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Measurement of the Respiratory Activity of Single Human Embryos by Scanning Electrochemical Microscopy

Hiroyuki Abe[1*], Masaki Yokoo[2], Takahiro Itoh-Sasaki[1], Megumi Nasu[3], Kaori Goto[3], Yoko Kumasako[3], Yasuhisa Araki[4], Hitoshi Shiku[5], Tomokazu Matsue[5], Takafumi Utsunomiya[3]

[1*]Graduate Program of Human Sensing and Functional Sensor Engineering, Graduate School of Science and Engineering, Yamagata University, Yonezawa, Japan; [2]Innovation of New Biomedical Engineering Center, Tohoku University, Sendai, Japan; [3]St-Luke Clinic, Oita, Japan; [4]The Institute for ARMT, Gunma, Japan; [5]Graduate School of Environmental Studies, Tohoku University, Sendai, Japan

*To whom correspondence should be addressed Fax: 81-238-26-3361; e-mail: abeh@yz.yamagata-u.ac.jp

Respiration is useful parameter for evaluating embryo quality as it provides important information about metabolic activity. In the present study, we employed scanning electrochemical microscopy (SECM) to accurately determine the oxygen consumption of single, identical human embryos at different developmental stages. The oxygen consumption rates of single embryos were low at 2-8-cell stages ($0.51 \pm 0.05 \times 10^{14}$/mol・$s^{-1}$, n=18) but increased by the morula ($0.61 \pm 0.11 \times 10^{14}$ /mol・s^{-1}, n=5) and early blastocyst ($0.72 \pm 0.06 \times 10^{14}$/mol・$s^{-1}$, n=14) stages. Later blastocysts exhibited even higher oxygen consumption rates ($1.00 \pm 0.19 \times 10^{14}$/mol・$s^{-1}$, n=4). Ultrastructural studies revealed that most mitochondria in embryos up to the 8-cell stage were immature and had a spherical or ovoid shape. However, by the morula stage, the mitochondria had elongated cristae, with the elongated morphology even more pronounced in mitochondria present in blastocysts. The maturation of mitochondria correlated with the increase of oxygen consumption rate during the development of embryos. The SECM technique may be a valuable tool for accurately assessing the mitochondrial function and quality of human embryos.

Key words: cell respiration; electrochemical miscroscopy; human embryo; culture

INTRODUCTION

Embryo quality is an important determinant of the success of embryo transfer and accurate evaluation of embryo quality improves the pregnancy rate for assisted reproduction of mammals, including humans. Several approaches have been used to evaluate embryo quality and viability. Morphological evaluation is the primary method. However, morphological evaluation is subjective and difficult, especially for embryos with intermediate morphological qualities [1]. Therefore, more objective selection criteria are needed.

The metabolic activity of embryos has been determined from the consumption of nutrients, such as glucose, pyruvate and amino acids [2]. Oxygen consumption is an ideal indicator of overall metabolic activity because adenosine triphosphate (ATP) is generated predominantly by oxidative phosphorylation, a process in which oxygen plays an essential role [3]. In previous paper we describe a novel cell respiration measuring system, scanning electrochemical microscopy (SECM). This technique is a useful method for evaluating embryo quality that correlates metabolic respiration with morphological quality, developmental potential, and pregnancy rate following embryo transfer [4]. In this study, we employed SECM to accurately determine the oxygen consumption of single, identical human embryos at different developmental stages.

MATERIAL AND METHODS

Oocyte retrieval and embryo culture

Ovarian stimulation was performed using with human menopausal gonadotropin (HMG; Nikken Kagaku, Tokyou, Japan), gonadotropin-releaseing hormone (GnRH) agonist (Buserecur, Fujiseiyaku, Tokyo, Japan), using the extended protocol. Human chorionic gonadotropin (HCG; Profasi, Serono, Switzerland, 10,000IU) was administrated when the diameter of the dominant follicle was 20 mm. Transvaginal oocyte aspiration was performed 34 h after HCG was administrated.

Oocytes were inseminated by conventional *in vitro* fertilization

(IVF) by 3-4 h after oocyte retrieval. Following an IVF-embryo transfer (ET) procedure, surplus embryos that patients preferred not to keep preserved were designated for our study. Informed consent for use of embryos in research was obtained from all patients. The embryos were cultured in Sydney IVF Cleavage Medium (Cook IVF, Brisbane, Australia) until Day 3, after which they were cultured in Sydney IVF Blastocyst Medium (Cook IVF). Embryos were evaluated by the Veeck method [5] at early developmental stage (day 3 after IVF) and by the Gardner method [6] at blastocyst stage based on their morphological features.

Scanning electrochemical microscopy measuring system

In this study, oxygen consumption was measured by SECM procedure [7,8]. This modified SECM system includes a measuring instrument on an inverted optical microscope stage, a potentiostat (Hokuto Denko Co., Tokyo, Japan), and a notebook computer as controller and analyzer (Fig. 1a-d). Pt-microdisk electrodes sealed in a tapered soft-glass capillary (Fig. 1e) were fabricated according to the method [9]. The tip potential was held at -0.6V versus Ag/AgCl with a potentiostat to monitor the local oxygen concentration in the solution. For the measurement of oxygen consumption, HFF99 medium (Fuso Pharmaceutical Industries, Osaka, Japan) was employed. Voltammetry of the Pt-microdisk electrode in HFF99 medium showed a steady-state oxygen reduction wave. No response from other electrochemically active species was observed near the oocyte surface. The tip scanning rate was 31.1 μm/s. The microelectrode with a Pt-disk radius less than 3 μm was selected so that the oxygen reduction current of the electrode was less than 1.0 nA. To easily handle many oocytes in a short time, a plate with cone-shaped microwells was used (Fig. 1f). The single human embryo was transferred into a cone-shaped microwell filled with HFF99 medium and embryo fell to the bottom of the well and remained at the lowest point (Fig. 1g). The microelectrode was scanned according to the z-direction from side point of sample (Fig. 1g, h). The motor driven XYZ-stage was located on the microscope stage for electrode tip scanning. The XYZ stage and potentiostat were controled by computer. The oxygen consumption rate of embryos is calculated by newly designed software based on spherical diffusion theory [7]. Measurements of each embryo were performed very rapidly (within 1 min).

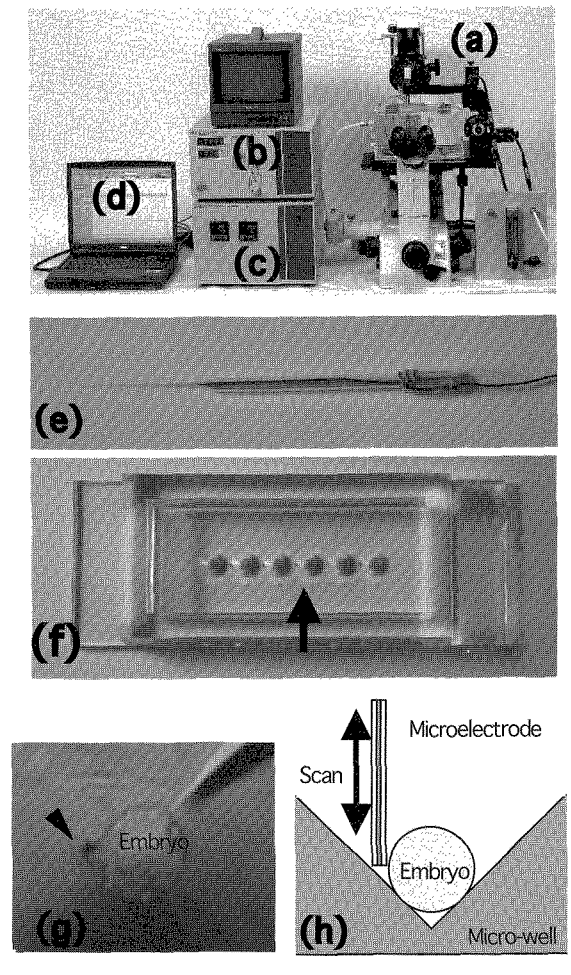

Fig. 1. A modified scanning electrochemical microscopy (SECM) system. SECM system includes a measuring instrument on the inverted optical microscope stage (a), potentiostat (b), controller (c), notebook computer (d), a microelectrode (e), and a plate (f) for measuring respiration activity of embryos. The plate has six cone-shaped microwells (arrow in f). Individual embryos are transferred into a microwell filled with medium. The embryo sinks down to the bottom of the well, remaining at the lowest point (g). Microelectrode (arrowhead in g) is scanned along the z-axis from the side point of embryo (h).

Electron microscopy

The electron microscopic study was carried out by the methods described previously [10]. Human embryos in various stages were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h at 0-4 ℃. Subsequently, the embryos were individually embedded in 1% agar. All samples were dehydrated by ethanol, substituted in

propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut with a diamond knife on an ultramicrotome (Reichert Ultracuts, Leica, Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (JEM-1210, Jeol, Tokyo, Japan).

RESULTS AND DISCUSSION

Morphology of human embryos

Figure 2 shows the morphological features of human embryos using differential interference contrast microscopy. The embryos showed various morphological features in each developmental stage. In this study, embryos classified as good quality based on their morphological features were selected and the oxygen consumption rates of individual embryos were measured by SECM system.

Oxygen consumption of human embryos in various developmental stages

Using a modified SECM measuring procedure, we successfully measured the respiration activity of single human embryos at several developmental stages (Table 1). The oxygen consumption rates of single embryos were low at 2-8-cell stages ($0.51 \pm 0.05 \times 10^{14}$/mol・$s^{-1}$, n=18) but increased by the morula ($0.61 \pm 0.11 \times 10^{14}$ /mol・s^{-1}, n=5) and early blastocyst ($0.72 \pm 0.06 \times 10^{14}$/mol・$s^{-1}$, n=14) stages. Later blastocysts exhibited even higher oxygen consumption rates ($1.00 \pm 0.19 \times 10^{14}$/mol・$s^{-1}$, n=4). These results demonstrate that SECM can detect differences in respiration activity of human embryos at several developmental stages.

The SECM system used in the present study can detect the oxygen concentration (ΔC) as small as a 1 μM difference between the bulk solution and the embryo surface: therefore, the system gives a precise and quantitative feature of oxygen consumption of single embryo [7]. Recently, SECM has been employed to quantify the respiration activity of embryos in several animal species [11]. SECM has been utilized to 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.

Ultrasructural features of mitochondria

Ultrastructural studies revealed that most mitochondria in embryos up to the 8-cell stage are immature and have a spherical or ovoid shape (Fig. 3a). However, by the morula stage, mitochondria have elongated cristae (Fig. 3b). This morphology is even more pronounced in mitochondria present in blastocysts (Fig. 3c). These results demonstrated that the maturation of mitochondria correlates with an increase of oxygen consumption rates during the development of human embryos. Similar findings have been reported in bovine embryos [4]. Mitochondria exhibited specific morphological changes as the respiration activity increased, because the number of mitochodria per cell and the number of cristae per mitochondrion are related to the energy requirement by respiration [12].

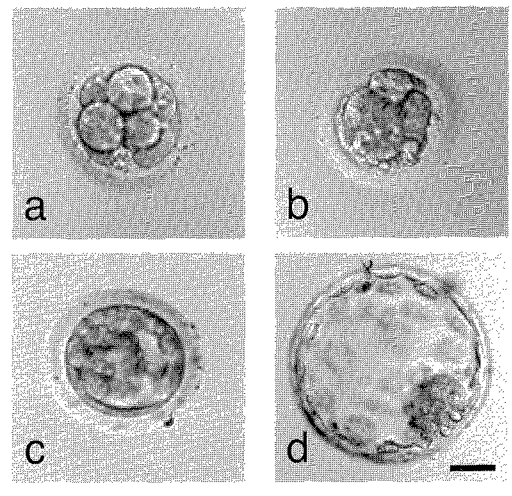

Fig. 2. Nomarski differential interference micrographs of 8-cell (a), morula (b), early blastocyst (c), and blastocyst (d) stages of human embryos developed from in vitro fertilized oocytes. Bar = 50 μm.

Table 1. Oxygen consumption rates of human embryos at various developmental stages

Embryonic stage	No. of embryos examined	O_2 consumption rate ($F \times 10^{14}$/mol・s^{-1})
2-8 cell	18	0.51 ± 0.05^{a}
Morula	5	0.61 ± 0.11^{ab}
Early blastocyst	13	0.72 ± 0.06^{b}
Blastocyst	4	1.00 ± 0.19^{c}

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

Mitochondria contribute a vital role to the metabolism of

energy-compounds in the cytoplasm to provide ATP for embryonic development. The development of mitochondria may be an important factor in embryo quality. There are conspicuous differences in the ultrastructural features of bovine embryos of high and low quality [13]. Morulae classified as low quality by morphological classification contained nucleoli with low transcriptional activity, a large number of lipid droplets, and immature mitochondria, consistent with these low quality embryos displaying low metabolic activities, including oxygen consumption. Thus, oxygen consumption associated with mitochondrial development is a reliable indicator of embryo quality.

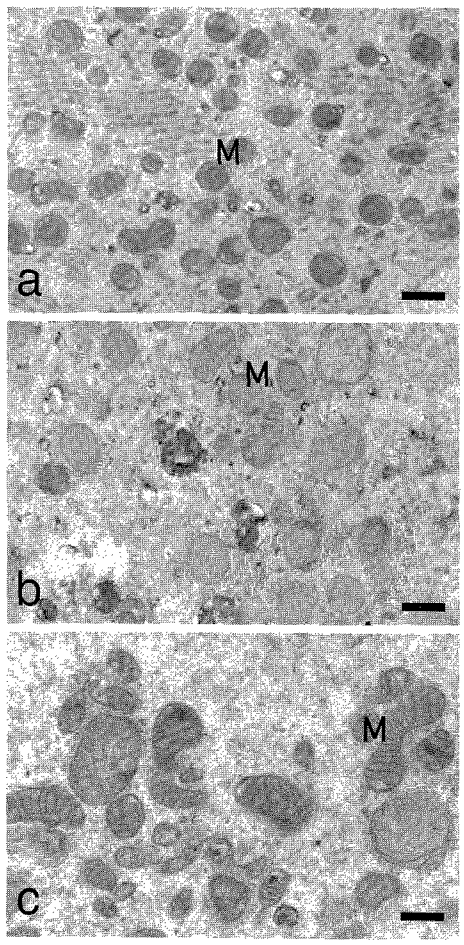

Fig. 3. Electron micrographs of human embryos at 2-cell (a), morula (b), and blastocyst (c). M, mitochondria. Bars = 0.5 μm.

CONCLUSIONS

SECM can non-invasively measure oxygen consumption by single, identical human embryos. This technique is a valuable tool for accurately assessing the mitochondrial function of human embryos. As respiration activity correlates with the quality of embryos, this technique may contribute to assessing the quality of human embryos in *in vitro* fertilization clinics.

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