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Multiple Analysis of Respiratory Activity in the Identical Oocytes by Applying Scanning Electrochemical Microscopy

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Scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode is used to scan and monitor the local distribution of electro-active species near the sample surface. In this study, we have studies on the SECM technique, to establish the accurate method for measurement of respiratory activity of single pig oocytes. The oxygen consumption rates of pig oocytes cultured in modified TCM199 medium were evaluated by the SECM system. After the measuring, distribution of active mitochondria and ATP content was investigated in the identical oocytes. The oocytes were classified in three types (Type-I, Type-II or Type-III) according to the pattern of active mitochondria distribution. There was no difference in the oxygen consumption rate ($F \times 10^{14}$/mol s^{-1}) between Type-II and Type-III (0.59 and 0.60, respectively). However, the ATP content (pmol/oocyte) was significantly higher in Type-III (2.38) compared with that of Type-II (1.53). Meanwhile, the oxygen consumption rate and ATP content of Type-I were very low (0.02 and 0.06, respectively). These results suggest that the oxygen consumption rate and ATP content of oocytes was significantly affected by category of mitochondrial distribution. In the present study, we succeeded in the multiple analysis of respiratory activity in the identical oocytes. This novel system may be a valuable tool for accurately assessing the mitochondrial functions.

Key words: cell respiration; electrochemical miscroscopy; pig oocyte; culture

INTRODUCTION

Oxygen consumption is an indicator of the overall metabolic activity and quality of a single embryo. Oxygen consumption of mammalian embryos has been with various methods such as Cartesian diver [1], spectrophotometrics [2,3], fluorescence [4,5] and electrochemical techniques [6], To establish the evaluation method for embryo quality based on the oxygen consumption activity, we have been studied on the scanning electrochemical microscopy (SECM) technique [7]. SECM technique has been successfully applied to investigate various biological systems including DNA [8], enzyme [9], antigen-antibodies [10,11], tissue [12], and cell [13], because of its non-invasive nature to quantitatively characterize localized chemical reaction under physiological conditions. In the previous study, we applied SECM technique to measure the oxygen consumption of single bovine embryos [14]. The oxygen consumption of individual bovine embryos produced in vitro fertilization (IVF) systems has been determined non-invasively and quantitatively by this technique. Furthermore, we have found that there was a close relationship between high oxygen consumption and developmental ability of bovine embryos [14-16]. The SECM procedures may be useful to assess the quality of embryos and contribute to improvements in reproductive technologies in mammals.

On the other hand, an accurate system to evaluate oocyte quality is lacking. The most convenient evaluation system for oocyte quality is based on oocyte morphology and status of oocyte-cumulus complexes (COCs). However, this evaluation method could be imprecise and subjective, since there is no clear correlation between oocyte quality and fertilization rates [17]. Oocyte quality could be one of the most important factor in determining successful fertilization and embryo development. Therefore, we attempt to establish an evaluation system for oocyte quality is based on the respiratory activity of oocytes. The aims of this study were: 1) to assess the oxygen consumption of single pig oocytes using SECM; 2) to examine the mitochondrial distribution and ATP content in the identical oocytes.

MATERIAL AND METHODS

Scanning electrochemical microscopy measuring system

In this study, oxygen consumption was measured by SECM procedure [16, 18]. 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).

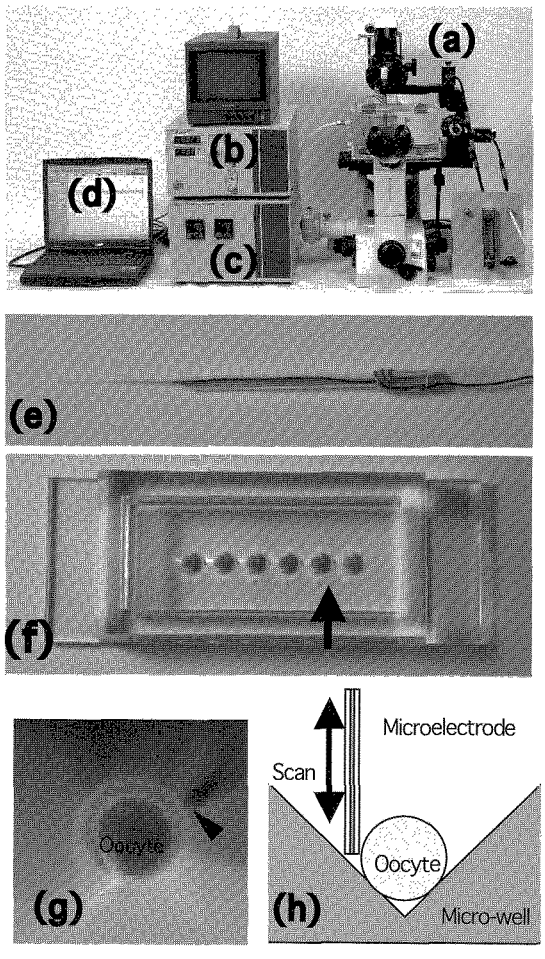

Fig. 1. A modified scanning electrochemical microscopy (SECM) system. SECM system includes a measuring instrument on the inverted optical microscope stage (a), potentiostat (b), controller (c), notebook computer (d), a microelectrode (e), and a plate (f) for measuring respiration activity of embryos. The plate has six cone-shaped microwells (arrow in f). Individual oocyte is transferred into a microwell filled with medium. The oocyte 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 oocyte (h).

Pt-microdisk electrodes sealed in a tapered soft-glass capillary were fabricated according to the method [19] (Fig. 1e). 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, modified human tubal fluid (HTF) medium [20] was employed. Its composition only includes salt electrolyte, glucose, sodium pyruvate, sodium lactate, HEPES and gentamicin sulfate. Voltammetry of the Pt-microdisk electrode in modified HTF 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 pig oocyte was transferred into a cone-shaped microwell filled with modified HTF medium and oocyte fell to the bottom of the well and remained at the lowest point. The microelectrode was scanned according to the z-direction from side point of sample (so-called "side-scanning": 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 oocytes is calculated by newly designed software.

Oocyte collection and maturation culture

Pig cumulus-oocyte complexes (COCs) were obtained from ovarian follicles 2-5 mm in diameter. COCs classified as good quality by morphological evaluation were cultured in TCM199 medium containing 2.2 mg/ml sodium pyruvate, 10 mg/ml bovine serum albumin (BSA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% pig follicular fluid for *in vitro* maturation (IVM) in a humidified atmosphere of 5% CO_2 in air at 37.0°C for 48 h. After the cultures, cumulus cells were completely removed by the pipetting. The oxygen consumption of single denuded oocyte was measured by SECM systems. After the measurement, oocytes were prepared for histological (mitochondrial distribution) and biochemical (ATP content) experiments.

Staining of mitochondria

Oocytes were stained by MitoTracker Orange (Invitrogen;

Carlsbad, CA). This dye becomes fluorescent once it accumulates in the membrane lipids of mitochondria with membrane potential and is an important tool for evaluating the distribution of active mitochondria. MitoTracker Orange was used at a concentration of 350 nM in HPM199 medium (based on TCM199) supplemented 0.5% BSA for 30 min at 37.0°C. Oocytes were washed three times, mounted in a drop of HPM199 medium and examined using a confocal laser scanning microscope (FV-300; Olympus, Tokyo, Japan).

Measurement of the ATP content of oocytes

The ATP content of completely denuded oocyte was measured using a commercial assay based on the luciferin-luciferase reaction (Promega, Sunnyvale, Ca). Oocytes were rinsed three times in phosphate buffered saline (PBS), and then transferred individually in 50 uL of PBS into plastic tubes. Then, 50 uL of BacTiter-Glo reagent was added to all tubes were incubated for 5 min at room temperature. The ATP content of the samples was measured using a liminometer (Luminometer 20/20n, Promega) with high sensitivity (0.001 pmol). A five-point standard curve (0-10 pmole/tube) was routinely included in each assay. The ATP content was determined from the formula for the standard curve.

RESULTS AND DISCUSSION

Oxygen consumption of pig oocytes before and after *in vitro* maturation

Oxygen consumption rates of immature oocytes (immediately upon recovery from ovary) were 0.44×10^{14}/mol s^{-1}. In the maturation culture, a higher oxygen consumption rate was found in matured oocytes (with polar body extrusion), whereas the oxygen consumption rate of non-matured oocytes (without polar body extrusion) decreased during oocyte maturation (Table 1). These results showed that the oxygen consumption of pig oocytes changed in maturation status of oocytes.

Table 1. Oxygen consumption rates of pig oocytes in different maturaton status

Maturation status	O_2 consumption rate ($F\times 10^{14}$/mol · s^{-1})
Immature	0.44 ± 0.03^{a}
Mature	0.44 ± 0.03^{a}
Non-mature	0.21 ± 0.03^{b}

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

Distribution of mitochondria in pig oocytes

Mitochondrial localization in oocytes after IVM is showed in Figure 2. The oocytes were classified in three types (Type-I, Type-II or Type-III) according to the pattern of active mitochondria distribution. Staining with MitoTracker orange revealed small mitochondrial clumps that were as a rule found in the periphery of the cytoplasm (Type-I). The Type-II oocytes showed the strong and homogeneous staining. In the oocytes classified as Type-III, mitochondrial clumps showing the strongest staining were seen in the central parts of the cytoplasm. The number of Type-II and Type-III oocytes gradually increased during the maturation culture. These results suggest that active mitochondria moved from the periphery to the central parts of the cytoplasm in oocytes during oocyte maturation. Similar mitochondrial reorganization has been reported in bovine oocytes before and after IVM [21].

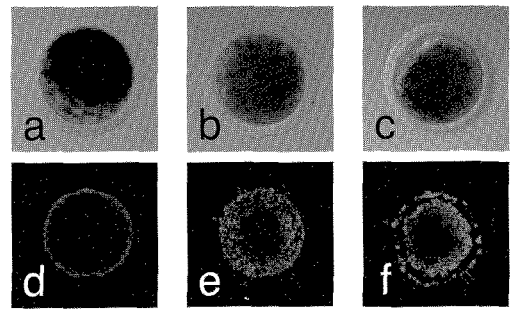

Fig. 2. Midline confocal sections of pig oocytes after maturation culture. a-c: Nomarski differential interference micrographs. d-f: Pig oocytes stained by MitoTracker orange. The oocytes are classified into three categories according to the mitochondria distribution, such as Type-I (a, d), Type-II (b, e), and Type-III (c, f).

Respiration rates and ATP content of different type of oocytes

The respiration rate and ATP content of Type-I oocyte were significantly lower than in Type-II and Type-III oocytes (Table 2). Although Type-II oocytes showed the high respiratory activity, they contained significantly less ATP than oocytes of Type-III oocytes. The average respiration rate and ATP content of Type-III

oocytes tended to be higher than that of other types of oocytes. These results demonstrated that the oxygen consumption rate and ATP content of pig oocytes was significantly affected by category of mitochondrial distribution. Type-II oocytes are thought to be an intermediate type between Type-I and Type-III oocyte in maturation status. This study suggests that there is a correlation between the respiratory activity and the maturation status of pig oocytes.

Table 2. Oxygen consumption rates of pig oocytes in different maturaton status

Category	O_2 consumption rate ($F\times10^{14}$/mol · s^{-1})	ATP content (pmol/oocyte)
Type-I	0.20 ± 0.10^{a}	0.08 ± 0.01^{a}
Type-II	0.54 ± 0.02^{b}	1.66 ± 0.11^{b}
Type-III	0.53 ± 0.01^{b}	2.08 ± 0.05^{c}

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

CONCLUSIONS

In this study, oxygen consumption by single pig oocytes was non-invasively and quantitatively determine by SECM measuring system. The biochemical and cytological studies strongly suggest that oxygen consumption is an important parameter to evaluate the competence of oocyte maturation in the pig. SECM measuring procedures can be used to accurately evaluate the metabolic activity and quality of pig oocytes.

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