

AC-electric-field-induced parthenogenesis of mouse oocyte

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To prevent parthenogenesis effect under embryo formation from sperms and oocyte, this study demonstrates that parthenogenesis of the oocyte of an ICR mouse can be induced with an AC electric field and without sperm insemination. The mouse oocytes were trapped with a positive dielectrophoretic force at the electrodes on an ITO-glass chip. The rates of parthenogenesis were tested under conditions of varied intensity of electric field from 33.35 kV/m to 266.8 kV/m and trapping durations 1, 3, 5 and 10 min. The results indicated that there is no significant statistical difference with varied trapping time ($p > 0.1$); with AC p-DEP electric activation, there is a significant statistical difference for 133.6 kV/m and 266.8 kV/m of electric field ($p = 0.0013$ and 0.0001 vs. control group respectively). The rate of parthenogenesis of the control group, oocytes with no electric activation, was $10.17 \pm 0.17\%$ ($n = 205$). The rates of parthenogenesis were $51.65 \pm 7.8\%$ ($n = 27$) at electric field 133.6 kV/m and $79.9 \pm 4.85\%$ ($n = 35$) at 266.8 kV/m. The viability of these oocytes treated with an electric field exceeded 80%; this method of electric activation is hence safe for a mouse oocyte.

1. Introduction: Parthenogenetic division (parthenogenetic development), also known as parthenogenesis, referring to sexual reproduction of animals and plants, is not subject to the formation of an embryo for an oocyte without sperm fertilisation under the extra activation, like AC electricity stimulation [1]. Three common parthenogenesis phenomena are sporadic parthenogenesis, constant parthenogenesis and cyclic parthenogenesis separately. The development of a parthenogenetic embryo of an infertile oocyte is much similar to the development of an embryo in vitro, except activation with various activation agents such as electric activation, chemical activation or stimulations of other types.

For activation of an oocyte during fertilisation, the concentration of intracellular calcium (Ca^{2+}) has repeatedly increased the regularity [2]. Free calcium at an appropriate concentration activates oocytes to promote a second polar body of oocyte meiotic discharge to form prokaryotic, then cleavage to be early embryo development.

Oocytes can be activated naturally, such as through sperm activation, temperature changes, osmotic pressure changes and intracellular free calcium. For artificial activation, other ways can include activation with added ethanol (6–9%) as an activator, electric activation, protein synthesis inhibit activation and cyclohexanesulphonamido amide activation [3–7].

In this research, we compared the effect of varying the trapping duration with an AC positive dielectrophoretic (p-DEP) force [8] and an applied AC electric field acting as inductive agents for parthenogenetic activation. The rates of parthenogenesis were defined based on the fraction of the two-cell stage for embryo development of mouse oocytes.

2. Materials and methods

2.1. Preparation of institute of cancer research (ICR) mouse oocytes: The ICR mice were treated in accordance with protocols approved by Animal Technology Laboratories of the Agricultural Technology Research Institute (ATRI-ATL). The oocytes were obtained from female mice (six weeks old). The females were superovulated with an intraperitoneal injection of Pregnant mare's serum gonatotropin (PMSG) (5 i.u.) to stimulate the development of ovarian follicles, followed by Human Chorionic Gonadotropin (hCG) (5 i.u.) 42–48 h later. After hCG administration, the superovulated mice were euthanised; the oocytes were obtained by

flushing the oviducts 10–13 h later. The oocytes were pre-treated with a micropipette and cultured in KSOM-AA.

2.2. Design and fabrication of a microfluidic system for fertilisation in vitro and observation:

(a) *ITO-glass electrode chip:* The fabrication process is shown in Fig. 1. First, we cleaned the Indium tin oxide (ITO)-glass substrate by washing it thoroughly with acetone, isopropanol and deionised water (DI). Hexamethyldisilazane (HMDS) vapour was deposited on the wafer for 5 min in the beaker to increase the adhesion between the photoresist and the surface of the glass. A positive photoresist (AZ5214) was spin-coated (3000 rpm, 30 s).

To remove the photoresist solvent, we set the temperature of soft baking at precisely 100°C for 1 min. After UV exposure, AZ 400K was used to develop the photoresist (AZ 400K: DI water = 1:5). The ITO-glass substrate, with $120 \Omega/\text{sq}$ square resistance, was then etched with aqua regia solution (DI water: nitric acid: hydrochloric acid = 1: 0.08: 1) at $45\text{--}46^\circ\text{C}$ for 40 s. To remove the residual AZ5214, ALEG-310 was heated to 60°C for 5 min for the completion of the ITO-glass electrode chip. The actual device is $5 \text{ cm} \times 3 \text{ cm}$, shown in Fig. 1b. The electrode has width $150 \mu\text{m}$; the gap between electrodes has width $100 \mu\text{m}$.

(b) *Observation platform:* The experiment was observed with an optical microscope (Olympus BX51). The ITO electrode pads were connected to a function generator (Agilent, 33220A) to regulate the AC voltage and frequency. A CO_2 incubator (NUAIRE, NU-5500) was applied to create a suitable environment (5% CO_2 , 37°C) for mouse embryo culture in vitro. The observation platform is shown in Fig. 2.

2.3. AC dielectrophoresis: The dielectrophoretic force for a spherical particle is calculated as [9]

$$F_{\text{DEP}} = 2\pi a^3 \epsilon_0 \epsilon_m \text{Re}[f_{\text{CM}}] |\nabla E_{\text{rms}}|^2 \quad (1)$$

in which a is the radius of a particle, ϵ_0 is the permittivity of vacuum ($8.854 \times 10^{-12} \text{ F/m}$), ϵ_m is the dielectric permittivity of a

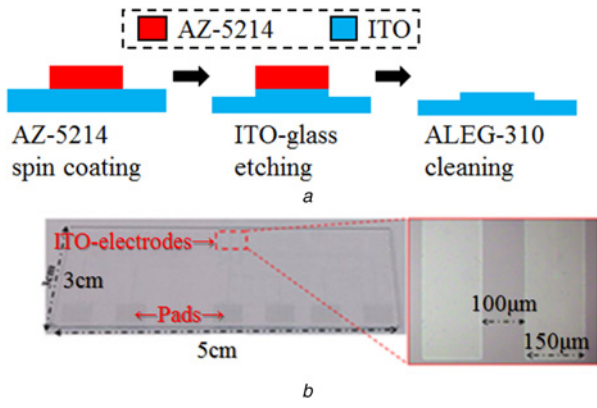


Fig. 1
a Fabrication of ITO-glass electrode chip. The pattern of the electrodes chip was fabricated with ITO glass etching
b Actual device (5 cm × 3 cm). Each gap between the electrodes has width 100 μm; each electrode has width 150 μm, thickness 50 nm, and square resistance 120 Ω/sq

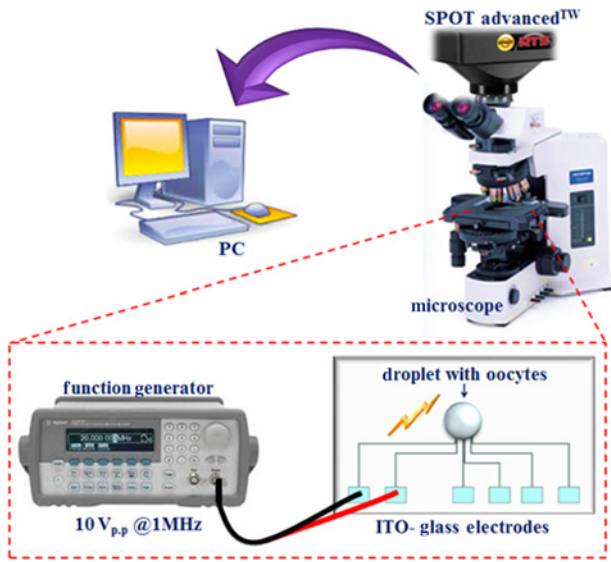


Fig. 2 Observation platform

medium, E_{rms} is the root-mean-square value of electric field and $\text{Re}[f_{CM}]$ is the real part of the Clausius-Mossotti factor, which is defined as

$$f_{CM} = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} \quad (2)$$

here ϵ_p is the dielectric permittivity of a particle; each complex permittivity takes the form $\epsilon^* = \epsilon - (j\sigma/\omega)$, in which $j = \sqrt{-1}$, and σ is the conductivity of the medium and particle. If $\text{Re}[f_{CM}] > 0$, the particle becomes pushed towards a region of strong electric field, a so-called p-DEP response, whereas if $\text{Re}[f_{CM}] < 0$, the particle is repelled from a region of strong electric field, a so-called negative dielectrophoretic response (n-DEP).

The expression of the protoplast model is represented as an effective protoplast permittivity (ϵ'_p), which is defined as

$$\epsilon'_p = \frac{C_{mem}^* R \epsilon_c^*}{C_{mem}^* R + \epsilon_c^*} \quad (3)$$

in which C_{mem}^* is the complex capacitance per unit area of a cell membrane, which takes the form $C_{mem}^* = C_{mem} - (jg_{mem}/\omega)$, g_{mem} is the conductance of a cell membrane, R is the radius of a cell and ϵ_c^* is the complex permittivity of cytoplasm. The value of $\text{Re}[f_{CM}]$ is important if we seek to predict precisely the dielectrophoretic response of a mouse oocyte.

Fig. 3a shows that oocytes are trapped and moved to a strong electric field under a p-DEP regime on applying an AC voltage. To predict the dielectrophoretic phenomenon of the oocyte, we used the protoplast model of Jones, shown in Fig. 3b. The dielectric data of a mouse oocyte were obtained from Choi *et al.* [10]. The relative permittivities of the DEP buffer solution and an oocyte were assumed to be 78.5 and 70. The radius of the mouse oocyte was 50 μm. The conductivity of the DEP buffer solution was 0.00056 S/m, the conductivity of the oocytes was assumed to be 0.02 S/m and the capacitance of the membrane was 1.25 μF/cm². We assumed also that the oocytes did not lyse under an applied electric field in this case. We thus calculated the transmembrane voltage of the oocyte as a spherical cell in an external field as follows [11].

If the induced transmembrane voltage exceeds 100 mV, electroporation occurs on the cell membrane. The electroporation voltage to prevent damage to the oocytes is estimated as

$$V_{tm} = \frac{1.5|E|a}{\sqrt{1 + (\omega\tau)^2}} \quad (4)$$

in which the time coefficient to charge the cell membrane is defined as

$$\tau_{mem} = a C_{mem} \left(\frac{1}{\sigma_{cyto}} + \frac{1}{2\sigma_{medium}} \right) \quad (5)$$

here a is the radius of a cell, $|E|$ is the intensity of the electric field and ω is the applied frequency. With conditions $E_{max} = 66.7$ kV/m and 1 MHz, τ_{mem} of oocyte is 0.59 ms; the transmembrane voltage

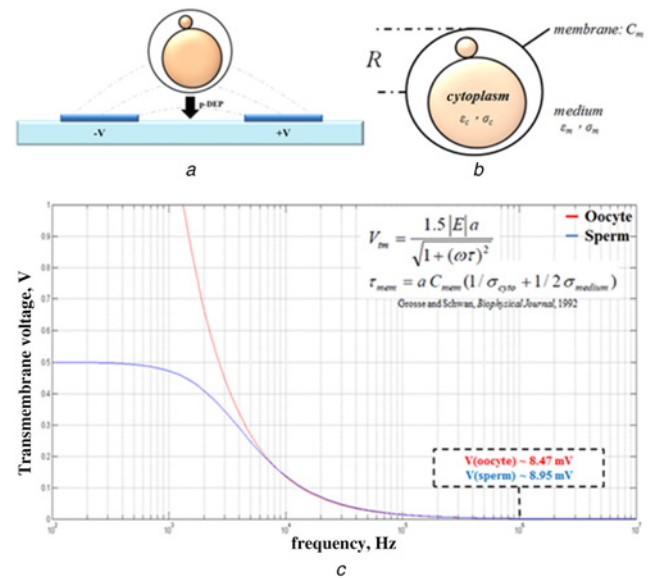


Fig. 3
a Illustration of mouse oocyte trapped with the p-DEP force under an AC electric field at the ITO-glass electrodes. The oocytes become trapped within the gap of the electrode
b Protoplast model for a mouse oocyte to predict the CM factor
c Transmembrane voltage (V_{tm}) of a mouse oocyte according to the frequency range from 10^2 to 10^7 Hz

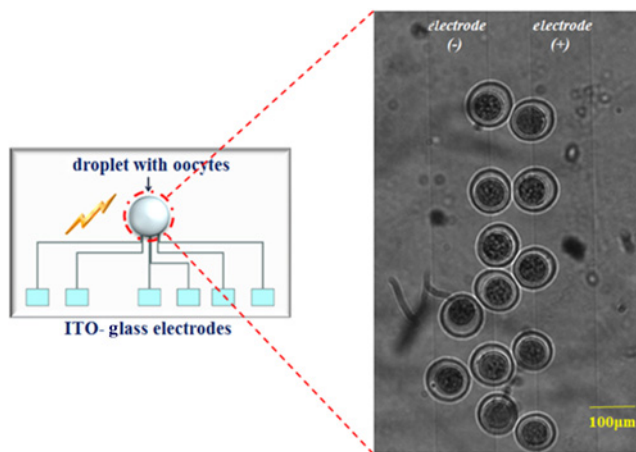


Fig. 4 Mouse oocytes were trapped with the p-DEP force between the gaps of the electrodes on ITO-glass at varied AC electric field in the range 33.75–266.7 kV/m

of oocyte is 8.47 mV, which is <100 mV. We hence assumed that it is not harmful for an oocyte in this case, as shown in Fig. 3c.

2.4. Experimental process of electric activation: The electric activation is illustrated in the following. The oocytes were washed three times in a DEP buffer solution from the KSOM-AA medium to maintain a small conductivity (<200 µS/cm). To manipulate the oocytes based on the p-DEP force, we set the condition of the AC bias pulse in the range of 5–40 V_{pp} at frequency 1 MHz.

Under an AC voltage at a frequency of order 1 MHz, the strong electric field induced a p-DEP force to trap mouse oocytes in the region of large intensity of electric field between the gaps of the ITO-glass electrodes, as shown in Fig. 4. The rates of parthenogenesis were measured on the ITO-glass electrode chip for varied trapping duration and electric activation. Electric intensities 33.35, 66.7, 133.4 and 266.8 kV/m of an AC electric field were applied for comparison with the control group, statically cultured in KSOM-AA (30 µl) of a droplet. The mouse oocyte becomes transferred into a DEP buffer droplet (10 µl) after activation of the p-DEP force for 1, 3, 5 and 10 min, respectively.

After AC electric activation, the oocytes were washed three times and transferred into a KSOM-AA medium from the DEP buffer solution. All these oocytes were cultured in another, fresh, pre-equilibrated, KSOM-AA droplet (30 µl) [12, 13] under mineral oil in a plastic Petri dish and incubated in a humidified incubator (CO₂ 5%, 37°C). The parthenogenesis was assessed one day after the AC electric activation and was defined strictly according to the occurrence of early cleavage for the two-cell stage of embryo development.

3. Results and discussion

3.1. Electric activation under varied trapping duration: An AC voltage 10 V_{pp} at 1 MHz, electric field about 66.7 kV/m, was applied to trap oocytes with a p-DEP force for varied trapping durations. The rates of parthenogenesis for 1, 3, 5 and 10 min, shown in Fig. 5, were $18.33 \pm 7.6\%$ ($n=32$), $33.22 \pm 16.7\%$ ($n=34$), $25.39 \pm 7.4\%$ ($n=39$) and $33.18 \pm 20.1\%$ ($n=41$), respectively. $P=0.2330$, 0.5082 , 0.2340 are the related numbers compared with the one trapped under 1 min for the trapping period. There is no significant statistical difference between the trapping durations ($P>0.1$). The viability of these oocytes was >80%, which means that the rates of parthenogenesis did not significantly increase when the trapping duration was increased under the p-DEP force.

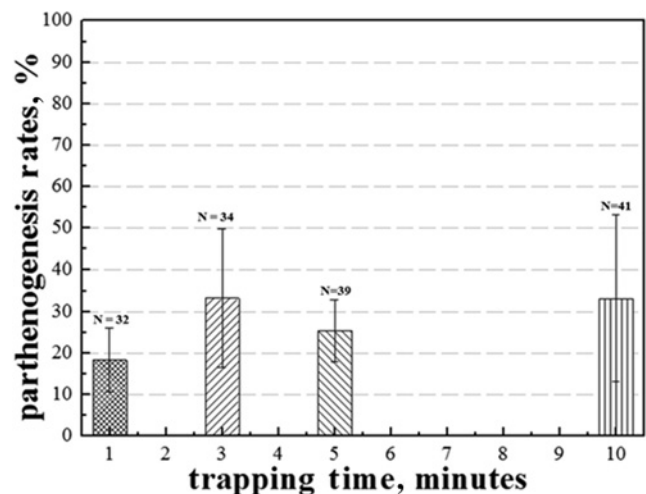


Fig. 5 At applied bias AC voltage 10 V_{pp} and frequency 1 MHz (electric field ~66.7 kV/m), the oocytes trapped with a p-DEP force at varied trapping duration (1, 3, 5 and 10 min). 'N' denotes the number of oocytes tested

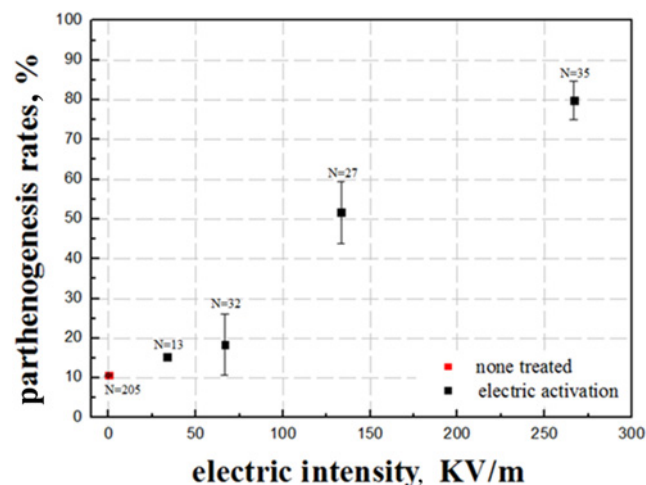


Fig. 6 At applied bias AC voltage and frequency 1 MHz in the electric field range 33.35–266.8 kV/m, the oocytes trapped with a p-DEP force for 1 min. A red point denotes the control group, in which the oocytes tested were not treated with electric activation. $P=0.5734$, 0.2445 , 0.0013 , 0.0001 versus control group, respectively

3.2. Electric activation under varied intensity of electric field: Fig. 6 shows the rates of parthenogenesis for oocytes with activation in an electric field in the range 33.35–266.8 kV/m. The oocytes were trapped with a p-DEP force for 1 min. The control group had no electric activation. The rates of parthenogenesis under activation with electric fields at 0, 33.35, 66.7, 133.4 and 266.8 kV/m were $10.17 \pm 0.17\%$ ($n=205$), 15.38% ($n=13$), $18.33 \pm 7.6\%$ ($n=32$), $51.65 \pm 7.8\%$ ($n=27$) and $79.9 \pm 4.9\%$ ($n=35$), respectively. The P values are 0.5734, 0.2445, 0.0013 and 0.0001 for comparison with the control group, respectively.

There is no significant statistical difference at applied electric fields 33.35 and 66.7 kV/m as P value was <0.1, but P was larger than 0.1 when electric fields 133.4 and 266.8 kV/m were applied to the trapping of oocytes. A strong electric field hence induces the parthenogenetic phenomena. An electric field should be carefully selected for embryo formation and development for fertilisation in vitro.

4. Conclusion: In this research, we have demonstrated that parthenogenesis of an ICR mouse oocyte is induced under an AC electric field without sperm insemination. The mouse oocytes were trapped with a p-DEP force at the ITO-glass electrodes for stimulation with the AC

electric field. The rates of parthenogenesis were evaluated with varied intensity of electric field and trapping duration. There was no significant statistical difference for varied trapping duration ($P < 0.1$), but the rates of parthenogenesis with 133.6 and 266.8 kV/m were $51.65 \pm 7.8\%$ ($n = 27$) and $79.9 \pm 4.85\%$ ($n = 35$). The significant statistical differences under varied electric fields 133.6 and 266.8 kV/m were $P = 0.0013$ and $P = 0.0001$ compared with the control group, respectively. According to our results, the viability of these oocytes that were treated with an AC electric field was $>80\%$, which proves that AC p-DEP would be a safe method using electric activation on a mouse oocyte. In the future, this AC p-DEP trapping method might be prospectively useful to improve the technique of artificial reproduction.

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