Embryo formation from low sperm concentration by using dielectrophoretic force

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A biochip system imitates the oviduct of mammals with a microfluidic channel to achieve fertilization *in vitro* of imprinting-control-region (ICR) mice. We apply a method to manipulate and to position the oocyte and the sperm of ICR mice at the same time in our microfluidic channel with a positive dielectrophoretic (DEP) force. The positive dielectrophoretic response of the oocyte and sperm was exhibited under applied bias conditions AC 10 V_{pp} waveform, 1 MHz, 10 min. With this method, the concentration of sperm in the vicinity of the oocyte was increased and enhanced the probability of natural fertilization. We used commercial numerical software (CFDRC-ACE+) to simulate the square of the electric field and analyzed the location at which the oocyte and sperm are trapped. The microfluidic devices were designed and fabricated with poly(dimethylsiloxane). The results of our experiments indicate that a positive DEP served to drive the position of the oocyte and the sperm to natural fertilization (average rate of fertilization 51.58%) in our microchannel structures at insemination concentration 1.5×10^6 sperm ml⁻¹. Embryos were cultured to two cells after 24 h and four cells after 48 h. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4915612>]

I. INTRODUCTION

With the growth of MEMS manufacturing technology in recent years, the scale of research thereon has tended toward development at the micro- and nanometer level.¹ These numerous techniques require an integration of many experts in varied fields of science. A biochip, which combines MEMS technology and biomedical technology, bestows the advantages of biocompatibility, great precision, modest cost, disposability, ease of production, etc. The miniaturization of a MEMS system and rapid parallel processing of biological samples enables one to treat many samples tested or a reaction on a small scale at the same time. Particle detection on a micro-scale can become more accurate and rapid. MEMS manufacturing technology can be achieved through a concept of a *lab on a chip* (LOC).²

With this progress, reproductive medicine is becoming an important part of the medical field. WHO statistics show that 10%–15% of couples have infertility problems. From those 15% reproductive disorders of couples, examination confirms 10%–30% male fertility disorder, 30%–40% female fertility disorder, and another 15%–30% of both fertility disorder.³ The major problem of infertility in women is typically caused by abnormal ovulation or tubal obstruction such that oocytes cannot normally await fertilization in the tube. Through the use of assisted

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reproductive technology (ART), part of the problem of infertility in women can be resolved by manual processing.⁴ The most typical methods of ART are fertilization *in vitro* (IVF) and intracytoplasmic injection of sperm (ICSI).⁴ IVF involves co-incubating the sperm and oocytes in a test tube or culture dish to natural fertilization with sperm at an appropriate concentration. After insemination, the embryo becomes transplanted into the woman's uterus. ICSI is an operating technique of fertilization in which a single sperm is injected directly into the oocyte to attain fertilization. Even if inactive sperm have chances of fertilization in this technique, it can be more than half the normal fertilization of oocytes, but up to 7% of oocytes might lyse after injection.^{4–6}

Before assisted reproductive technology is applied, the oocyte and sperm are generally pre-treated with a manual method before the oocyte and sperm achieve the purpose of fertilization. Oocyte positioning can greatly enhance the opportunities for the sperm to fertilize so that ICSI improves the rate of pregnancy. The traditional IVF technique generally requires medical experts to select appropriate sperm and to fix oocytes with microscopic operation. This process is a serious drain on medical manpower; traditional methods might damage the quality of the oocytes. In this work, we imitate the oviduct, so effectively to position the oocytes with a dielectrophoretic (DEP) microchannel chip technique.^{7–10} The biochip increases the opportunity of sperm in natural fertilization and directly replaces the traditional microinjection.

The techniques of using a microfluidic chip for fertilization *in vitro* have been developed for several years.¹¹ In the manipulation of an oocyte, a DEP force is used to trap and to select a healthy oocyte on applying a waveform (AC 3 V, 1 MHz, 15 s). It showed that a selected group of oocytes had superior blastocyst developmental potential.¹² The requirement of a limited concentration of sperm within the microfluidic device is another important issue. Suh discussed the implications of a total quantity of sperm and their concentration within a microfluidic device.¹³ If we can achieve IVF in a microfluidic chip with limited sperm or fewer sperm, the Oligozoospermia patient is helped. Our goal is thus to develop a LOC system based on microfluidic techniques and dielectrophoresis to assist oocyte insemination. In this work, we applied a positive dielectrophoretic force to capture oocytes in a microzone between two electrodes to enhance the quantity of sperm around the oocytes and to increase the concentration of sperm to form mouse embryos.

II. MATERIALS AND METHODS

A. Preparation of ICR mouse oocytes and sperm

The ICR mice in these experiments were treated in accordance with protocols approved by the Animal Technology Institute Taiwan (ATIT).

1. Oocyte collection

The oocytes were obtained from female ICR mice of age six weeks. The females were superovulated with an intraperitoneal injection of pregnant mare serum gonadotropin (PMSG, 5IU) to stimulate the development of ovarian follicles, followed by human chorionic gonadotropin (hCG, 5IU) 42–48 h later. After hCG administration, the superovulated mice were euthanized; the oocytes were obtained on flushing the oviducts 10–13 h later. The oocytes were pre-treated with a micropipette and cultured in a KSOM medium.

2. Sperm collection

The sperm were obtained from male ICR mice of age six weeks. The males were euthanized with cranial/cervical dislocation. The cauda portion of the epididymis was minced with scissors and removed into the medium. After allowing sperm to swim for 1 h, the sperm for insemination were collected and incubated at 37 °C in an atmosphere of CO₂ (5%) in air. The concentration of sperm in all experiments was about 1.5×10^6 sperm ml⁻¹. The concentration of one epididymis generally contains 1×10^7 sperm/ml. For traditional IVF, the minimum concentration, according to Han *et al.*,¹⁴ was diluted to 1×10^6 sperm/ml. In this work, the

concentration of a traditional method is 1.5×10^6 sperm/ml, so containing 45 000 sperm in a $30 \mu\text{l}$ droplet. We applied the same total quantity of sperm in the dielectrophoretic microfluidic method.

B. Design and fabrication of a microfluidic biochip for fertilization *in vitro* and an observation platform

Our fabricated microfluidic IVF biochip combines an electrode chip and a SU8-3050 mold of the microfluidic IVF biochip; a further step involves bonding the electrode chip and the polydimethylsiloxane (PDMS) microstructures with oxygen-plasma bonding.

1. Electrode chip

We initially cleaned the glass substrate by washing it rigorously with propanone, isopropanol (IPA), and deionized water, and immersed it into a piranha solution (sulfuric acid: hydrogen peroxide = 7:1) for 10 min at 90°C . HMDS (Hexamethyldisilazane) vapor was deposited on the wafer for 5–10 min to increase the adhesion between the photoresist and the surface of the glass wafer. The positive photoresist (AZ5214) was spin-coated at 3000 rpm for 30 s. To remove most photoresist solvent, the temperature of soft baking must be precisely set at 100°C for 1 min. After UV exposure, AZ400K was used to develop the photoresist (AZ400K: DI (deionized) water = 1:5). When the pattern of the electrodes was made, Cr and Au were deposited (thicknesses 20, 150 nm) on a glass substrate with an E-gun evaporator. The final step is lift-off to produce the electrode chip.

2. Mold of the SU8–3050 microchannel

Soft lithography was used to fabricate the SU8-3050 structure mold. We initially cleaned the glass substrate by washing it rigorously with propanone, IPA, and deionized water; we then immersed it into a piranha solution (sulfuric acid: hydrogen peroxide = 7:1) for 10 min at 90°C .

The negative photoresist (SU8-3050) was spin-coated at two speeds, first at 500 rpm for 10 s and then at 1000 rpm for 30 s. The purpose of the first speed is to let the photoresist become coated on the entire silicon wafer and of the second speed is to determine the final thickness of the photoresist. The total uniform thickness of the SU8 3050 mold is about $140 \mu\text{m}$. To remove slowly most photoresist solvent, we set the soft baking parameter on heating by increasing 5° per 3 min to 65°C (heating 30 min) and 95°C (heating 1 h). After UV exposure, a SU8 developer was used to develop the photoresist. When the SU8 structure was finished, we used a standard cleaning procedure (propanone, isopropanol, and deionized water) to clean the silicon wafer.

We poured the PDMS (A: B = 10 g:1 g) into a structure mold and cured it under vacuum to remove the bubbles for about 30 min at 85°C .

3. Bonding

The PDMS microfluidic-microstructures and the glass substrate with Cr/Au electrodes were bonded to each other with an oxygen plasma¹⁵ to complete the fabrication of our microfluidic IVF biochip, before heating it on a hotplate for 1 h at 85°C . At the end of the process, we punched two holes in the PDMS on both sides of the microchannel (diameter 3.5 mm) to serve as inlet and outlet reservoirs. Of three pairs of electrodes, the purpose of each pair is to produce a nonuniform electric field to induce a dielectrophoretic force. The entire microfluidic IVF biochip fabrication and the actual device ($5 \text{ cm} \times 3 \text{ cm}$) are shown in Figure 1.

4. Observation platform

The movements of sperm and oocytes were observed and recorded with an optical microscope (Olympus BX51). The electrodes of the microfluidic IVF chip connect the function generator (Agilent, 33220A) to regulate the AC voltage and the frequency. The outlet of the microfluidic IVF chip is connected to a syringe pump (KD Scientific Syringe Pumps, KDS 220)

FIG. 1. (a) Fabrication of our microfluidic IVF biochip. The pattern of the electrode chip was fabricated with a lift-off technique. The structure mold of the microchannel was fabricated with SU8 soft lithography on a silicon wafer, with PDMS to copy the mold. The PDMS was bonded with the electrode chip with an oxygen plasma. (b) Actual device (5 cm $\times$ 3 cm). Two punched holes (diameter 3.5 mm) serve as inlet and outlet reservoirs. The outlet reservoir is connected to a syringe pump to control the rate of volume flow.

to apply a steady flow field and to control the rate of volume flow. The CO₂ incubator (NUAIRE, NU-5500) provided a suitable environment (5% CO₂, 37 °C) for use with the mouse embryo culture *in vitro*. The observation platform is shown in Figure 2.

C. Experimental process of standard IVF control group

DEP buffer is an aqueous solution containing sucrose (236 mM) and glucose (59 mM). To ensure that the DEP buffer solution is usable for the oocyte and sperm of ICR mice for fertilization *in vitro*, we compared the rate of fertility and the developmental stage of the embryo between the culture media (KSOM) and DEP buffer solution. The experimental process is described in the following statement.

The total volumes of micro-droplet media for the culture media (KSOM) and DEP buffer solution insemination were 30 μ l in the culture dishes at the desired sperm concentration (1.5×10^6 sperm ml⁻¹); the oocytes were loaded into microdroplets using fine pipettes; about 20 oocytes were added to each microdroplet. The sperm and oocytes were co-incubated in a humidified incubator (CO₂ 5%, 37 °C, 1 h).

FIG. 2. Observation platform. An optical microscope is used to observe the entire experiment. The electrodes of our microfluidic IVF biochip connect the function generator to regulate the AC voltage and the frequency. The outlet is connected to a syringe pump to apply a steady flow field and to control the rate of volume flow.

After insemination, all oocytes of these two groups were washed three times in KSOM medium, transferred into another fresh KSOM microdroplet ($30\ \mu\text{l}$) under mineral oil in a plastic Petri dish, and incubated in a humidified incubator (CO_2 5%, 37°C).¹² Fertilization was assessed one day after insemination and was defined rigorously when early cleavage of the embryo development occurred to the two-cell stage. The experimental protocol of standard IVF control groups is shown in Figure 3.

D. Experiment of microfluidic IVF

To manipulate the oocytes and sperm based on a positive DEP, we set the condition of an AC pulse at 10 V and frequency 1 MHz.^{12,13} With a manual method, the oocyte samples were fertilized *in vitro* and cultured as follows. The oocytes were washed three times in DEP buffer solution before the experiment.

The chip outlet was connected to a syringe pump to provide a steady flow; the rate of volume flow was about $1\ \mu\text{l}\ \text{min}^{-1}$; the oocytes were pulled at the same time. With voltage $10\ \text{V}_{\text{pp}}$

FIG. 3. Co-incubation of sperm and oocytes was conducted during 1 h for insemination, after which the oocytes of two groups were transferred into another fresh KSOM microdroplet ($30\ \mu\text{l}$) under mineral oil in a Petri dish and incubated in a humidified incubator (CO_2 5%, 37°C).

and a 1-MHz sine wave to generate a strong electric field, the sine wave induced a positive DEP to trap oocytes at the region of large electric field in the microchannel. We loaded the oocytes at the inlet of the reservoir with a pipette. When an oocyte passed through the electrode pads it was trapped between the two electrode pads with the positive DEP force. The sperm was pulled in the same way by the syringe pump in the microchannel to fertilize the trapped oocyte, as shown in Figure 4(a). As shown in Figure 4(b), the oocytes were initially trapped between the electrode pads to await the sperm. As soon as the sperm increased around the oocyte, the syringe pump was stopped to await fertilization and to make the fluid stable in the microchannel. That step lasted about 20 min to keep the loading sperm continuously. In addition to turn off the syringe pump, the function generator was also turned off to remove the dielectrophoretic force. After insemination, the oocytes were washed three times in KSOM medium and cultured in a microdroplet ($30\ \mu\text{l}$)^{16,17} under mineral oil in a plastic Petri dish and incubated in a humidified incubator (CO_2 5%, 37°C).

III. RESULTS

A. Standard IVF control group

The experiments included two control groups to ensure that the DEP buffer solution had no effect during fertilization *in vitro*. For insemination ($1\ \text{h}$, $1.5 \times 10^6\ \text{sperm ml}^{-1}$), the rate

FIG. 4. (a) Illustration of experiment of microfluidic IVF. The concentration of sperm in the inlet reservoir is about $1.5 \times 10^6\ \text{sperm ml}^{-1}$. The sperm and oocytes become trapped in the region of the electrodes. (b) (1)–(4) At applied AC voltage $10\ \text{V}_{\text{pp}}$ and frequency $1\ \text{MHz}$, the oocytes were trapped with a positive dielectrophoretic force between two electrode pads. The scale bar is $300\ \mu\text{m}$. The red arrow shows the direction of the flow. (3) After trapping the oocyte, the sperm were loaded into the inlet reservoir of the microchannel. (4) Many sperm swim around the oocyte (red arrow).

of fertility for these two groups of embryos was comparable between the KSOM group and the DEP buffer solution. In Figure 5(a), the rate of fertility of a standard IVF control group in DEP buffer solution (64%, $n = 58$) shows no significant difference from a standard control group in KSOM medium (73%, $n = 79$). Blastocyst development of mouse embryo culture *in vitro* between these two groups (10%, KSOM; 11%, DEP buffer solution) was also similar, as shown in Figure 5(b).

B. Microfluidic IVF

Commercial CFD (CFD-RC, CFD-ACE+) numerical software was used to calculate the electric field of our dielectrophoretic microfluidic IVF system. With the non-uniform electric fields, the oocytes and sperm were manipulated and positioned. Figure 6(a) presents a case as follows: applied voltage 10 V_{pp}, frequency 1 MHz, microchannel height 140 μm and width 520 μm , gap 100 μm between two electrodes, and width of each electrode 150 μm . A black line represents that the electrodes have height 1 μm . The gradient of the square of the electric field intensity was used to predict the strength of the electric field, as the oocytes and sperm became trapped and moved to the strong electric field under a positive DEP regime. The maximum value of E^2 is $4.44 \times 10^9 \text{ V}^2 \text{ m}^{-2}$ and of electric-field intensity is about $6.67 \times 10^4 \text{ V m}^{-1}$; the curve of the gradient of the square of electric field is shown in Figure 6(b). The position of the electrodes in Figure 6(b) is from 0.0008 to 0.00095 and from 0.00105 to 0.0012. The values of

FIG. 5. (a) Rates of fertilization between the two groups (73% KSOM ($n = 79$); 64% DEP buffer solution ($n = 58$)). (b) The normal embryo development of these two groups at five times.

FIG. 6. (a) Numerical results (CFDRC-ACE+) of the squared electric field of our dielectrophoretic microfluidic IVF system (prospective view). The applied voltage is 10 V_{pp} and the frequency is 1 MHz. The maximum electric-field intensity is 6.67×10^4 V/m. The microchannel has height 140 μm and width 520 μm ; the gap between two electrodes is 100 μm and the width of each electrode is 150 μm . A black line represents that the electrodes have height 1 μm . (b) Numerical results (CFDRC-ACE+) for the curve of distribution of the gradient of the squared electric field; the maximum value of electric field is 66.7×10^4 kV/m. (c) Real part of Clausius-Mossoti factor.

E^2 at $x=0.0008$ and $x=0.0012$ are about $2.5 \times 10^8 \text{ V}^2 \text{ m}^{-2}$, which is smaller than the least value ($5 \times 10^8 \text{ V}^2 \text{ m}^{-2}$) in the graph. Figure 6(c) represents the real part of the polarizability parameter (Clausius-Mossoti, CM factor, f_{CM}). The equation of dielectrophoretic force affecting a spherical particle of radius a in a medium with dielectric permittivity ϵ_m is

$$F_{\text{DEP}} = 2\pi a^3 \epsilon_m \text{Re}[f_{\text{CM}}] \nabla E^2.$$

The equation of the CM factor¹⁸ is

$$Re[f_{CM}] = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*}.$$

ϵ_p^* and ϵ_m^* are complex permittivities of the particle and of the medium. Of dielectrophoretic force of two types, one is a positive dielectrophoretic force with a positive CM factor that can induce the particle toward a strong electric field; the other is a negative dielectrophoretic force of negative CM factor that performs a contrasting behavior. The relative permittivity of the DEP buffer solution was 78.5, and the radius of the oocytes was assumed to be $50\ \mu\text{m}$. The conductivity of the DEP buffer solution was $0.00056\ \text{S/m}$. Here, the relative permittivity was assumed to be 70 and the conductivity of the oocytes to be $0.02\ \text{S/m}$. In this case, a frequency about $1\ \text{MHz}$ is not harmful for the oocyte and sperm.

Figure 6(c) shows that, at applied AC voltage $10\ \text{V}_{\text{pp}}$ and frequency $1\ \text{MHz}$, the oocytes and sperm were trapped with a positive dielectrophoretic force between the two electrode pads. The result of the microfluidic IVF shows that the concentration of sperm was increased with this trapping method. The concentration of sperm in the vicinity of the oocytes was increased from $1.5 \times 10^6\ \text{sperm ml}^{-1}$ to about $6.0 \times 10^7\ \text{sperm ml}^{-1}$, as shown in Figure 4(b-4). In Figure 7(a), the rate of fertility of microfluidic IVF in the DEP buffer solution is 51.58% ($n=75$) at the insemination concentration ($1.5 \times 10^6\ \text{sperm ml}^{-1}$). Figure 7(b-1) shows that the oocytes developed to the two-pronuclei stage (2PN) at the sixth hour after fertilization. On the second day after

FIG. 7. (a) Total statistics of microfluidic IVF experiments in our biochip. The average rate of fertility is about 51.58% ($n=75$) at the insemination concentration ($1.5 \times 10^6\ \text{sperm ml}^{-1}$). (b) Embryo development tracking; magnification $40\times$. (1) Normal oocyte. (2) Normal development of a zygote after 6h. Two pronuclei are marked with dashed circles. (3) Normal development of a two-cell embryo at E1.5. (4) Normal development of an eight-cell embryo at E2.5.

fertilization, the embryos had developed to the two-cell stage as shown in Figure 7(b-2); the same embryos kept for another 12 h developed to the four-cell stage as shown in Figure 7(b-3), and after a further 12 h to an eight-cell stage as shown in Figure 7(b-4).

IV. CONCLUSIONS

We demonstrate a microfluidic IVF biochip that can imitate the oviduct and enhance the rate of fertilization of mouse fertilization *in vitro*. We manipulated and positioned the oocytes and sperm with a positive DEP force in the microchannel at the same time. On applying voltage $10 V_{pp}$ and a 1-MHz sine wave to generate a strong electric field, a positive DEP was induced to trap the oocytes and sperm in a region of large electric field in the microchannel. The result of the microfluidic IVF shows that the concentration of sperm was increased with this trapping method. The concentration of sperm in the vicinity of the oocytes was increased from 1.5×10^6 sperm ml^{-1} to about 6.0×10^7 sperm ml^{-1} , as shown in Figure 4(b-4). The rate of fertilization with the microfluidic chip in a DEP buffer solution was about 51.58%. In the future, we shall continue to test the limit of sperm concentration that is still able to achieve mouse fertilization *in vitro*, to integrate the embryo culture *in vitro* into our biochip and then to transplant embryo into the uterus of the ICR mice. Our goal is to develop a concept of a lab-on-chip^{11,14,19–22} for assisted reproduction techniques *in vitro*.

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