

medium (HFF99; Fuso Pharmaceutical Industries) at 37°C in an atmosphere of 5% CO₂ until the sperm were injected.

Procedures of ICSI and mouse oocyte activation The round-headed sperm of this patient were injected into the mouse oocytes by a piezo-driven unit using the methods reported elsewhere (13–15). The oocyte membranes were broken by applying one or two faint piezo pulses, and the sperm were injected into each ooplasm. It took approximately 20 minutes to inject a group of about 15 oocytes. The injected oocytes were kept for 20–30 minutes at room temperature. During this interval, the oocytes that had been classified into the activation treatment group were activated in a Ca-free Toyoda, Yokoyama, Hoshi (TYH) medium containing 10 mM SrCl₂ for 60 minutes at 37°C in an atmosphere of 5% CO₂. These oocytes were transferred one by one into a single 10-μL drop of HFF99 medium with 10% SSS under mineral oil in a plastic dish, and the dish was incubated at 37°C in an atmosphere of 5% CO₂. Approximately 7–11 hours after injections and the activation treatment, the oocytes were observed under an inverted microscope and were rotated to determine whether or not the second polar body and two pronuclei were present.

For the statistical analysis, the obtained data were analyzed by the χ^2 -test.

Ovarian Stimulation, Oocyte Collection, and Oocyte Activation in Patient

Ovarian stimulation was conducted using a combination of GnRH agonist (Nasanyl, Yamanouchi, Japan) and hMG (Pergoreen; Serono, Geneva, Switzerland). An injection of 5000 units of hCG (Profasi, Serono) was administered when the dominant follicle reached a mean diameter of 20 mm. Vaginal ultrasound-guided follicle puncture was conducted 36 hours after the hCG injection. The retrieved oocytes were cultured for several hours in Quinn's Advantage Cleavage medium (Sage, Pasadena, CA) at 37°C in an atmosphere of 6% CO₂, 5% O₂, 89% N₂, under humidified conditions, and all oocytes were denuded enzymatically with 70 IU/mL SynVitto Cumulase (MediCult, Copenhagen, Denmark) for 30–60 seconds, followed by mechanical denudation. The motile round-headed sperm were injected into the oocytes in metaphase II, and these oocytes were activated by calcium ionophore A23187 (Sigma; 10 μM/mL) for 5 minutes after ICSI. Subsequently, the oocytes were rinsed several times in culture medium and incubated overnight to recognize 2 pronuclei at 37°C in an atmosphere of 6% CO₂, 5% O₂, and 89% N₂ under humidified conditions. For blastocyst cultivation, we transferred the embryos to a sequential medium Quinn Advantage (Sage) or Multi-Blasto Medium (Irvine, CA) on day 3.

Vitrifying and Warming of Blastocysts

All blastocysts were cryopreserved using the vitrification method as described in Kyono et al. (16) to prevent severe ovarian hyperstimulation syndrome (OHSS). The blastocysts were equilibrated for 8–10 minutes in 7.5% ethylene glycol

(EG) and 7.5% dimethyl sulfoxide (DMSO) and then into cryoprotectant 15% EG, 15% DMSO, and 0.5 M sucrose (Kitazato Supply Ltd., Japan) for 1 minute. After that, they were immediately put on a Cryotop and inserted into liquid nitrogen. Warming was performed at room temperature by the rapid thawing method following the market procedure (Kitazato Supply Ltd.). Cryoprotectant was removed by stepwise reduction of the EG and DMSO concentration containing 1.0, 0.5, and 0 M sucrose.

After a single vitrified-warmed blastocyst (grade 5BA) was transferred, a clinical pregnancy was determined by the presence of a gestational sac (GS) and a fetal heartbeat via a transvaginal ultrasound.

RESULTS

The couple did not achieve a pregnancy in spite of repeatedly attempted IUI. We diagnosed the husband as having globozoospermia, which is defined as round-headed sperm lacking an acrosome by the Kruger test and transmission electron microscope (Fig. 1). MOAT was tried but still resulted in no fertilization under piezo-ICSI without activation. As the fertilization rate after SrCl₂ activation was increased to 68.4%, it was strongly recommended to pursue the necessary artificial activation to increase the fertilization rate (Table 1).

Round-headed sperm were injected into 17 oocytes during metaphase II. Among the injected oocytes, 15 oocytes were activated (15/17, fertilization rate 88.2%). By day 5, 10 embryos had developed, and another blastocyst developed on the sixth day. Seven of 11 blastocysts were of good morphology, and four blastocysts did not fit the criteria for fair quality. Two months later, one vitrified blastocyst (grade 5BA) was warmed and transferred to the female patient on the fifth day after hormone treatment with P 50 mg (Fuji Pharmaceutical Co., Ltd., Tokyo, Japan). The luteal phase was supported by daily injections of P. After 10 days, pregnancy was established by positive β-hCG in the serum, and the fetal heartbeat was later confirmed by ultrasound. The couple did not desire amniocentesis so we did not perform it. Fortunately, the female patient remained in good condition throughout the full-term pregnancy. Finally, a healthy boy weighing 3180 g was delivered at 40 weeks of gestation by cesarean section.

DISCUSSION

ICSI is a useful technique for men who are infertile because of round-headed sperm. ICSI has led to fertilization, embryo development, pregnancy, and delivery of infants (1–9). However, several investigators have reported low to absent fertilization (2, 4, 5, 17, 18). Rybouchkin et al. (11) discovered that fertilization was improved when a calcium ionophore was added. They suggested that a sperm-associated oocyte-activating factor that normally causes the Ca²⁺ flux required for fertilization might be absent or down-regulated in globozoospermic sperm, a suggestion that was subsequently confirmed by several studies (11, 19, 20). Subsequently, Rybouchkin et al. in 1997 (18), Kim et al. in 2001 (21),

TABLE 1

Results of injected round-headed sperm to mouse oocytes and activation.

	Activation (+)	Activation (-)
No. of oocytes with ICSI	22	20
No. of surviving oocytes	21	19
Normal morphology	19	19
Abnormal morphology	0	0
2PN 2PB (+)	13 (68.4)	0
2PN 2PB (-)	1 (5.3)	0
1PN 2PB (+)	3 (15.8)	0
3PN 2PB (-)	2 (10.5)	0
0PN 2PB (+)	0	0
0PN 2PB (-)	0	19 (100)
Total activated oocytes	19 (100)	0 (0)

Note: Data in parentheses are percents. Activation (+): The activation treatment group was activated in a Ca-free TYH medium containing 10 mM SrCl₂ for 60 minutes at 37°C in an atmosphere of 5% CO₂. The details are mentioned in Materials and Methods. 2PN = two pronuclei formation; 2PB = first and second polar body.

Kyono. Birth from a globozoospermic patient. Fertil Steril 2009.

Heindryckx et al. in 2005 (15), and Tajera et al. in 2008 (22) reported successful pregnancies and deliveries using ICSI with calcium ionophore oocyte activation. On the other hand, Tesarik et al. in 2002 (14) reported that the possibility of using a simple modification of the standard ICSI micromanipulation technique, instead of ionophores, alleviates concerns about the possible harmful effects to human embryos of these insufficiently tested drugs. Vigorous aspiration of oocyte cytoplasm and repeated in-and-out movements of the microinjection needle within the human oocyte during the ICSI produce a considerable influx of calcium ions from the surrounding culture medium into the oocyte. It is also possible that mechanical disruption of endoplasmic reticulum during the movements of the microinjection needle in the oocyte cytoplasm and repeated aspiration releases the calcium stored in this organelle. The modified ICSI technique increases the intracellular concentrations of the free calcium ion in the injected oocyte compared with the standard ICSI technique.

Electrical stimulation, ethanol, SrCl₂, Ca ionophore, and vigorous ICSI have been reported as AOA. Ca ionophore, ethanol 8%, and SrCl₂ (from our data) displayed the efficacy of AOA for globozoospermia in heterologous ICSI models. Ca ionophore and vigorous ICSI were reported to have the

effectiveness of AOA for globozoospermia in human ICSI clinically, but electrical stimulation, ethanol, and SrCl₂ are not yet reported to be effective. More studies are needed to examine which method is truly the best for AOA in globozoospermia.

This couple had tried IUI three times; however, they did not achieve pregnancy. First, we diagnosed the husband as having globozoospermia. Second, we compared the results with and without oocyte activation using SrCl₂ on mouse oocytes with round-headed sperm injection. From the outcomes of MOAT, we concluded that round-headed sperm might lack the ability of oocyte activation. We reported the efficacy and the safety of SrCl₂ in the patients with low fertilization by ICSI (23). However, the SrCl₂ activator is not yet approved for use with human oocytes in the case of globozoospermia. Third, in this case, we tried calcium ionophore A23187 treatment, which was also reported by several investigators (18, 19, 22, 23) to activate human oocytes injected with round-headed sperm. The fertilization rate in our case is 82.2% (15 of 17), which may be attributed to the AOA with calcium ionophore A23187 treatment. We believe that oocyte activation after ICSI using round-headed sperm is vital.

Edirisinghe et al. (24) studied the cytogenetics of unfertilized oocytes after ICSI. Failure of activation and the consequent failure of the oocyte to complete meiosis II were likely to have contributed to the high rate of premature chromatin condensation seen in their cases. These data suggest that round-headed sperm lack the capacity to penetrate oocytes and may also be deficient in their oocyte-activating ability.

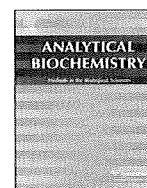
Generally, round-headed sperm were observed to have a comparatively higher chance of abnormal aneuploidy chromosome (25–27), and chromatin structure and centrosomal function had activated abnormally in globozoospermia (28–30).

Although karyotypes of round-headed sperm have not been discussed in many nuclear studies in great detail, several articles have reported on aneuploidy, centrosomal dysfunction, and chromatid structure abnormalities. More studies regarding the karyotypes of rounded-headed sperm are necessary in the future. Round-headed sperm are deficient in oocyte-activation capacity, and this deficiency is independent of the variation in morphology of the sperm heads. The most probable reason for this deficiency is the absence or down-regulation of the sperm oocyte-activating factor in these sperm. Further investigation of globozoospermia would give valuable information on the general mechanism of spermatogenesis; different aspects of sperm-egg interaction; and physiology, etiology, and chromosome aneuploidy of globozoospermia.

In summary, we report a successful pregnancy and delivery from a transfer of a single vitrified-warmed blastocyst after performing ICSI with round-headed sperm and AOA in a globozoospermic patient.

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A microfluidic dual capillary probe to collect messenger RNA from adherent cells and spheroids

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ABSTRACT

Collection of bioanalytes from single cells is still a challenging technology despite the recent progress in many integrated microfluidic devices. A microfluidic dual capillary probe was prepared from a theta (θ)-shaped glass capillary to analyze messenger RNA (mRNA) from adherent cells and spheroids. The cell lysis buffer solution was introduced from the injection aperture, and the cell-lysed solution from the aspiration aperture was collected for further mRNA analysis based on reverse transcription real-time PCR. The cell lysis buffer can be introduced at any targeted cells and never spilled out of the targeted area by using the microfluidic dual capillary probe because laminar flow was locally formed near the probe under the optimized injection/aspiration flow rates. This method realizes the sensitivity of mRNA at the single cell level and the identification of the cell types on the basis of the relative gene expression profiles.

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Hydrodynamics is an important aspect for controlling the mass transfer of reagents and has been combined with various analytical tools such as electrochemical [1–5], electrophoretic [6–8], and chromatographic [9] instruments. Recently, the concept of a micro-total analysis system (μ TAS)¹ has been receiving attention in analytical chemistry to realize integration of parallel and sequential operations on a solid substrate [10–13]. Microfluidic devices have rendered possible the guiding of chemicals based on laminar flow [14–18]. This technology has been applied for local patterning of proteins [14], targeted delivery of cells, and removal of attached cells [15,16]. However, for most chip designs, microfluidics has currently been constructed in “closed” channels based on the lab-on-a-chip concept. Delamar and coworkers invented the remarkably innovative microfluidic probe (MFP) that can be applied in “open” space and, therefore, can form a focused laminar flow at any site for protein patterning, cell lysing, and injecting fluorescent dyes into target

cells [19,20]. The original MFP possessed a mesa structure prepared with a silicon chip and two apertures for injection and aspiration.

In the current study, we first applied the MFP technology for collection of messenger RNA (mRNA) from single adherent cells. The probe has been constructed with a dual pipette and comprised a theta (θ)-shaped glass capillary. Pulled glass capillaries [21–24] and dual pipette probes [25–30] have been used in various analytical tools with micromanipulators, including physiology, capillary electrophoresis, and electrochemistry, as well as for various scanning probe microscopies (SPMs). An exclusive characteristic of MFP allows the aspirated sample solution to be used for further analysis, similar to that in perfusion or microdialysis. We collected mRNA from monolayer-cultured cells, namely the human breast cancer cell line MCF-7 and the malignant human mammary epithelial cell line HMT-3522 T4-2 (T4-2) [31,32], using the probe for injecting a lysis buffer and aspirating the lysed cytosol. The collection efficiency of the mRNA was evaluated quantitatively using real-time PCR. This technique has a significant impact in the research field of single cell analysis because the collection process is the bottleneck to automatically perform sequential chemical processing on an integrated μ TAS device. We focused on the expression levels of β 1-integrin, a major protein to regulate cell–cell and cell–ECM (extracellular matrix) interactions, because the microfluidic dual capillary probe is principally applicable to collect

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¹ Abbreviations used: μ TAS, micro-total analysis system; MFP, microfluidic probe; mRNA, messenger RNA; SPM, scanning probe microscopy; T4-2, HMT-3522 T4-2; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; RNase, ribonuclease; RT, reverse transcription; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

adherent cells and spheroids at any position. It is already known that $\beta 1$ -integrin expression for MCF-7 is significantly smaller than that for T4-2. Also, it is known that $\beta 1$ -integrin expression for spheroid culture is larger than that for monolayer culture. Therefore, as a model system, MCF-7 and T4-2 cell lines cultured in monolayer and spheroid cultures were selected for the mRNA analysis samples.

Materials and methods

Microfluidic dual capillary probe

A theta-shaped borosilica glass tube (1.5 mm o.d., 1.02 mm i.d., TST150-6, World Precision Instruments) was cut to a length of 50 mm and pulled with a capillary puller (PN-3, Narishige). The top of the dual pipette capillary was then planed with a diamond grinder (EG-6, Narishige) to fabricate a disk-shaped tip with an outer diameter of 40 to 100 μm (Fig. 1). It was difficult to prepare the probes with precisely the same orifice size.

The bottom of the dual pipette probe was connected to the two silica capillary tubes (25 cm in length, 0.375 mm o.d., 0.075 mm i.d., 31942, GL Science) by using epoxy glue. The two microsyringes, syringe 1 (used for injection) and syringe 2 (used for aspiration), were connected tightly with the silica capillary tube with a special adaptor set (Upchurch Scientific).

Cell culture

Methods for monolayer culture [33] and three-dimensional culture on reconstructed basement membrane (on-top culture) [34] have been described elsewhere [35]. The human breast cancer cell line (MCF-7) was donated by the Cell Resource Center for Biomedical Research (Tohoku University). The malignant human mammary epithelial cell line (T4-2) [32] was obtained from the American Type Culture Collection. RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 50 $\mu\text{g ml}^{-1}$ penicillin (Gibco), and 50 $\mu\text{g ml}^{-1}$ streptomycin (Gibco) was used for MCF-7 culture. Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco) containing 250 ng ml^{-1} insulin (Boehringer

Mannheim), 10 $\mu\text{g ml}^{-1}$ transferrin (Sigma), 2.6 ng ml^{-1} sodium selenite (Collaborative Research), 0.1 nM estradiol (Sigma), 1.4 μM hydrocortisone (Collaborative Research), and 5 $\mu\text{g ml}^{-1}$ prolactin (Sigma) was used for culturing T4-2. For monolayer culture, The bottom of a 35-mm polystyrene culture dish (BD Biosciences) was coated with type 1 collagen solution (Research Institute for the Functional Peptides) by incubation for more than 30 min. Cells were seeded onto the collagen-coated dish as a single cell suspension. For on-top culture, the bottom of a 35-mm polystyrene culture dish was initially coated with 30 μl of Matrigel (BD Biosciences). The Matrigel coated on the culture dish was solidified at 37 $^{\circ}\text{C}$ for 10 min. Single cells were seeded on the layer of the Matrigel with 50 μl of 1 to 5×10^4 cells/ml cell suspension in medium solution containing 2% Matrigel. Single cells were adherent onto the Matrigel layer after 30 to 60 min incubation at 37 $^{\circ}\text{C}$. The culture dish was further filled with the medium containing 2% Matrigel. Cells proliferate and form spheroids after 3 days.

Cell lysis and mRNA collection with the microfluidic dual capillary probe

The probe was silanized with a 50- μl drop of 4,4,5,5,6,6,6-nonafluorohexyltrichlorosilane (LS-912, Shin-etsu Chemical) evaporated in a closed 200-ml beaker under a nitrogen atmosphere at room temperature for 2 h. The probe and the two syringes were washed with 99.6% ethanol. The probe was set on an XYZ stage, and the two syringes were controlled with syringe pumps (KDS200&KDS230, Muromachi). The distance between the probe and the sample was defined by gently touching the bottom of the culture dish, and the probe was retracted 20 μm apart from the bottom using a motor-driven XYZ stage and a stage controller (D73MS, Suruga Seiki). Then the probe was located at the center of the microscope view.

Cell extraction using microfluidic dual capillary probe

Fig. 1 shows a scheme of the experimental setup. The lysis buffer RLT (50 μl , RNeasy Micro Kit, Qiagen) was roared into the injection syringe (syringe 1) and the aspiration syringe (syr-

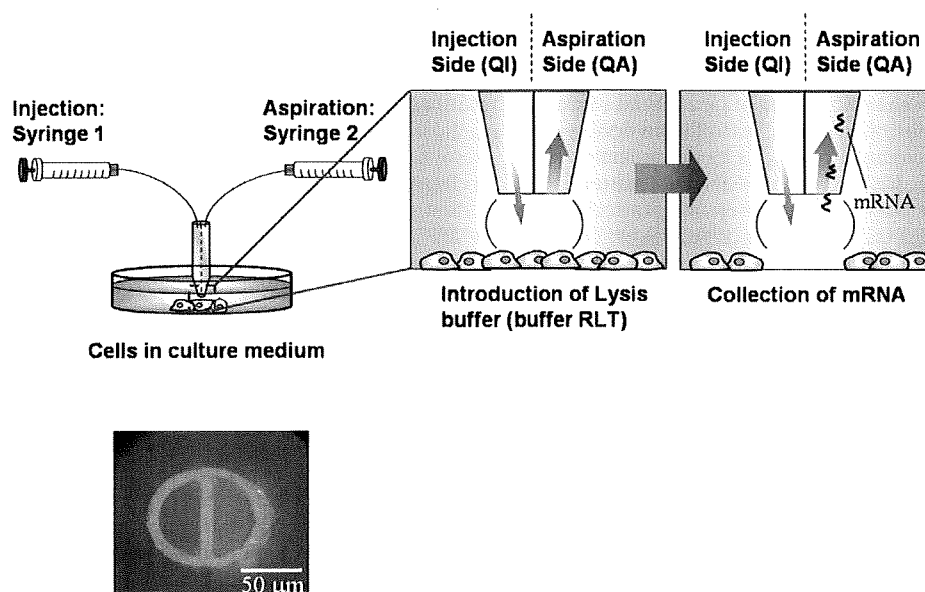


Fig. 1. Scheme of the experimental setup. QI (injection flow rate) and QA (aspiration flow rate) were set at 40 and 360 nl/min, respectively. The flow rate ratio (QA/QI) was set at 9. A bottom view of the theta-shaped microfluidic dual capillary probe is also shown.

inge 2). mRNA was collected from MCF-7 monolayer cells by using the microfluidic dual capillary probe. The injection (QI) and aspiration (QA) flow rates were set at 40 and 360 nl/min, respectively. The flow rate ratio (QA/QI) was 9 [19]. After the observation of cellular lysis and removal from the culture dish, the probe was transferred into another dish filled with 2 ml of buffer RLT and further aspirated at 5 μ l/min for 10 min. Initially, syringe 2 was loaded with 50 μ l of buffer RLT; thus, the final volume of the collected cell-lysed solution with syringe 2 resulted in approximately 150 μ l. The collected cell lysate solution was poured out into a 0.2-ml polymerase chain reaction (PCR) tube. Then 5 μ l of the 4 ng/ μ l carrier RNA (Qiagen) was added into the PCR tube for preventing the degradation of the targeted RNA originating from contaminated ribonuclease (RNase). The carrier RNA also guards the adsorption of the targeted RNA onto the PCR tube.

The solution in the PCR tube was then purified using an RNeasy Micro Kit (Qiagen). Reverse transcription (RT) reaction and real-time PCR were performed using a QuantiTect Reverse Transcription Kit (Qiagen) and a LightCycler FastStart DNA Master Kit (Roche), respectively. The real-time PCR (LightCycler 1.5, Roche) was performed for at least two genes using 2 μ l of the sample complementary DNA (cDNA) taken from 20 μ l of the synthesized source cDNA solution. The running conditions have been described elsewhere [35]. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank Accession No. M33197) and β 1-integrin (GenBank Accession No. NM_133376) were designed and synthesized by Nihon Gene Research Laboratories. The actual sequences, apricon sizes, and annealing temperatures of the primers were listed as follows: GAPDH, forward 5'-TGA ACG GGA AGC TCA CTG G-3', reverse 5'-TCC ACC CTG TTG CTG TA-3', 307 bp, 62 °C; β 1-integrin, forward 5'-GTC CAA CCT GAT CCT GTG TC-3', reverse 5'-GCA ACC ACA CCA GCT ACA AT-3', 167 bp, 66 °C. The precise GAPDH copy number was determined using a standard plasmid DNA-containing GAPDH synthesized by Nihon Gene Research Laboratories. We also performed bulk measurement using 10^5 to 10^6 cells as a starting sample. In this case, an RNeasy Mini Kit (Qiagen) was used for RNA purification. For the monolayer cultured cell sample, the medium (RPMI 1640 or DMEM/F12) of the culture dish was changed to the cell lysis buffer RLT (Qiagen), and the lysate was further analyzed to estimate the relative β 1-integrin expression level normal to GAPDH for each cell type. The process for spheroid lysis and mRNA extraction was basically the same as that for the monolayer adherent cells.

Results and discussion

Fig. 2A shows the sequential photographs of the sample MCF-7 monolayer cultured on a 35-mm polystyrene dish before and after the cellular collection using the dual capillary probe at a probe-sample distance of 20 μ m. Initially, syringe 2 was turned on to aspirate the culture medium from aperture 2 at a flow rate of 360 nl/min (QA). Syringe 1 was immediately used to inject the cell lysis buffer from aperture 1 at a flow rate of 40 nl/min (QI). This protocol ensured that cell lysis buffer solution never spilled out of the targeted area. As seen in Fig. 2A, the target cells were removed within 30 s and the number of lysed cells was found to be 11. The margins of the removed cells are nearly intact, ensuring that the cell lysis buffer attacks only the targeted area during laminar flow when introduced and never leaks out of the targeted area. In the same manner, one cell collection was demonstrated from adherent MCF-7 cells seeded with a lower cellular density (Fig. 2B).

Fig. 3 shows the time course during the real-time PCR operation and the melting curve analysis performed after the real-time PCR

for β 1-integrin mRNA sample collected from MCF-7 monolayer. These results were obtained from 11 cells and 1 cell using the microfluidic dual capillary probe corresponding to the photographs shown in Fig. 2A and B, respectively. The peak in the melting curve was well correlated with the signal obtained for the bulk of cellular samples with 10^5 to 10^6 cells.

The copy number of the GAPDH, which was collected from the MCF-7 monolayer (including 1–17 cells, $n=6$) by using the probe, was 76.7 copies/cell. This value corresponded to the apparent collection efficiency 7.6% because the copy number estimated using bulk measurement ($\sim 10^6$ cells) was 1014 copies/cell. This low efficiency is due to the instability of the mRNA; the expected collection efficiency with QA/QI = 9 was approximately 100%, as shown in the literature [19]. In our case, the collection efficiency of approximately 100% was achieved for a QA/QI value larger than 8 when a solution containing 10^5 particles/ml of 6- μ m-diameter microspheres (Polysciences) was introduced through the microfluidic dual capillary probe. The concentration of the product (mRNA) should be high; thus, the QA/QI value cannot be set at an undesirably larger value for our objective, that is, mRNA collection. The smaller the gap distance becomes, the larger the expected collection efficiency increases. The orifice size did not seem to affect the collection efficiency of the mRNA. The microfluidic dual capillary probe with less than 10 μ m diameter can be fabricated, and we observed that a part of the MCF-7 spheroid was lysed with the smaller probe. However, in the current stage, we have not succeeded in RNA analysis with the smaller probe.

The retention time of the cell-lysed solution including mRNA and buffer RLT within the aspiration side compartment of the probe is 38 min, which is estimated from the volume of the aspiration side compartment (13.7 μ l, a half-volume of the dual capillary probe) and the QA value (360 nl/min). This time scale is significantly large compared with the actual time period taken for the process of cell lysis (30 s), implying that the collected mRNA remains in the probe compartment but does not reach syringe 2. During the cell lysis, buffer RLT is diluted to approximately 10% of the original concentration, and this may be the main reason for the mRNA degradation given the roles that buffer RLT plays in RNase deactivation and RNA stabilization. After the cell lysis process, the probe was transferred into a buffer RLT pool and further aspirated with 50 μ l (see Materials and Methods). In this step, the cell-lysed sample surely reaches syringe 2, where the concentration of buffer RLT recovers to the original one because the volume of the cell-lysed sample (180 nl) is negligibly small compared with the total volume of buffer RLT (150 μ l).

Although the mRNA collection efficiency was low in the current stage, comparison of the gene expression at individual cell level was possible. Expression of β 1-integrin for MCF-7 and T4-2 collected using the microfluidic dual capillary probe was surveyed normal to that of GAPDH for each. Fig. 4 reveals the relative β 1-integrin expression for a small number of adherent cells (1 to 17 MCF-7 cells [$n=6$] and 1 T4-2 cell [$n=3$] cultured with monolayer). The β 1-integrin expression for the T4-2 cells was approximately 10-fold greater than that for the MCF-7 cells. The results obtained using the current method with the microfluidic dual capillary probe are similar to those obtained using a conventional method based on the collection of bulk cells ($\sim 10^6$ cells), indicating that the current method provided quantitative information regarding the expression levels of the target gene in the two cell lines. T4-2 expresses β 1-integrin at substantially higher level than does MCF-7 [31]. Although our theta-shaped probe was very easy to fabricate, the performance was comparable to that achieved by a sophisticated MFP [19].

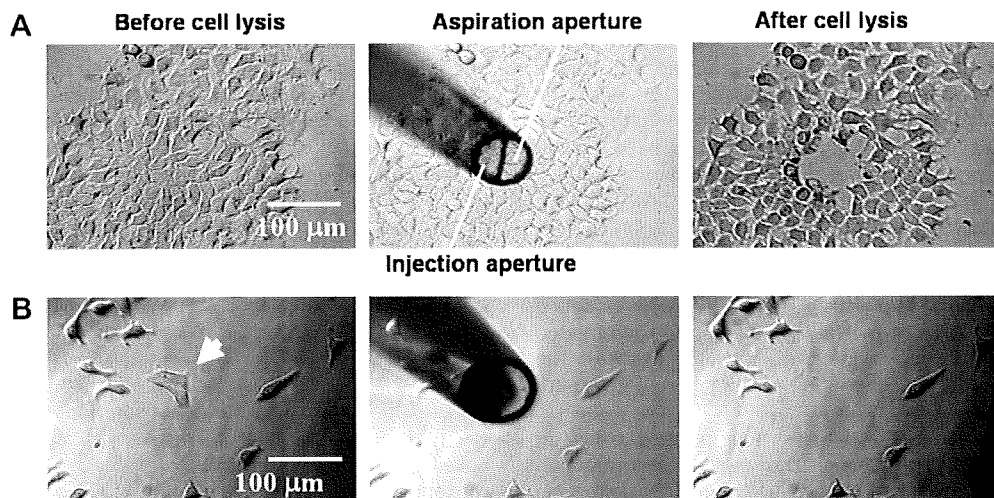


Fig. 2. (A) Sequential photos of MCF-7 monolayer before and after the RNA collection using the microfluidic dual capillary probe. The injection (QI) and aspiration (QA) flow rates were set at 40 and 360 nl/min, respectively. The distance between the probe and the bottom of the culture dish was set at 20 μm . It takes 10 to 30 s for the cell lysis to be completed, and the total volume of the cell-lysed solution was approximately 150 μl . From this picture, 11 cells were collected to further mRNA analysis. (B) In the same manner, the single cell collection was available from adherent MCF-7 cells seeded with a lower cellular density.

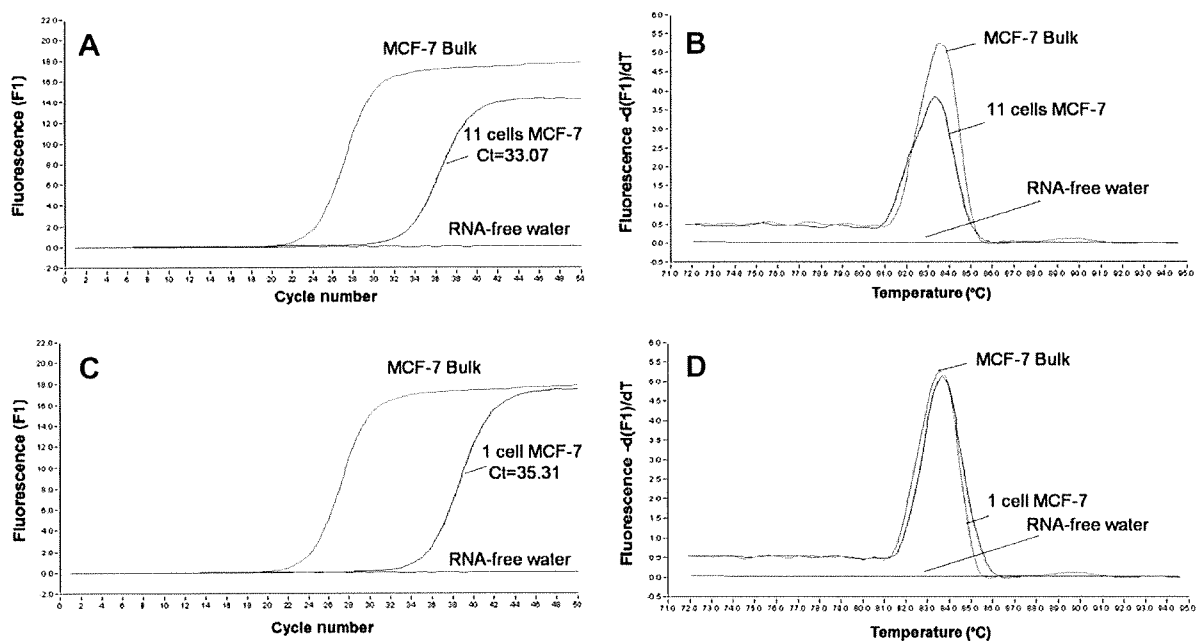


Fig. 3. Time course of the fluorescence intensity during the real-time PCR operation (A and C) and melting curve analysis performed after real-time PCR (B and D) for the $\beta 1$ -integrin mRNA sample collected from MCF-7 monolayer (11 cells [A and B] and 1 cell [C and D] shown in Fig. 2) using the microfluidic dual capillary probe.

Moreover, our method enabled collection of mRNA from a single spheroid, namely, an aggregated cellular mass in a three-dimensional cultures system. MCF-7 and T4-2 cells were cultured onto a Matrigel sheet to form a spheroid 3 days after the single cell seeding. Initially, we tried to pick one cell or several cells from a spheroid using the microfluidic dual capillary probe, but it was difficult to lyse a part of the single spheroid with a diameter of 100 μm . Fig. 5 shows the relative $\beta 1$ -integrin expression from single spheroids (MCF-7 [$n = 4$] and T4-2 [$n = 2$]). The $\beta 1$ -integrin expression for the spheroid was identical to, or a little bit larger than, that cultured with monolayer for both cell types. The results obtained for bulk measurement (~ 8000 spheroids) were in good

agreement with those obtained for individual spheroids collected using the theta-shaped probe. Again, the results of the relative gene expression profiles clearly identified the differences between the two cell types.

Conclusions

In conclusion, MFP technology has been applied, for the first time, for the mRNA collection and analysis of single adherent cells and single spheroids. The collection efficiency was estimated as 7.6% using the GAPDH mRNA from single MCF-7 cells. Using the current method, a quantitative discussion is possible to distinguish

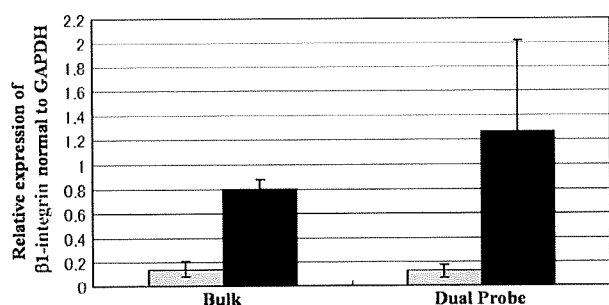


Fig. 4. Relative expression levels of $\beta 1$ -integrin normal to GAPDH for MCF-7 cells (gray bars) and T4-2 cells (filled bars) cultured with monolayer. mRNA collected from bulk ($\sim 10^6$ cells) (left) and the microfluidic dual capillary probe (right).

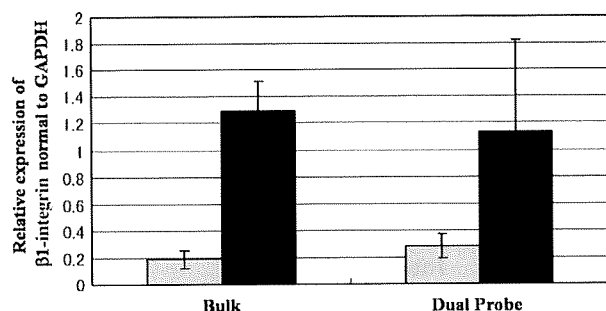


Fig. 5. Relative expression levels of $\beta 1$ -integrin normal to GAPDH in spheroids for MCF-7 cells (gray bars) and T4-2 cells (filled bars) three-dimensionally cultured for 3 days onto a Matrigel sheet. mRNA was collected from bulk (~ 8000 spheroids) (left) and the microfluidic dual capillary probe (1 spheroid) (right).

the difference in the gene expression levels between the two cell lines: MCF-7 and T4-2. The current method is also applicable to mRNA analysis in a three-dimensional culture system.

Acknowledgments

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—Mini Review—

Morphological Evaluation and Measurement of the Respiration Activity of Cumulus-oocyte Complexes to Assess Oocyte Quality

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Abstract: Scanning electrochemical microscopy (SECM) is a non-invasive and sensitive technique for measuring cellular respiration. In this paper, we review the SECM technique, to establish it as an accurate method for measuring the respiratory activity of single cumulus-oocyte complexes (COCs) and oocytes in animals as well as in humans. Oxygen consumption rates of COCs are influenced by the surrounding cumulus volume and the mitochondrial activity of the cumulus cells. An increase in the oxygen consumption rate was found in bovine oocytes, whereas the oxygen consumption of human oocytes tends to decrease during *in vitro* maturation (IVM). To analyze the metabolic activity of mitochondrial respiration, ATP content and mitochondrial distribution in bovine oocytes have been examined. An electron microscopic study confirmed mitochondrial reorganization in bovine oocytes during oocyte maturation. These results show that the respiratory activity of oocytes changes with maturation status during IVM and mitochondrial reorganization may partly influence respiratory activity. The SECM procedure is therefore a useful technique for evaluating the metabolic activity and quality of oocytes and cumulus cells in the IVM process.

Key words: Cumulus cells, Oocyte maturation, Mitochondria, Oxygen consumption, Electrochemical measurement

Introduction

The *in vitro* maturation (IVM) of human oocytes is an attractive technique that provides a patient-friendly approach to assisted reproductive technology. IVM is relatively simple with a shorter period of treatment and lower costs than conventional *in vivo* fertilization (IVF). For anovulatory patients with polycystic ovaries (PCO), a decrease in the dose of ovarian stimulating drugs lowers the risk of ovarian hyperstimulation syndrome. IVM has been successfully applied to animals [1, 2]. Cha *et al.* were the first group to show the success of IVM in human beings using immature donor oocytes retrieved from antral follicles [3]. Recent studies have demonstrated that the results from IVM are comparable to those achieved with contemporary IVF [4, 5]. The applicability and development of IVM technology is dependent on the improvement of *in vitro* culture systems. During *in vitro* culture, cumulus cells play an important role in oocyte maturation. If provided with the several factors that are essential for normal nuclear and cytoplasmic maturation, oocytes can mature and develop to an embryo after fertilization [6]. Therefore, an appropriate evaluation of cumulus-oocyte complexes (COCs) is indispensable for evaluating the quality of oocytes and improving of the results of IVM.

Over the years, several approaches have been used to evaluate COCs. Morphological evaluation is the main technique used to assess COC quality and to predict the subsequent maturation of oocytes in the IVM process. However, morphological evaluations are subjective and difficult, especially for COCs with

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intermediate morphological qualities. Therefore, more objective evaluation criteria are needed. Previous studies have suggested that a greater understanding of the metabolic respiration of cumulus cells might yield new strategies for evaluating the quality of bovine oocytes [7, 8]. In this paper we describe the morphological evaluation of COCs and the application of a novel cell respiration measuring system using scanning electrochemical microscopy (SECM) to the assessment of the metabolic activity of cumulus cells and oocytes in bovine and human specimens.

Morphological Evaluation of Cumulus-oocyte Complexes and Oocytes

An appropriate evaluation of COCs is indispensable for the improvement of the IVM system, because cumulus cells play an important role in oocyte quality. For a morphologically precise evaluation of human COCs, size of an oocyte is an important parameter. The precise evaluation of human COCs is needed to predict the competence of oocyte maturation. Prior research has indicated that the diameter of immature oocytes is one of most reliable parameters for predicting oocyte quality. Eppig and Schroeder reported that mice oocytes isolated from females younger than 13 days of age are capable of spontaneous break down of the germinal vesicle (GVBD) when the mean diameter is greater than 60 μm [9]. In a study of porcine immature oocyte, progression to metaphase II was observed in 40% of oocytes that were over 110 μm in diameter, whereas no oocyte less than 90 μm in diameter resumed meiosis [10]. In rhesus monkey oocytes, in which meiotic competence occurs late during oocyte development, oocyte diameters appear relatively constant as the competence to undergo GVBD increases. These phenomena suggest that there is no association between oocyte diameter and maturation [11].

In comparison to animal systems, little is known about humans. Based on data from unstimulated polycystic ovary syndrome (PCOS) patients, Cavilla *et al.* deduced that an oocyte diameter of 81 μm at the time of retrieval was the threshold for GVBD, whereas oocytes of more than 103 μm would mature to metaphase II [12]. They also noted that, during *in vitro* human oocyte maturation, an increase in the average diameter of only 3 μm (from 106 to 109) represents a large change in the cytoplasmic volume (increasing an astonishing 8% during culture), suggesting that oocyte diameter provided valuable information about oocyte potential

during IVM. In a proposal for the precise evaluation of human immature oocytes, the two-dimensional area of a depiction image was calculated using image analysis software "Image J".

Another important parameter of oocyte morphological quality is the volume of the human cumulus mass. During IVM, cumulus cells are known to maintain the oocyte nucleus at the GV stage [13]. Expanded human cumulus-oocyte complex patterns have a higher expression of LH receptor mRNA and are associated with more efficient oocyte maturation [14]. Early reports supported the idea that cumulus expansion during IVM improves the developmental capacity [15, 16]. In mice, the fertilization rate of IVM matured oocytes is correlated with the quantity and quality of the expanded cumulus mass [17]. The mechanical loss or spontaneous loss of cumulus cells from COCs has been shown to correlate with a loss of fertilizability [18]. Therefore, the quantity of cumulus mass is a factor influencing the success of IVM. For the morphological classification of cumulus mass, some researchers have separated the cumulus patterns into multilayered and expanded [19, 20].

In this review, we have estimated the multilayered and expanded cumulus mass as the consecutive change and made objective evaluations utilizing an image analysis software program. The COC area was calculated by tracing the edge of the cumulus mass. If the edge was not clear (usually observed in the expanded cumulus mass), the image was analyzed using an edge enhancement mode. The multiple layer formation of the cumulus mass was presented as the C-ratio (area of COC / area of immature oocyte). For the morphological evaluation of human COCs, forty-two human COCs, retrieved from eight women with the PCOS during an IVM program, were used. All COCs were aspirated 36 hours post-hCG between the 10th and 12th day of the menstrual cycle and cultured for 26 hours in TCM199 medium with 10% patient serum, 100 IU/L human chorionic gonadotropin and 75 IU/L follicle stimulating hormone under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. The parameters analyzed were (1) *Area*; area of the immature oocyte and (2) *Layer*; multiple layer formation of the cumulus mass, presented as the C-ratio. As shown in Fig. 1, these two morphological parameters were compared between the immature (germinal vesicle: GV or metaphase I: MI) and mature (metaphase II: MII) oocyte groups after *in vitro* culture. In the *Area* comparison, the mean level of *Area* was significantly higher in the mature group ($8,886 \pm 184$ vs. $9,806 \pm 161$, Mean \pm SEM, $P < 0.05$). In the

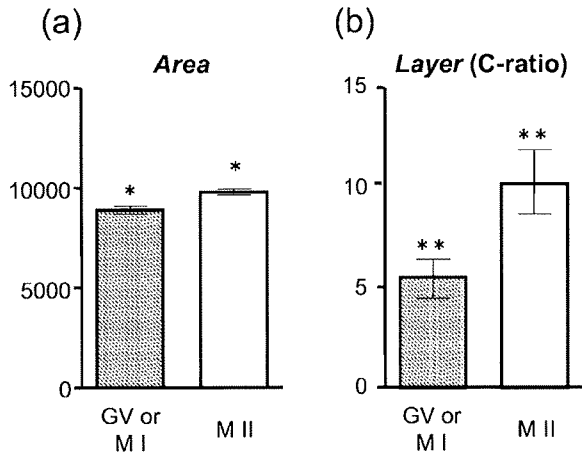


Fig. 1. Comparison of morphological parameters of human immature oocyte and COCs. a: *Area*, Area of immature oocyte; b: *Layer*, Multiple layer formation of cumulus mass was evaluated as C-ratio (Area of COC / Area of immature oocyte). Area and Layer were analyzed before *in vitro* culture and compared prospectively between mature (MI) and immature (MI or GV) group. *, **: significantly different ($P < 0.05$).

Layer comparison, the mean level of the C-ratio was significantly higher in the mature group (5.8 ± 0.8 vs. 10.3 ± 1.6 , Mean \pm SEM, $P < 0.05$). These results suggest the C-ratio is a useful parameter for predicting the maturation status of oocytes in the IVM process.

Subsequently, we examined the relationship between

the cumulus mass morphology and the oocyte quality. Human COCs were classified into five grades based on cumulus mass morphology as follows: Grade 1 (G1), cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; Grade 2 (G2), cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; Grade 3 (G3), regular round oocytes with cumulus cells covering half of the domain; Grade 4 (G4), naked oocytes without cumulus mass; Grade 5 (G5), naked and irregular shaped oocytes (Fig. 2). High maturation rates of immature oocytes were detected in G1 and G2 (70.0% and 64.3%, respectively) in contrast to the lower maturation rate of 17.7% (mean percentage from G3 to G5).

Cumulus cells are a production site of steroids, growth factor, proteins and other compounds that contribute to cytoplasmic maturation of oocytes. Beneficial effects of cumulus cells on microtubule dynamics and/or chromatin stability, oocyte maturation and early embryonic development have been reported in many species, including humans [21–23]. Cumulus cells are also known to play an important role in the regulation of the meiotic progression of oocytes. During the growth and development of meiotic competence of an oocyte, the cumulus cells are responsible for maintenance of nuclear arrest at the germinal vesicle (GV) stage by transfer of an inhibitory signal through gap junctions which elevates the intracellular cyclic adenosine monophosphate (cAMP) level in the oocytes

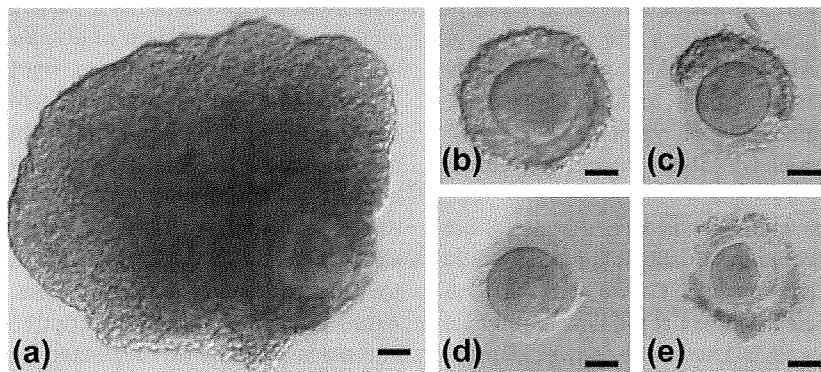


Fig. 2. Light micrographs of human COCs classified by morphological evaluation. (a) Grade 1, cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; (b) Grade 2, cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; (c) Grade 3, regular round oocytes with cumulus cells covering half of the domain; (d) Grade 4, naked oocytes without cumulus mass; (e) Grade 5, naked and irregular shaped oocytes. Bars = 20 μ m.

[24, 25]. Although the precise regulation mechanism of meiotic progression is still controversial [26], it has been suggested that well-developed cumulus cells have the capacity to regulate appropriate maturation and the development of immature oocytes.

Evaluating the Quality of Embryos and Oocytes with Measurement of Respiration Activity with an Electrochemical Measuring Technique

The metabolic activity of embryos and oocytes has been determined from the consumption of nutrients, such as glucose, pyruvate and amino acids [27–30]. Oxygen consumption is an idea indicator of overall metabolic activity because adenosine triphosphate (ATP) is predominantly generated by oxidative phosphorylation, a process in which oxygen plays an essential role [31–33]. Oxygen consumption by embryos and oocytes has been studied with various measuring techniques, such as the Cartesian diver [34, 35], spectrophotometry [36, 37], ultrafluorescence measurements [38, 39], and self-referencing microelectrodes [40–43].

Electrochemical measurement using scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species, such as oxygen near the sample surface [44]. This technique can measure the concentration profile of a metabolic product around a spherical sample, such as an embryo, with a probe microelectrode. We have employed the SECM technique to examine oxygen consumption by single embryos [45]. Using a modified SECM measuring procedure, we quantified the respiration activity of embryos in several animal species including humans [46]. SECM can non-invasively measure the respiration activity of single embryos from livestock, such as cattle and pigs, as well as those from small rodents, all with high reproducibility. We recently demonstrated that bovine embryos with high oxygen consumption are better candidates for further development into good quality embryos and yielded higher pregnancy rates after embryo transfer. The respiration activity correlates with the embryo quality. SECM is a highly sensitive and non-invasive method for measuring cellular respiration and may be a valuable tool for accurately assessing the quality of embryos, which could contribute to improved outcomes in assisted reproduction, including human IVF. On the other hand, an accurate method for evaluating the respiratory activity of oocytes remains to be developed.

Table 1. Oxygen consumption rates ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of bovine COCs and denuded oocytes in oocyte maturation cultures

Maturation status	COC (n)	Oocyte (n)
Immature	5.48 ± 0.82 (16) ^a	0.67 ± 0.02 (16) ^c
Mature	3.15 ± 0.42 (20) ^b	1.10 ± 0.05 (20) ^d

Values with different superscripts in each column differ significantly ($P < 0.05$).

Oocyte quality could be the most important factor in determining successful fertilization and embryo development. Therefore, we attempted to establish an evaluation system for oocyte quality based on the respiratory activity of oocytes.

In previous studies, we evaluated the SECM technique, to establish an accurate method for measuring the respiratory activity of single bovine and porcine oocytes [8, 48]. With the SECM procedure, oxygen consumption of bovine COCs and denuded oocytes was monitored (Table 1). Oxygen consumption rates ($\times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of immature COCs and oocytes (immediately after recovery from an ovary) were 5.48 and 0.67, respectively. Although the respiration rate of denuded oocytes was lower than that of cumulus cells, the oxygen consumption rate by a single bovine oocyte was quantitatively measured by SECM.

Oxygen consumption has been monitored in COCs and oocytes cultured in serum-free medium for oocyte maturation. An increase in the oxygen consumption rate was found in oocytes [1, 10], whereas the oxygen consumption by COCs [3, 15] decreased during IVM. To analyze the metabolic activity of mitochondrial respiration, the ATP content and mitochondrial distribution in oocytes were examined. The ATP content of oocytes after maturation culture was significantly higher than that of immature oocytes (Fig. 3). In immature oocytes, staining with MitoTracker Orange revealed mitochondrial clumps with a strong signal in the periphery of the cytoplasm (Fig. 4). After IVM, the mitochondrial clumps were located more toward the center of the cytoplasm. An electron microscope study confirmed mitochondrial reorganization in bovine oocytes during oocyte maturation (Fig. 5). These results show that the respiratory activity of bovine oocytes increases during IVM and mitochondrial reorganization may thus be partly due to the respiratory activity. Therefore, we consider the SECM procedure is a useful technique for evaluating the metabolic activity and quality of single oocytes.

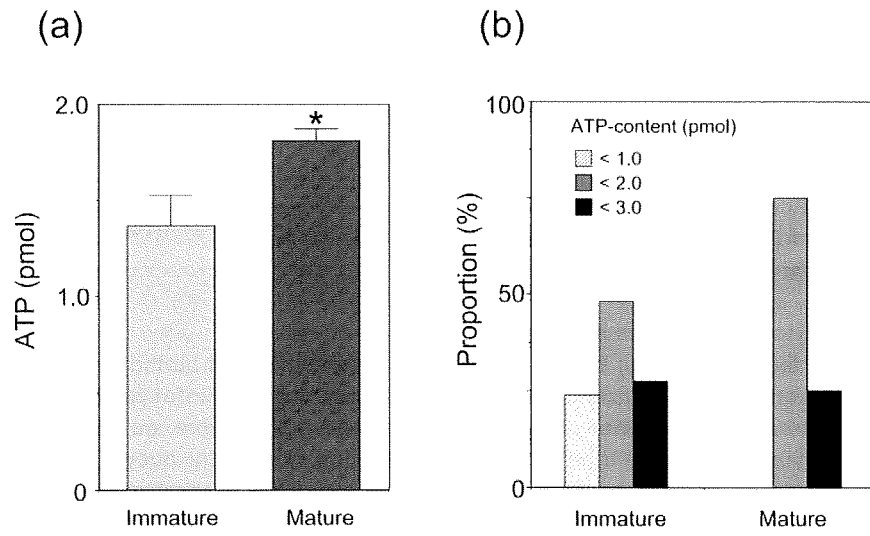


Fig. 3. (a) The ATP content and (b) proportion of oocytes categorized by ATP content: immature and mature bovine oocytes. *: significantly different ($P < 0.05$).

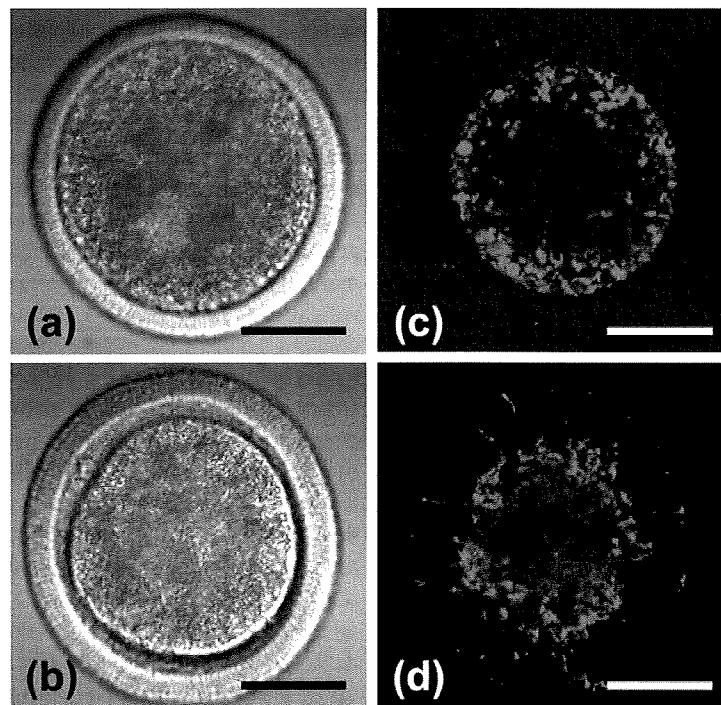


Fig. 4. Midline confocal sections of (a, b) immature and (c, d; cultured in IVMD101 medium) mature bovine oocytes stained by MitoTracker orange. Bars = 50 μ m.

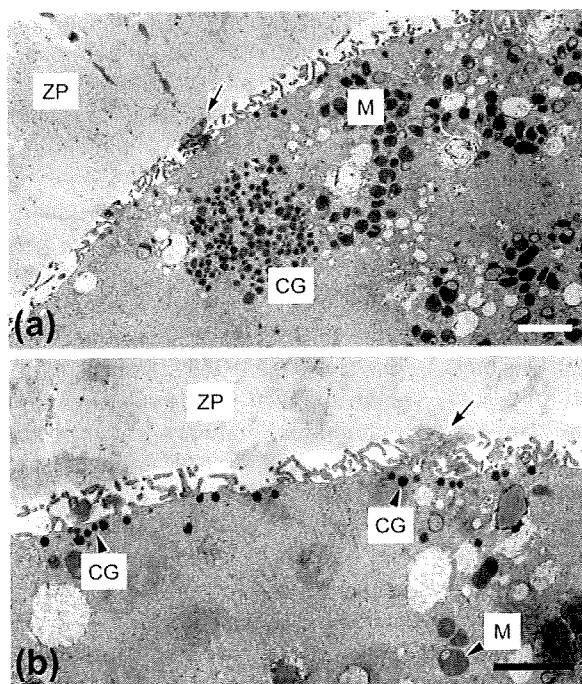


Fig. 5. Electron micrographs of (a) immature and (b) cultured in serum-free maturation medium mature oocytes. a: Many aggregates of mitochondria (M) and cortical granules (CG) were present in the cortex cytoplasm. b: Cortical granules were distributed in the periphery of the cytoplasm, but aggregates of mitochondria were not found. ZP, zona pellucida. Arrows: microvilli. Bars = 2 μ m.

Measuring the Respiration Activity of Human COCs and Oocytes

In this part, we review the respiration activity of human COCs and oocytes. Eighty-five human COCs retrieved from eighteen women with the PCOS during an IVM program were examined. Informed consent for the use of the COCs in this study was obtained from all the patients. Thirteen of the eighteen women were administered a short course of follicular stimulating hormone (FSH) and five women accomplished an IVM program without the use of FSH before hCG administration. All COCs were aspirated 36 h post-hCG between the 10th and 12th day of the menstrual cycle and cultured 26 h in TCM199 medium with 10% patient serum, 100 IU/L human chorionic gonadotropin and 75 IU/L follicle stimulating hormone under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Cellular unevenness of the cumulus mass has an influence on SECM measurement results. Therefore,

Table 2. Oxygen consumption rates ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of human COCs classified by morphological evaluation

Category	Pre-culture (n)	Post-culture (n)
G1	7.79 ± 1.00 (50)	6.11 ± 0.74 (50)
G2	1.46 ± 0.15 (25)	1.63 ± 0.33 (25)
G3	1.26 ± 0.35 (8)	1.60 ± 0.55 (8)
G4	0.86 ± 0.30 (2)	0.79 ± 0.11 (2)
G5	0.77 (1)	0.35 (1)

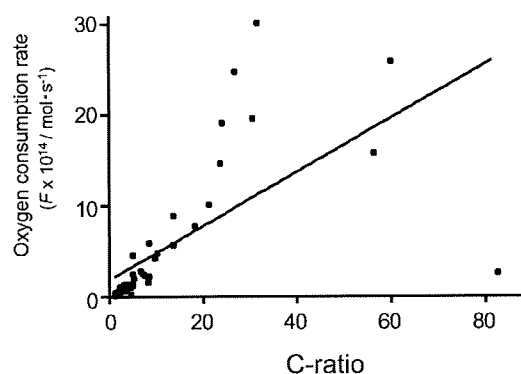


Fig. 6. Correlation between oxygen consumption rate and C-ratio.

the oxygen consumption rate was measured three times for each COC and the mean was used as the measured value.

Relationship between the oxygen consumption rates and morphological categories of human COCs in the pre-culture and post 26 hours-culture stages is shown in Table 2. A linear correlation between the oxygen consumption rate and the C-ratio was shown in Fig. 6 (correlation coefficient: $r^2 = 0.423$, $P < 0.01$). The respiration activity measured by SECM showed that the respiration activity of human COCs with multi-layer cumulus cells (G1) was higher than in the other categories (G2–G5). Ultrastructural studies revealed that the cumulus cells in G1 COCs, which showed high respiration activity, contained many well-developed mitochondria. In contrast, undeveloped mitochondria were scattered in the cumulus cells in G3 COCs (Fig. 7). These results suggest that respiration rates are directly influenced by the surrounding cumulus volume and mitochondrial activity in cumulus cells.

A comparison of the oxygen consumption fluctuation between the pre-culture stage and after 26 hours of culture is shown in Fig. 8. In the FSH administration group, the mean oxygen consumption rate tended to

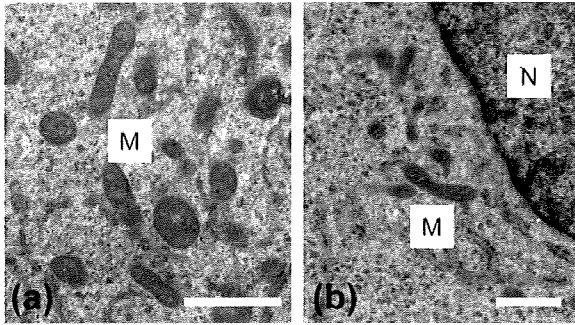


Fig. 7. Electron micrographs of human cumulus cells. Many well-developed mitochondria (M) are present in the cumulus cells of the Grade 1 COC (left image). In contrast, mitochondria showing small size are scattered in the cumulus cells of the Grade 3 COC. N: Nucleus. Bars = 1 μ m.

decrease after 26 h of culture (5.62 ± 0.83 vs. 4.25 ± 0.58). In contrast, the mean oxygen consumption rates were similar between the two stages in the non-FSH administration group (4.07 ± 1.19 vs. 4.17 ± 1.02). There was no clinical advantage gained by extending the FSH pre-treatment from 3 to 6 days to produce follicles more than 10 mm in diameter [49]. On the other hand, Wynn *et al.* demonstrated a higher maturation rate in a FSH treatment group [50]. In their study, the maturation rate to metaphase II was higher in the FSH

administration group (68.3% vs. 61.3%, in comparison to the non-FSH administration group, unpublished data). The benefit of FSH pre-treatment remains controversial and the development competence cannot be evaluated because of the limitations of the current study protocol.

The efficacy of the administration of hCG remains controversial. In hCG protocol, all patients are administered hCG before oocyte retrieval. After the LH surge, oocytes resume the first meiotic division and enter the second division [51]. At the same time, cumulus mass begins to change to the expanding form. Cumulus expansion may influence a variety of fundamental developmental changes which occur during fertilization. Regarding the use of hCG in bovine, the cumulus cells from antral follicles as small as 5 mm have mRNA transcripts for LH receptors and may respond to hCG stimulation [52]. This finding provides evidence of a mechanism by which hCG begins the maturation process of small antral oocytes *in vivo* and facilitates the completion of meiosis *in vitro*. Chian *et al.* demonstrated that the percentage of oocytes achieving maturation after 48 h *in vitro* culture was significantly higher in the hCG-primed group than in the non-hCG-primed group during human IVM-IVF [53].

Finally, the results of the respiration measurement of single human oocytes using a SECM system are listed in Table 3. The oxygen consumption rate of pre-cultured oocytes (GV stage) was $0.49 \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$,

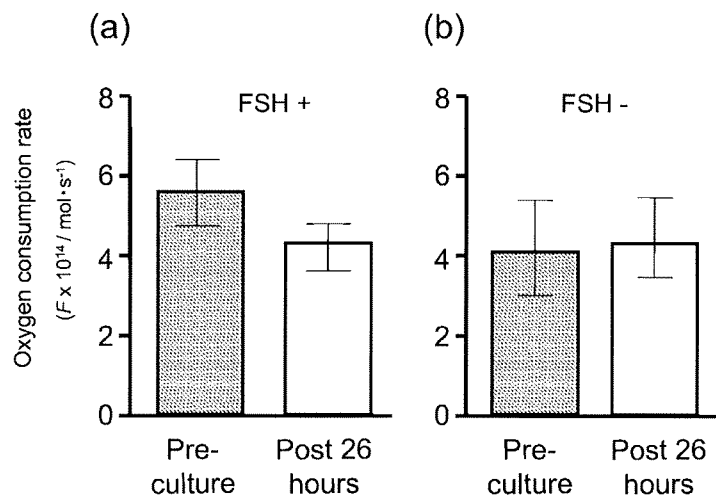


Fig. 8. Comparison of oxygen consumption with or without FSH administration at the pre-culture stage and after 26 h of culture. (a) oxygen consumption rate in the FSH administered group and (b) in the non-FSH administered group.

Table 3. Oxygen consumption rates ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of human denuded oocytes in oocyte maturation cultures

Maturation status	Oxygen consumption rate (n)
GV (Pre-culture)	0.49 ± 0.07 (10)
GV or MI (Post-culture)	0.40 ± 0.21 (19)
MI (Post-culture)	0.41 ± 0.15 (30)

whereas the oxygen consumption rate tended to decrease in matured MII oocytes ($0.41 \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$). These results suggest that the respiration activity of human oocytes changes with maturation status of oocytes, although the mechanism of this fluctuation needs to be confirmed with further studies.

Conclusions

The SECM technique is a non-invasive and sensitive method for measuring the oxygen consumption of individual COCs and oocytes in animal species including humans. The respiration activity of COCs is directly influenced by the surrounding cumulus cell volume and the mitochondrial activity of cumulus cells. Biochemical and cytological studies strongly suggest that oxygen consumption is an important parameter for evaluating the competence of oocyte maturation. It may be feasible to monitor the profile of an oocyte's mitochondrial activity by measuring its oxygen consumption, and select the oocytes that can sustain fertilization and the development of embryos. Therefore, the SECM technique may have a future in clinical application as a predictor of oocyte quality which could be used for determining to develop into good quality embryos.

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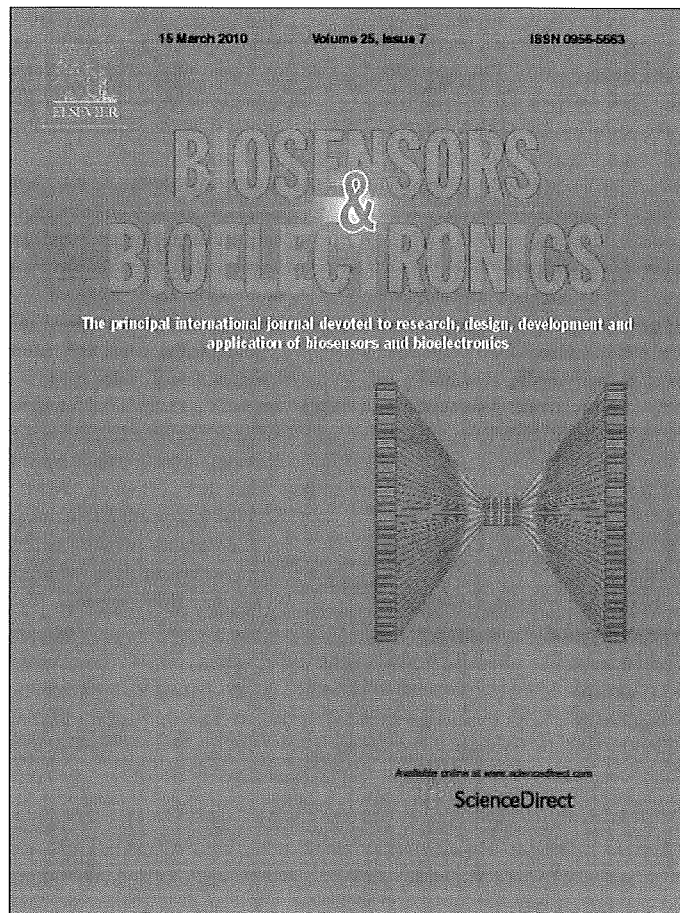
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Electrochemical monitoring of hydrogen peroxide released from leucocytes on horseradish peroxidase redox polymer coated electrode chip

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ABSTRACT

We developed an electrochemical-sensing device for continuous monitoring extracellular hydrogen peroxide (H_2O_2). The device consists of an indium-tin-oxide electrode coated with osmium-polyvinylpyridine gel polymer containing horseradish peroxidase (Os-HRP) and a poly-dimethyl siloxane well to house the cells on the chip. Granulocyte-like differentiated HL-60 cells were accommodated in the well and stimulated with phorbol 12-myristate 13-acetate (PMA), which triggered the generation of H_2O_2 . The extracellular H_2O_2 released from the cells was enzymatically reduced at the Os-HRP-modified electrode chip using Os(II) as an electron donor, resulting in reduction current responses by the device. The reduction current increased immediately upon PMA stimulation and this current transient was similar to that obtained by conventional chemiluminescence assays using sodium luminol. Apocynin, an inhibitor of NADPH oxidase activation, eliminated both the electrochemical and chemiluminescence signals. On the other hand, superoxide dismutase (SOD) increased the amperometric signals and catalase (CAT) decreased, whereas SOD decreased luminescence emission and CAT did not. These results were in accordance with the expected reaction mechanism, and strongly indicate that this new electrochemical-sensing device successfully detects extracellular H_2O_2 production.

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1. Introduction

Reactive oxygen species (ROS) are key mediators of the immune and inflammation processes. Leucocytes produce superoxide anion ($\bullet O_2^-$), a primary ROS generated through the activation of NADPH oxidase (Rossi, 1986). $\bullet O_2^-$ is unstable and rapidly disproportionated to hydrogen peroxide (H_2O_2) and oxygen in the presence or absence of superoxide dismutase (SOD) (Griendling and Harrison, 1999). H_2O_2 is relatively stable and permeable through cell membranes. Thus, the noninvasive measurement system, such as electrochemical detection, for H_2O_2 production from the leucocytes can provide essential information in understanding of the immune and inflammation processes. In the research of ROS production of the cells, phorbol myristate acetate (PMA) is widely used for triggering the respiratory burst by mimicking a physiological activator, diacylglycerol, and is known to induce the activation of NADPH oxidase (Hardy et al., 1991). The activation of NADPH oxidase and concomi-

tant ROS production are efficiently inhibited by apocynin, with an IC50 value of $10 \mu M$ in activated human neutrophils (Stefanska and Pawliczak, 2008).

Among the enormous studies for the measurement of ROS, luminol-amplified chemiluminescence techniques are widely used for the measurement of ROS because of their high sensitivity and simple handling (Dahlgren and Karlsson, 1999). However, luminol reacts with several ROS including H_2O_2 , $\bullet O_2^-$, NO, OH^- and ONO_2^- via complicated reaction pathways to emit chemiluminescent signal (Dahlgren and Karlsson, 1999; S. Kasai et al., 2005; Li et al., 1999; Radi et al., 1993). Of these substrates, the main chemiluminescence activity in respiratory bursts is believed to be derived from $\bullet O_2^-$ (S. Kasai et al., 2005; Lundqvist and Dahlgren, 1996), but it is very difficult to estimate the real contribution of ROS species. Electrochemical techniques have also been employed for real-time detection of the release of ROS from living cells. Amatore et al. (2006) simultaneously detected multiple ROS from a single macrophage using a carbon microelectrode. Pt electrodes have been commonly used for detecting H_2O_2 since Pt shows electrocatalytic activity for the oxidation of H_2O_2 at a potential of approximately +0.7 V vs. Ag/AgCl (Amatore et al., 2006; S. Kasai et al., 2005; Liu and Zweier, 2001). The use of horseradish peroxidase (HRP)-modified electrodes allows us to determine the H_2O_2

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