduate School of Engineering, Toyohashi University of
Technology, Toyohashi, Aichi 441-8580, Japan
[3)Laboratory of Veterinary Virology, Departments of Veterinary Medicine, School of Veterinary Medicine,
Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[4)Laboratory of Animal Therapeutics, Department of Veterinary Science, School of Veterinary Medicine,
Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

ABSTRACT. Instrument cost is a major problem for the transduction of DNA fragments and proteins into cells. Water-in-oil droplet electroporation (droplet-EP) was recently invented as a low-cost and effective method for the transfection of plasmids into cultured human cells. We here applied droplet-EP to livestock animal cells. Although it is difficult to transfect plasmids into bovine fibroblasts using conventional lipofection methods, droplet-EP enabled us to introduce an enhanced green fluorescent protein (EGFP)-expressing plasmid into bovine earlobe fibroblasts. The optimal transfection condition was 3.0 kV, which allowed 19.1% of the cells to be transfected. For swine earlobe fibroblasts, the maximum transfection efficacy was 14.0% at 4.0 kV. After transfection with droplet-EP, 69.1% of bovine and 76.5% of swine cells were viable. Furthermore, droplet-EP successfully transduced *Escherichia coli* recombinant EGFP into frozen-thawed bovine sperm at 1.5 kV. Flow cytometry analysis revealed that 71.5% of spermatozoa exhibited green fluorescence after transfection. Overall, droplet-EP is suitable for the transfection of plasmids and proteins into cultured livestock animal cells.

KEY WORDS: bovine cell, electroporation, sperm, swine cell

Transfection is a non-viral procedure for the delivery of exogenous nucleic acids and proteins into cultured cells or individuals to potentiate or disrupt cellular functions [3, 22]. Numerous studies reported various transfection procedures, such as the phosphate calcium method [21], diethylaminoethyl-dextran method [31], and lipofection [11]. Although several reports have demonstrated that lipofection successfully transfected plasmids to bovine or swine-derived cultured cells [20, 34], we and other investigators found that transfection efficiency by lipofection was markedly lower in bovine fibroblasts compared with that observed in human or mouse fibroblasts [19, 20, 47]. It is therefore necessary to establish efficient transfection methods for livestock animals.

Electroporation (EP) is another popular transfection method, rendering the plasma membrane transiently permeable under a strong electric pulse. This effect results in the transfer of membrane-impermeant substances into cells [35]. EP offers several advantages (such as versatile application, low cytotoxicity, and simple operation) over other transfection methods [14, 29]. Moreover, EP is applicable to the transfection of various materials (e.g., DNA and RNA fragments, plasmids, and recombinant proteins) using a single apparatus [9, 13, 35]. On the other hand, the high cost of the apparatus is a major problem hindering the widespread use of this method. In 2019, the cost of most commercially available electroporators was >10,000 USD.

Droplet-EP is a novel EP approach involving an entirely different mechanism from conventional EP [24, 25]. In this method, the cells are encapsulated in a medium droplet. The droplet is then floated in silicone oil, and charged by applying a direct current (DC) electric field to create pores on the plasma membrane. The cost of a droplet-EP apparatus (DC power source) is approximately one-tenth to one-fifth of the cost of a commercially available electroporator. It is of note that droplet-EP has been shown to effectively introduce plasmids into the human embryonic kidney cell line HEK293 [24, 25]. However, thus far the applicability of droplet-EP to livestock cells has not been evaluated. In this study, we attempted to transfect a plasmid and a

recombinant protein to bovine and swine cells by using droplet-EP.

MATERIALS AND METHODS

Bovine ear-derived fibroblasts and swine ear-derived fibroblasts

Bovine earlobe fibroblasts have been previously reported [19]. Pieces of swine earlobes were obtained from the Rakuno Gakuen Field Education Center (Ebetsu, Japan) when pigs (aged 3 days) were ear-notched. The swine earlobe tissue blocks were cut into fragments, and vigorously washed with phosphate-buffered saline (PBS) containing 200 U/ml penicillin, 200 μg/ml streptomycin, and 500 ng/ml amphotericin B (Nacalai Tesque, Kyoto, Japan). Subsequently, the tissues were treated with 2 mg/ml collagenase type I (Wako Pure Chemical, Osaka, Japan) in Tyrode's solution and cut into small pieces using scissors. The tissue slices were further digested for 30 min at 37°C with strong agitation. After extensive washing with PBS and centrifugation, the pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 200 U/ml penicillin, 200 μg/ml streptomycin, and 500 ng/ml amphotericin, and filtrated with a cell strainer (70 μm; Corning, New York, NY, USA) to remove debris. The cells were cultured in DMEM supplemented with 10% FCS at 37°C, 5% carbon dioxide, and 95% humidity.

Enhanced green fluorescent protein (EGFP)-encoding plasmid

The pEGFP-N2 plasmid was purchased from Takara Bio (Kusatsu, Japan). The plasmid was prepared from *Escherichia coli* (*E. coli*) DH5α using a FavorPrep Plasmid DNA Extraction Midi Kit (Favorgen, Ping-Tung, Taiwan).

Water-in-oil droplet EP of EGFP-encoding plasmid to bovine or swine fibroblasts

Droplet-EP was performed as previously reported with some modifications [24, 25]. One well of a 24-well cell culture plate was layered with 1 ml silicone oil (KF96-1; Shin-Etsu Chemical, Tokyo, Japan) on 1.5 ml of fluorocarbon oil (Fluorinert FC-96; 3M, Maplewood, MN, USA). The anode and cathode were set at a 6 mm interval. The parameters for each experiment are shown in Table 1. Bovine and swine fibroblasts were detached by treatment with trypsin, and washed with DMEM supplemented with 10% FCS twice. After counting cell number, the cell were resuspended in DMEM without FCS. To date, we employed DMEM as a vehicle of droplet-EP when we collected basic transfection data of mouse and human cells. Therefore, we used DMEM for transfection of the plasmid to bovine or swine fibroblasts. A droplet including the cells and transfected materials was gently added into the silicone oil phase in the well. Through a strong charge applied using a DC High Voltage power supply (HAR-30R10; Matsusada Precision, Kusatsu, Japan), the droplet was elongated and attached to both electrodes, resulting in the generation of a short circuit. Subsequently, the droplet was retrieved and transferred to the culture medium.

Microscopic observation

Fibroblasts seeded on a collagen-coated cover glass slide were fixed with 4% paraformaldehyde for 10 min at room temperature. After a thorough wash with PBS, the cells were stained with Hoechst33342 (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at room temperature. The cover glass was embedded on the cell-seeded glass slide with 50% glycerol, and the cells were observed using a C2 confocal microscope (Nikon, Tokyo, Japan). Scanned images were analyzed using ImageJ software [42]. Transfection efficiency was determined as the ratio of the number of EGFP-positive cells to Hoechst33342-positive cells.

Cell viability

Cell viability was evaluated immediately after droplet-EP or conventional EP. Dead cells were stained with 0.5% trypan blue (Nacalai Tesque). Cell viability was calculated as the ratio of the number of non-stained cells to total cells.

Transfection of EGFP-encoding plasmid to bovine or swine fibroblasts using NEPA21

As a reference of gene transfection, we performed transfection of the *EGFP*-encoding plasmid using a NEPA21 electroporator (Nepagene, Chiba, Japan) according to the protocol provided by the manufacturer. Briefly, 1.0×10^6 trypsinized bovine or swine fibroblasts and 10 μg of pEGFP-N2 plasmid in 100 μl of DMEM (Thermo Fisher Scientific) were placed in a 2 mm-gap cuvette (Nepagene) and pulsed using NEPA21. The parameters for the transfection pulse were as follows: poring pulse voltage: 275 V; pulse length: 2.5 msec; pulse interval: 50 msec; number of pulses: 2; decay rate: 10%; polarity +, and transfer pulse voltage: 20 V; pulse length: 50 msec; pulse interval: 50 msec; number of pulses: 5; decay rate: 40%; and polarity +/−. The cells were then rapidly transferred to a cell culture plate filled with DMEM containing 10% FCS.

Table 1. Transfection condition

Transfected cell	# of Cell ($\times 10^5$)	Plasmid/Protein	Size of plasmid/protein	Amount of plasmid/ protein (pmol)	Solvent	Droplet volume (μl)
Bovine fibroblast	1.0	pEGFP-N2	4.7 kb	0.121	DMEM	3.0
Swine fibroblast	1.0	pEGFP-N2	4.7 kb	0.121	DMEM	3.0
Murine sperm	5.0	EGFP protein	27 kDa	0.605	Opti-MEM®	10.0
Bovine sperm	5.0	EGFP protein	27 kDa	0.605	Opti-MEM®	10.0

DMEM, Dulbecco's Modified Eagle Medium; EGFP, enhanced green fluorescent protein.

Transfection of EGFP plasmid to bovine fibroblasts by lipofection

Bovine earlobe fibroblasts were transfected with pEGFP-N2 using Lipofectamine 2000 (Thermo Fisher Scientific), Lipofectamine LTX (Thermo Fisher Scientific), Fugene 6 (Promega, Madison, WI, USA), or Fugene HD (Promega) according to the instructions provided by the manufacturer. Briefly, 0.2, 0.1, 0.1, and 0.1 μg of the *EGFP*-encoding plasmid was encapsulated with Lipofectamine 2000, Lipofectamine LTX, Fugene 6, and Fugene HD, respectively. The liposome-and DNA complexes were subsequently supplemented to 1×10^4 adherent bovine fibroblasts. After 4 hr incubation, the medium was exchanged to DMEM supplemented with 10% FCS. The cells were observed using a fluorescence inverted microscopy IX71 (Olympus, Tokyo, Japan) with a cooled CCD camera DP73 (Olympus) at 24 hr after the transfection.

Preparation of recombinant EGFP

Recombinant EGFP was prepared as previously described [48]. The pET-His6-GFP-TEV-LIC plasmid was obtained from Addgene (Cambridge, MA, USA). The plasmid was introduced to *E. coli* BL21 (DE3) (BioDynamics Laboratory, Tokyo, Japan). The plasmid-introduced *E. coli* clone was treated with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Nacalai Tesque) for 3 hr. After centrifugation at 3,000 ×g for 20 min, the bacterial pellet was lysed with 20% Triton X-100 on ice, followed by sonication using a VP-050 sonicator (Taitec, Koshigaya, Japan). After centrifugation at 13,000 × g for 30 min at 4°C, the supernatant was collected. For the purification of EGFP, HisPur Cobalt Resin (Thermo Fisher Scientific) was used according to the instructions provided by the manufacturer.

Droplet-EP of recombinant EGFP protein to mouse or bovine sperm

Mature (aged 12–15 weeks) male C57BL/6J mice (SLC, Hamamatsu, Japan) were sacrificed through cervical dislocation under anesthesia with 100 mg/kg of sodium pentobarbital. The sperm of mice was collected from the cauda epididymides. Frozen bovine sperm in straws was kindly gifted by Genetics Hokkaido Association (Sapporo, Japan). To recover sperm activity, both mouse and bovine sperm in Opti-MEM (200 μl), covered with liquid paraffin (Wako Pure Chemical), was pre-incubated at 37°C for 1.5 hr [45]. After centrifugation at 1,000 × g for 5 min, the sperm was resuspended in a drop of 10 μl Opti-MEM. To minimize lot difference, we selected Opti-MEM for a pre-conditioning and transfection medium. A droplet (3 or 10 μl) including the sperm and EGFP was gently floated in the silicone oil layer in the well. Immediately after droplet-EP was performed, the droplet was retrieved. The parameters for droplet-EP of EGFP protein to the sperm are listed in Table 1. The procedures of the animal experiments were approved by the Animal Ethics Committees of Rakuno Gakuen University (Permit Number: VH19A8).

Bovine sperm viability

Viability of bovine sperm was determined by the eosin-nigrosin staining method [4]. Briefly, the sperm was stained with 0.5% eosin Y (Muto Pure Chemical, Tokyo, Japan) for 15 sec, and subsequently stained with 0.5% nigrosin (Wako Pure Chemical) for 15 sec. The sperm was smeared on a glass slide and then air-dried. Since dead sperm was dyed red, sperm viability was calculated as the ratio of the number of non-stained cells to total cells. Viability was determined immediately after transfection.

Flow cytometry analysis

The transfected sperm was analyzed by flow cytometry to evaluate the efficacy of droplet-EP for the transfection of EGFP into sperm. The sperm was fixed in 4% paraformaldehyde for 10 min at room temperature. After washing twice with PBS, they were analyzed using a FACS Verse system (BD Biosciences, San Jose, CA, USA). The data were further analyzed using Flow Jo 10 (FlowJo, Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed via one-way ANOVA corrected for multiple comparisons with the Holm-Bonferroni method, using Kaleida Graph software (Hulinks, Tokyo, Japan).

RESULTS

Droplet-EP efficiently transfects EGFP plasmid DNA into bovine fibroblasts

We previously reported that the transfection efficiency of plasmids into bovine fibroblasts was extremely low using commercially available lipofection reagents [19]. Hence, we initially attempted to transfect a mammalian expression plasmid into bovine fibroblasts using the droplet-EP technique. As previously reported [24, 25], we floated a droplet including the cells and plasmids in silicone oil (Fig. 1). Under the lower current, the droplet frequently moved back and forth between the electrodes. An increase in the electric field caused morphological changes to the droplet (flattening). Eventually the droplet attached to both electrodes and created a short circuit. For the transfection into bovine fibroblasts, we encapsulated 1.0×10^5 cells and 0.12 pmol plasmid in a 3.0 μl droplet, and applied a voltage of 2.0, 3.0, 3.5, or 4.0 kV. Previously, we transfected plasmids into human embryonic kidney cells using droplet-EP [25]. In this series, we found that droplet size is negatively correlated with transfection efficiency when voltage, cell number, and DNA amount were constant. Since 2–3 μl droplet was most effective for transfection of plasmids into 1.0×10^5 of the cells, we first tried to transfect the *EGFP*-encoding plasmid using a 3.0 μl droplet. Electric current <2.0 kV failed to generate a short circuit, and did not result in transfection (Fig. 2A). In contrast, 3.0 kV caused a short circuit, followed by the expression of EGFP in 27.3 ± 9.7% of live cells (Fig. 2A and 2B). Both 3.5 and 4.0 kV transduced the plasmid into the cells to a significant

degree, despite the inversely proportional relationship between the transfection efficiency and voltage. Notably, cell viability was also inversely proportional to the voltage (Fig. 2C). Cell viability was 82.2 ± 2.5, 69.0 ± 4.1, 57.9 ± 0.9, and $49.4 \pm 1.8\%$ when the cells were subjected to droplet-EP at 2.0, 3.0, 3.5, and 4.0 kV, respectively. In a further step, we multiplied the efficiency and
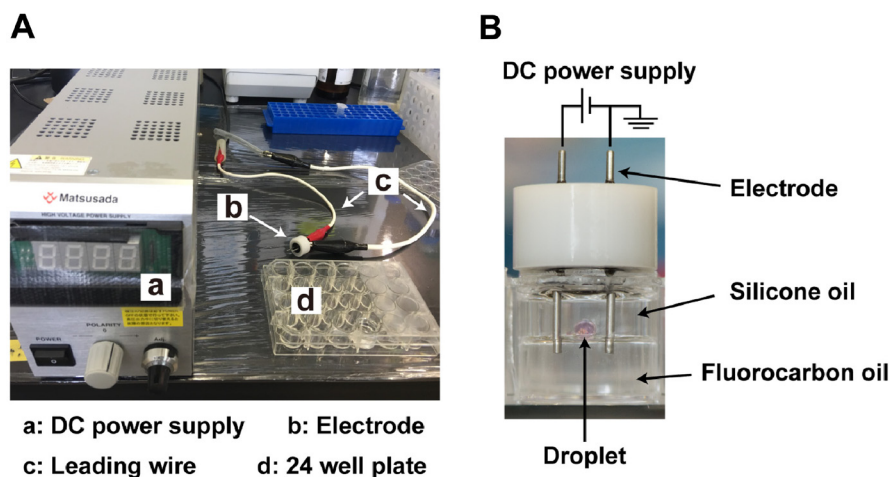

a: DC power supply b: Electrode
c: Leading wire d: 24 well plate

Fig. 1. Configuration of a droplet-electroporation (EP) apparatus. (A) Positive and negative electrodes were connected to a high voltage direct current (DC) power supply. (B) A medium droplet containing cells and plasmids or recombinant proteins was floated in silicone oil and placed between the electrodes.
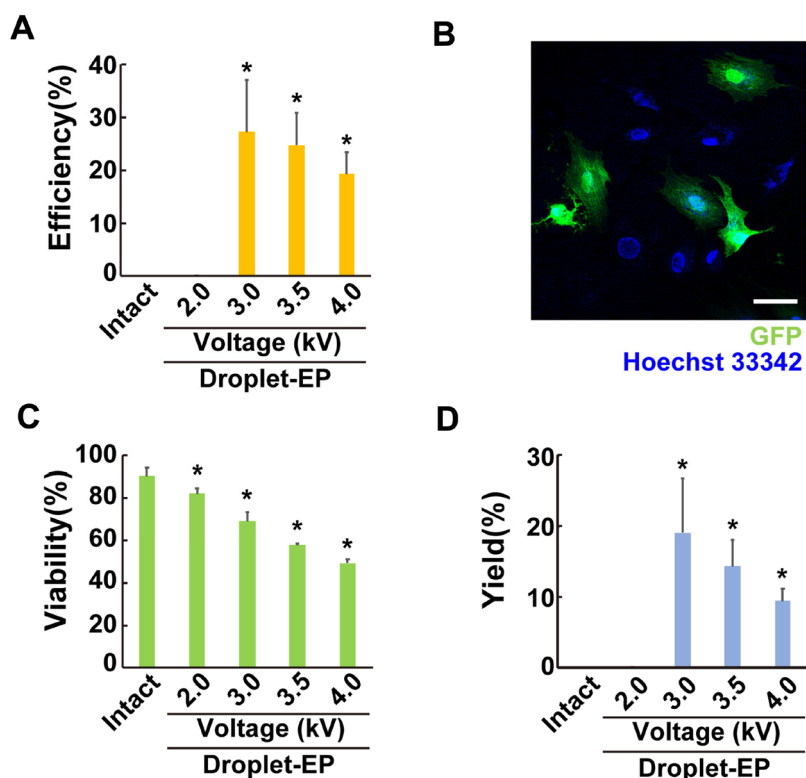

Fig. 2. Efficiency, viability, and yields of transfection of the pEGFP-N2 plasmid into bovine fibroblasts by droplet-EP. A total of 4×10^5 bovine earlobe fibroblasts were subjected to droplet- electroporation (EP) at the following settings: 2.0, 3.0, 3.5, or 4.0 kV. Cell viability was determined immediately after charging the cells through droplet-EP. Subsequently, the transfected cells were cultured for 24 hr. After staining the nuclei with Hoechst33342, the transfection efficiency was determined using a laser-scanning microscope. (A) Transfection efficiency was determined based on the proportion of enhanced green fluorescent protein (EGFP)-expressing cells in Hoechst33342-positive cells. (B) A representative image of EGFP-transfected cells. Scale bar=50 μm. (C) Cell viability was determined using the trypan blue staining method. (D) The yields of transfections were determined by multiplying the efficiency and cell viability obtained at each voltage. "Intact" indicates that droplet-EP was not performed. Values are means ± one standard deviation over three wells. *$P<0.05$ vs. intact cells.

cell viability at each voltage to more precisely determine the effectiveness of droplet-EP. These indices, namely transfection yield, resulted in transfection at each setting. As shown in Fig. 2D, 3.0 kV resulted in the greatest yield (19.1 ± 7.9%, n=3), followed by 3.5 kV and 4.0 kV (14.4 ± 3.7% and 9.5 ± 1.8%, respectively). On the other hand, transfection yield and cell viability of EP using the commercially available electroporator NEPA21 at the optimal setting was 40.2 ± 5.0 and 76.9 ± 2.6%, respectively. We also transfected the *EGFP*-encoding plasmid to the bovine fibroblasts by lipofection. Neither Lipofectamine 2000, Lipofectamine LTX, Fugene 6, nor Fugene HD effectively transfected the plasmid to the cells: Transfection efficacies of Lipofectamine 2000, Lipofectamine LTX, Fugene 6, and Fugene HD were 0.72 ± 0.87% (n=5), 0.00 ± 0.00% (n=5), 0.16 ± 0.38% (n=5), and 1.63 ± 0.79% (n=5), respectively. Collectively, these results indicate that the optimal condition of droplet-EP effectively transfects plasmid DNA into bovine fibroblasts at a comparable level with that reported using a commercially available electroporator.

Droplet-EP efficiently transfects EGFP plasmid DNA into swine fibroblasts

We subsequently attempted to transfect plasmids into swine cells. For this purpose, we isolated fibroblasts from pieces of swine earlobes using a method previously described [1]. Morphologically, swine earlobe fibroblasts were flatter than bovine earlobe fibroblasts, and were proliferative for ≥20 passages (Fig. 3A). In accordance with bovine fibroblasts, swine fibroblasts were not transfected with the pEGFP-N2 plasmid when droplet-EP was performed at the 2.0 kV setting (Fig. 3B). On the other hand, transfection efficiency was increased in a directly proportional manner to the voltage at ≥4.0 kV. At 4.0 kV, 18.3% of cells were transfected with the plasmid. Even at this intense voltage setting, >75% cells were alive, although cell viability was slightly higher at lower voltage settings (Fig. 3C; 90.5 ± 2.3%, 87.9 ± 1.3%, and 82.6 ± 2.2% at the 2.0, 3.0, and 3.5 kV settings, respectively). Given that viability of bovine earlobe fibroblasts was ~50% at the 4.0 kV setting, swine earlobe fibroblasts were resistant to relatively higher voltage. Figure 3D exhibits the yields of transfection to swine earlobe fibroblasts. The 4.0 kV setting resulted in the highest yield (14.0 ± 5.9%), followed by the 3.0 and 3.5 kV settings (11.3 ± 6.7% and 12.7 ± 6.2%, respectively). The transfection yields of the NEPA21 at the optimal condition were ~3-fold higher (40.6 ± 9.0%) than those obtained with droplet-EP. Meanwhile, cell viability (78.3 ± 3.8%) after EP using a NEPA21 was slightly less than that of droplet-EP at 4.0 kV setting. These results show that droplet-EP sufficiently transfected a plasmid into swine fibroblasts but that transfection efficacy was lower than that obtained with the commercially available electroporator.
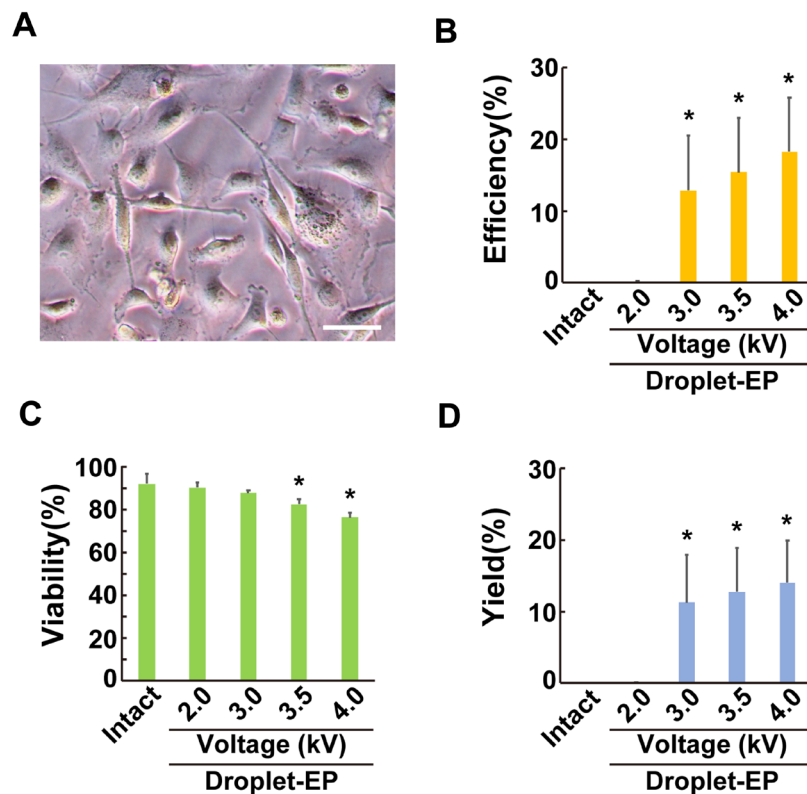

Fig. 3. Efficiency and cytotoxicity of transfection of the pEGFP-N2 plasmid into swine fibroblasts by droplet-electroporation (EP). Swine fibroblasts were isolated from earlobe skin, and subjected to transfection by droplet-EP. (A) Morphology of swine earlobe fibroblasts. Scale bar=50 μm. (B–D) Transfection efficiency (B), cell viability (C), and yields of transfection (D) were determined (as shown in Fig. 1). Values are means ± one standard deviation over three wells. *$P<0.05$ vs. intact cells.

Droplet-EP introduces EGFP protein into mouse and bovine spermatozoa

In the dairy scientific fields, gonadal cells were recently manipulated by gene editing [6, 46]. This requires that exogenous proteins or nucleotides are introduced into the cells. Notably, sperm cannot survive under cell culture conditions for >1 day [7]. Considering that cells typically require 24 hr to produce a sufficient amount of recombinant proteins, the introduction of a plasmid may not be applicable to sperm. Therefore, we attempted to introduce a purified protein into sperm using the droplet-EP approach. We are aware of no previous study investigating the transfection of recombinant protein to cells through droplet-EP. The present study was the first to introduce recombinant EGFP into mouse sperm which have previously been subjected to the introduction of proteins through conventional EP [40]. Droplet-EP at the 3.0 kV setting, which is the lowest voltage producing a short circuit using a 3.0 μl of sperm suspension, killed 91.3 ± 3.3% of total sperm (n=15) although 77.0 ± 9.1% (n=15) of sperm was alive without EP. To reduce the voltage producing a short circuit, we increased the size of the droplet to 10 μl, which included 2.7 μg of purified recombinant protein and 5.0×10^5 spermatozoa. In this case, 1.5 kV sufficed to generate a short circuit through droplet-EP. Reduction of voltage to 1.5kV increased sperm viability to 55.2 ± 15.6% (n=15). Figure 4A shows a microscopic image of a sperm after transfection at this setting. A number of spermatozoa exhibited an intense green florescent signal in the head, middle piece, and tail part, suggesting the successful transfection of EGFP through the droplet-EP method. In agreement with this finding, flow cytometric analysis demonstrated that 24.9% of the cells showed a stronger green fluorescent signal compared with untransfected cells (Fig. 4B).

We subsequently examined whether droplet-EP may be applicable to protein transfection into bovine sperm. As with the transfection into mouse sperm, we encapsulated frozen-thawed bovine sperm (5.0×10^5 cells) and 2.7 μg of recombinant EGFP in 10 μl of Opti-MEM, and applied a voltage (1.5 kV). Similar to the mouse sperm, a proportion of spermatozoa exhibited bright green fluorescence after droplet-EP with the EGFP (Fig. 4C). Moreover, flow cytometry demonstrated that 71.5% of the transfected cells showed a green fluorescent signal (Fig. 4D), and 24.9% showed intense green fluorescence, suggesting that the transfection efficacy was not constant among the sperm samples. Nevertheless, droplet-EP may be applied to the transfection of proteins into animal sperm.
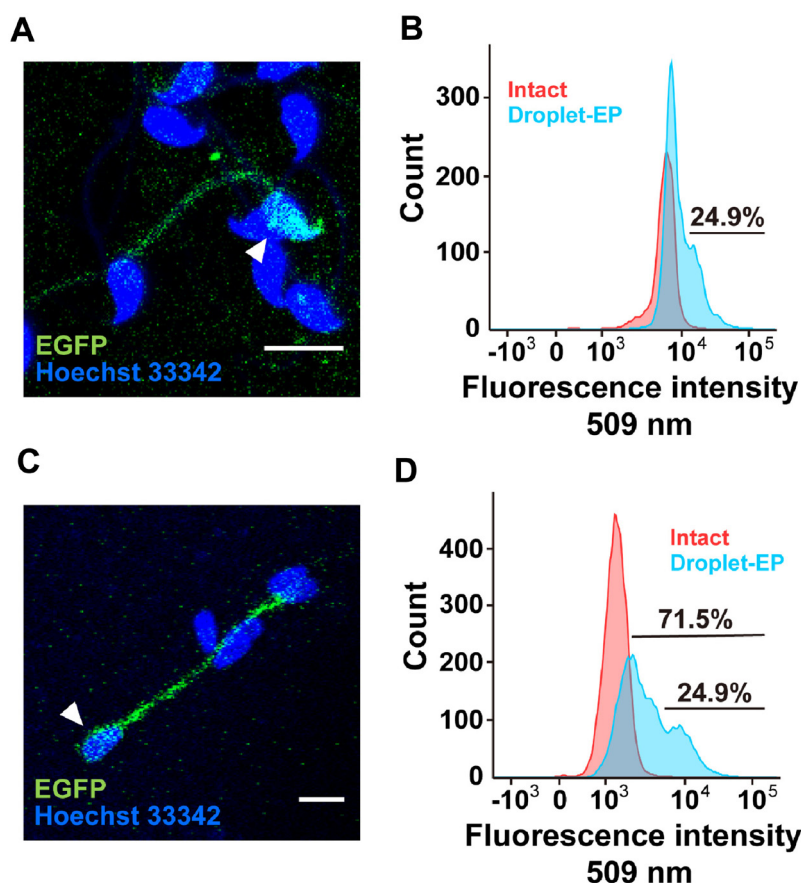

Fig. 4. Introduction of recombinant enhanced green fluorescence protein (EGFP) to mouse and bovine sperm. *E. coli* recombinant EGFP was transduced into mouse (A, B) and bovine (C, D) sperm by droplet- electroporation (EP) with a 10 μl droplet at the 1.5 kV setting. The nuclei were counterstained with Hoechst33342. (A, C) Representative images of mouse (A) or bovine (C) sperm transfected with recombinant EGFP. Arrowheads indicate EGFP-transfected spermatozoa. Scale bar=10 μm. (B, D) Histograms of the flow cytometric analysis of EGFP in mouse (B) or bovine (D) sperm. Mouse or bovine sperm were mixed with EGFP followed by droplet-EP (blue-filled area, Droplet-EP) or mixed with EGFP without droplet-EP (red-filled area, Intact). Representative data from three experiments are shown.

DISCUSSION

Transfection of gene fragments or recombinant proteins is currently widely performed in veterinary basic and clinical research. EP is one of the most effective methods for transfection. It is notable that EP allowed us to transfect plasmids into bovine cells, whereas lipofection methods were scarcely able to introduce them into the cells [19]. A major problem hindering the utilization of EP is cost. For example, the cost of NEPA21, one of the most widely used instruments for EP in livestock animal cells [17], is approximately 20,000 USD. Other popular instruments also typically cost 10,000–25,000 USD. The manipulation of cells with exogenous genes or proteins through EP is thus limited to research laboratories with relatively substantial funds. In contrast, droplet-EP only requires a DC power supply, which costs approximately 2,000 USD. Considering that droplet-EP does not require any special expendables, the present study provides basic information regarding a novel direction for transfection with high efficiency and low cost.

Although the proliferation of most somatic cells is determined by the Hayflick limit [15], numerous fibroblast-lined cells are still able to divide after 10 passages under appropriate culture conditions [23]. Moreover, fibroblasts express a relatively wide variety of signaling molecules. Thus, they are frequently used as atypical models for research into intracellular signaling cascades. For example, mouse derived-NIH3T3 and Swiss3T3 cells have been used to investigate the insulin, calcium, and Notch signals [32, 39, 43]. In these experiments, gene manipulations (e.g., overexpression, gene knockdown, gene knockout, and gene editing) were conducted by introducing exogenous gene fragments or recombinant proteins [3, 22]. On the other hand, non-negligible variations in cell signaling exist, which are derived from differences between species [2, 33]. Therefore, it is necessary to establish transfection methods for several species-derived fibroblasts. In this study, we determined the optimal setting of droplet-EP for bovine and swine fibroblasts; both are difficult to transfect through conventional lipofection [20, 34], and droplet-EP thus facilitates the study of cell signaling events in bovine and swine cells.

In previous studies, droplet-EP introduced plasmids into human fibroblasts cells at a relatively low voltage without generating a short circuit [25]. In contrast, we found that higher voltage was required for transfection into bovine and swine fibroblasts in the present study. Transfection of the plasmid was only observed in droplets which generated a short circuit between the electrodes. Thus, the generation of a short circuit rather than high voltage appears to be the crucial factor in introducing DNA and protein to livestock animal cells. We also observed the effective transfection of recombinant protein into mouse and bovine sperm by generating a short circuit at a low voltage setting, supporting this notion.

So far, a conventional electroporation was reported to effectively introduce recombinant Cas9 protein into mouse sperm [46]. Since transfection efficiency as well as viability of sperm have not been described in this report, we are not able to compare effectiveness of conventional EP and droplet-EP regarding protein transfection to sperm. In this report, 55% spermatozoa were alive after droplet-EP. Given that 23% sperm died during a freeze-thawing process, droplet-EP virtually damaged less than only one-third of the cells using an optimal setting (1.5kV). Additionally, we could introduce recombinant EGFP protein into significant proportion (mouse, 24.9%; cattle, 71.5%) of sperm. Nevertheless, we have to evaluate whether efficacy of droplet-EP is enough to proceed downstream applications such as genome editing and fertility treatments.

One of the disadvantages of droplet-EP might be limitation of cell number for transfection. This limitation is attributed to size of droplet. We had to set less than 10 μl droplet between the electrodes at a 6 mm interval. In the droplet, we concentrated 1.0×10^5 fibroblasts, which is comparable to the number of cells for lipofection at 35 mm dish scale. Taking advantage of lipofection and conventional electroporation techniques, plasmids can be introduced into more than 1.0×10^6 cells by a single transfection. Thus, flexibility of cell numbers is desirable for droplet-EP. In this study, we found that generation of short circuit is a critical factor to introduce plasmids into the cells by droplet-EP. In other words, if we can make short circuit in a larger droplet, we might introduce plasmids to larger number of cells. To achieve this, we have to perform droplet-EP in a larger droplet between more than 6 mm interval of electrodes.

The reasons for the markedly lower transfection efficacy observed in bovine cells remain unclear [37]. Transfection efficiency is highly dependent on membrane permeability, which is determined by the size and number of pores on the plasma membrane [41]. In addition, the duration of high permeability is also a critical factor influencing transfection [44]. These factors are highly dependent on membrane flexibility, which is determined by the lipid constituents of the plasma membrane. Previous reports showed that the content of cholesterol is somewhat higher in bovine cells than in human and mouse cells [8, 27]. Cholesterol confers inflexibility to the plasma membrane [38]. Therefore, temporary deprivation of cholesterol may improve the transfection efficiency in bovine cells by droplet-EP as well as other transfection techniques.

Addition of electrolytes or dimethyl sulfoxide is considered another option for improving the transfection efficiency by droplet-EP [5, 36]. Alternatively, repeated generation of a short circuit may increase the transfection efficiency in livestock animal cells. Since swine fibroblasts are relatively resistant to high voltage, the repeated generation of short circuits appears to be particularly suitable for these cells. Further improvements may increase the effectiveness of nucleotide and protein transfection into livestock animals (e.g., bovine, swine, and chicken) by droplet-EP.

In this study, we transfected bovine and swine cells with an EGFP-expressing plasmid. The molecular weight of the EGFP-expressing plasmid was ~4.7 kb. Transfection efficiency is critically influenced by the size of the introduced materials [12]. For example, introduction of a 3.5 kb and a 10.9 kb plasmid to mesenchymal stem cells through conventional EP yielded a 42% and 5% transfection, respectively [26]. Further studies are warranted to optimize the transfection of larger products into bovine and swine cells.

In Japan, livestock animals are bred mainly through artificial insemination. A decrease in the rate of conception is one of the

greatest challenges especially for dairy farmers [10, 18]. The low conception rate may be attributed to the fertilizing capacity of sperm [28]. To date several lines of study have attempted to improve male fertility by introducing exogenous gene fragments or proteins into sperm. For instance, Ma *et al*. reported that the introduction of miR-26a-5p into swine sperm potentiated fertility [30]. Moreover, recent advances in gene editing using CRISPR/Cas9 system technologies may confer high fertilizing capacity to sperm [16, 40]. Hence, the development of transfection methods with simple operation and low cost is warranted. In the present study, droplet-EP transfected the EGFP into one-quarter of the frozen-thawed bovine spermatozoa simply and cost-effectively. Droplet-EP may therefore be applicable to the manipulation of bovine sperm in veterinary basic and clinical research.

REFERENCES

1. Ahn, K. S., Kim, Y. J., Kim, M., Lee, B. H., Heo, S. Y., Kang, M. J., Kang, Y. K., Lee, J. W., Lee, K. K., Kim, J. H., Nho, W. G., Hwang, S. S., Woo, J. S., Park, J. K., Park, S. B. and Shim, H. 2011. Resurrection of an alpha-1,3-galactosyltransferase gene-targeted miniature pig by recloning using postmortem ear skin fibroblasts. *Theriogenology* **75**: 933–939. [Medline] [CrossRef]
2. Barreiro, L. B., Marioni, J. C., Blekhman, R., Stephens, M. and Gilad, Y. 2010. Functional comparison of innate immune signaling pathways in primates. *PLoS Genet.* **6**: e1001249. [Medline] [CrossRef]
3. Bharali, D. J., Klejbor, I., Stachowiak, E. K., Dutta, P., Roy, I., Kaur, N., Bergey, E. J., Prasad, P. N. and Stachowiak, M. K. 2005. Organically modified silica nanoparticles: a nonviral vector for in vivo gene delivery and expression in the brain. *Proc. Natl. Acad. Sci. USA* **102**: 11539–11544. [Medline] [CrossRef]
4. Björndahl, L., Söderlund, I. and Kvist, U. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum. Reprod.* **18**: 813–816. [Medline] [CrossRef]
5. Chafai, D. E., Mehle, A., Tilmatine, A., Maouche, B. and Miklavčič, D. 2015. Assessment of the electrochemical effects of pulsed electric fields in a biological cell suspension. *Bioelectrochemistry* **106** Pt B: 249–257. [Medline] [CrossRef]
6. Choi, D., Cheung, S., Xie, P., Parrella, A., Rosenwaks, Z. and Palermo, G. D. 2018. Feasibility of gene knockout in human sperm cells using CRISPR-Cas9. *Fertil. Steril.* **110**: e301. [CrossRef]
7. Christian, N., Songsasen, S. and Leibo, S. P. 1993. Presence of motile sperm in mice 24 hours postmortem. *Theriogenology* **39**: 201. [CrossRef]
8. Dawaliby, R., Trubbia, C., Delporte, C., Noyon, C., Ruysschaert, J. M., Van Antwerpen, P. and Govaerts, C. 2016. Phosphatidylethanolamine is a key regulator of membrane fluidity in eukaryotic cells. *J. Biol. Chem.* **291**: 3658–3667. [Medline] [CrossRef]
9. Deora, A. A., Diaz, F., Schreiner, R. and Rodriguez-Boulan, E. 2007. Efficient electroporation of DNA and protein into confluent and differentiated epithelial cells in culture. *Traffic* **8**: 1304–1312. [Medline] [CrossRef]
10. Dochi, O., Kabeya, S. and Koyama, H. 2010. Factors affecting reproductive performance in high milk-producing Holstein cows. *J. Reprod. Dev.* **56** Suppl: S61–S65. [Medline] [CrossRef]
11. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**: 7413–7417. [Medline] [CrossRef]
12. Fischer, D., Bieber, T., Li, Y., Elsässer, H. P. and Kissel, T. 1999. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* **16**: 1273–1279. [Medline] [CrossRef]
13. Gehl, J. 2003. Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol. Scand.* **177**: 437–447. [Medline] [CrossRef]
14. Gothelf, A. and Gehl, J. 2012. What you always needed to know about electroporation based DNA vaccines. *Hum. Vaccin. Immunother.* **8**: 1694–1702. [Medline] [CrossRef]
15. Hayflick, L. and Moorhead, P. S. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**: 585–621. [Medline] [CrossRef]
16. Huguet, E. and Esponda, P. 2000. Generation of genetically modified mice by spermatozoa transfection in vivo: preliminary results. *Mol. Reprod. Dev.* **56** Suppl: 243–247. [Medline] [CrossRef]
17. Ikeda, M., Matsuyama, S., Akagi, S., Ohkoshi, K., Nakamura, S., Minabe, S., Kimura, K. and Hosoe, M. 2017. Correction of a Disease Mutation using CRISPR/Cas9-assisted Genome Editing in Japanese Black Cattle. *Sci. Rep.* **7**: 17827. [Medline] [CrossRef]
18. Irikura, N., Uematsu, M., Kitahara, G., Osawa, T. and Sasaki, Y. 2018. Effects of service number on conception rate in Japanese Black cattle. *Reprod. Domest. Anim.* **53**: 34–39. [Medline] [CrossRef]
19. Ishino, T., Hashimoto, M., Amagasa, M., Saito, N., Dochi, O., Kirisawa, R. and Kitamura, H. 2018. Establishment of protocol for preparation of gene-edited bovine ear-derived fibroblasts for somatic cell nuclear transplantation. *Biomed. Res.* **39**: 95–104. [Medline] [CrossRef]
20. Jin, L., Kim, D. and Roh, S. 2014. Comparison of various transfection methods in human and bovine cultured cells. *Int. J. Oral Biol.* **39**: 177–185. [CrossRef]
21. Jordan, M. and Wurm, F. 2004. Transfection of adherent and suspended cells by calcium phosphate. *Methods* **33**: 136–143. [Medline] [CrossRef]
22. Kim, T. K. and Eberwine, J. H. 2010. Mammalian cell transfection: the present and the future. *Anal. Bioanal. Chem.* **397**: 3173–3178. [Medline] [CrossRef]
23. Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Tabara, N., Barber, M. and Yang, X. 2000. Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc. Natl. Acad. Sci. USA* **97**: 990–995. [Medline] [CrossRef]
24. Kurita, H., Takahashi, S., Asada, A., Matsuo, M., Kishikawa, K., Mizuno, A. and Numano, R. 2015. Novel parallelized electroporation by electrostatic manipulation of a water-in-oil droplet as a microreactor. *PLoS One* **10**: e0144254. [Medline] [CrossRef]
25. Kurita, H., Takao, Y., Kishikawa, K., Takashima, K., Numano, R. and Mizuno, A. 2016. Fundamental study on a gene transfection methodology for

mammalian cells using water-in-oil droplet deformation in a DC electric field. *Biochem Biophys Rep* **8**: 81–88. [Medline]

26. Lesueur, L. L., Mir, L. M. and André, F. M. 2016. Overcoming the specific toxicity of large plasmids electrotransfer in primary cells in vitro. *Mol. Ther. Nucleic Acids* **5**: e291. [Medline] [CrossRef]

27. Levitan, I., Christian, A. E., Tulenko, T. N. and Rothblat, G. H. 2000. Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. *J. Gen. Physiol.* **115**: 405–416. [Medline] [CrossRef]

28. Lieberman, D., McClure, E., Harston, S. and Madan, D. 2016. Maintaining semen quality by improving cold chain equipment used in cattle artificial insemination. *Sci. Rep.* **6**: 28108. [Medline] [CrossRef]

29. Luft, C. and Ketteler, R. 2015. Electroporation knows no boundaries: the use of electrostimulation for siRNA Delivery in Cells and Tissues. *J. Biomol. Screen.* **20**: 932–942. [Medline] [CrossRef]

30. Ma, J., Fan, Y., Zhang, J., Feng, S., Hu, Z., Qiu, W., Long, K., Jin, L., Tang, Q., Wang, X., Zhou, Q., Gu, Y., Xiao, W., Liu, L., Li, X. and Li, M. 2018. Testosterone-Dependent miR-26a-5p and let-7g-5p Act as Signaling Mediators to Regulate Sperm Apoptosis via Targeting *PTEN* and *PMAIP1*. *Int. J. Mol. Sci.* **19**: e1233. [Medline] [CrossRef]

31. Mack, K. D., Wei, R., Elbagarri, A., Abbey, N. and McGrath, M. S. 1998. A novel method for DEAE-dextran mediated transfection of adherent primary cultured human macrophages. *J. Immunol. Methods* **211**: 79–86. [Medline] [CrossRef]

32. Matsuno, Y., Kiwamoto, T., Morishima, Y., Ishii, Y., Hizawa, N. and Hogaboam, C. M. 2018. Notch signaling regulates cell density-dependent apoptosis of NIH 3T3 through an IL-6/STAT3 dependent mechanism. *Eur. J. Cell Biol.* **97**: 512–522. [Medline] [CrossRef]

33. Metcalfe, H. J., La Ragione, R. M., Smith, D. G. and Werling, D. 2014. Functional characterisation of bovine TLR5 indicates species-specific recognition of flagellin. *Vet. Immunol. Immunopathol.* **157**: 197–205. [Medline] [CrossRef]

34. Nakayama, A., Sato, M., Shinohara, M., Matsubara, S., Yokomine, T., Akasaka, E., Yoshida, M. and Takao, S. 2007. Efficient transfection of primarily cultured porcine embryonic fibroblasts using the Amaxa Nucleofection system. *Cloning Stem Cells* **9**: 523–534. [Medline] [CrossRef]

35. Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P. H. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1**: 841–845. [Medline] [CrossRef]

36. Niakan, S., Heidari, B., Akbari, G. and Nikousefat, Z. 2016. Comparison of different electroporation parameters on transfection efficiency of sheep testicular cells. *Cell J.* **18**: 425–437. [Medline]

37. Osorio, J. S. and Bionaz, M. 2017. Plasmid transfection in bovine cells: Optimization using a realtime monitoring of green fluorescent protein and effect on gene reporter assay. *Gene* **626**: 200–208. [Medline] [CrossRef]

38. Raffy, S. and Teissié, J. 1999. Control of lipid membrane stability by cholesterol content. *Biophys. J.* **76**: 2072–2080. [Medline] [CrossRef]

39. Ribeiro, C. M., Reece, J. and Putney, J. W. Jr. 1997. Role of the cytoskeleton in calcium signaling in NIH 3T3 cells. An intact cytoskeleton is required for agonist-induced [Ca2+]i signaling, but not for capacitative calcium entry. *J. Biol. Chem.* **272**: 26555–26561. [Medline] [CrossRef]

40. Sato, M. and Nakamura, S. 2019. Possible production of genome-edited animals using gene-engineered sperm. *In*: Gene Editing−Technologies and Applications (Chen, Y. C. and Chen, S. J. eds.), IntechOpen, London.

41. Saulis, G. and Saulė, R. 2012. Size of the pores created by an electric pulse: microsecond vs millisecond pulses. *Biochim. Biophys. Acta* **1818**: 3032–3039. [Medline] [CrossRef]

42. Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**: 671–675. [Medline] [CrossRef]

43. Shepherd, P. R., Withers, D. J. and Siddle, K. 1998. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem. J.* **333**: 471–490. [Medline] [CrossRef]

44. Tsong, T. Y. 1991. Electroporation of cell membranes. *Biophys. J.* **60**: 297–306. [Medline] [CrossRef]

45. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. and Kopf, G. S. 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121**: 1139–1150. [Medline]

46. Wang, J., Parrella, A., Xie, P., Rosenwaks, Z. and Palermo, G. D. 2019. A step toward gene remodeling of mammalian spermatozoa by CRISPR-Cas9. *Fertil. Steril.* **112**: e262. [CrossRef]

47. Wang, T., Larcher, L. M., Ma, L. and Veedu, R. N. 2018. Systematic screening of commonly used commercial transfection reagents towards efficient transfection of single-stranded oligonucleotides. *Molecules* **23**: 2564. [Medline] [CrossRef]

48. Yamamoto, M., Harada, Y., Suzuki, T., Fukushige, T., Yamakuchi, M., Kanekura, T., Dohmae, N., Hori, K. and Maruyama, I. 2019. Application of high-mannose-type glycan-specific lectin from Oscillatoria Agardhii for affinity isolation of tumor-derived extracellular vesicles. *Anal. Biochem.* **580**: 21–29. [Medline] [CrossRef]