

—Mini Review—

A Non-invasive and Sensitive Method for Measuring Cellular Respiration with a Scanning Electrochemical Microscopy to Evaluate Embryo Quality

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Abstract: Respiration is useful parameter for evaluating embryo quality as it provides important information about metabolic activity. In this paper, we describe a scanning electrochemical microscopy (SECM) technique that is a non-invasive and sensitive method for measuring oxygen consumption by individual embryos. SECM measuring system can non-invasively measure respiration activity by single embryos of several species including human. The bovine embryos with higher oxygen consumption are better candidates to further development into good quality embryos and yielded higher pregnancy rates after embryo transfer. Respiration activity correlates with the embryo quality. SECM technique may be a valuable tool for accurately assessing the quality of embryos and thereby contribute to improving outcomes associated with assisted reproduction, including human in vitro fertilization.

Key words: Cellular respiration, Mitochondria, Embryo quality, Electrochemistry

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, 2]. Therefore, more objective selection criteria are needed. Previous

studies have suggested that a greater understanding embryo metabolism could yield new strategies for evaluating the quality of individual embryos [3, 4].

The metabolic activity of embryos has been determined from the consumption of nutrients, such as glucose, pyruvate and amino acids [5–8]. 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 [9–11]. In this paper we describe a novel cell respiration measuring system, scanning electrochemical microscopy. 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.

Measuring the Oxygen Consumption of Embryos

Oxygen consumption by mammalian embryos has been studied with various techniques. The earliest studies used a Cartesian diver technique to measure the oxygen consumption by large groups of rabbit oocytes [12] and preimplantation mouse embryos [13]. Subsequently, spectrophotometry was employed to measure the oxygen consumption of single human oocytes and blastocysts [14] as well as mouse blastocysts [15]. This technique indirectly measures oxygen consumption by determining the amount of oxyhaemoglobin converted to haemoglobin. Overstrom *et al.* [3] have developed a multi-channel sensor with solid-state oxygen electrodes to measure oxygen consumption by individual mouse and bovine embryos. However, this technique has not achieved widespread use because it has limited sensitivity and is time

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consuming [4]. An ultramicrofluorescence technique also has been employed to measure the oxygen consumption by small groups of mouse [16] and bovine embryos [9]. This technique exploits the fluorescent characteristics of pyrene to indirectly measure oxygen consumption by embryos cultured in the presence this polycyclic aromatic hydrocarbon over a period 4–6 h. Unfortunately, this technique is not sensitive enough to measure respiration by single embryos and the fluorophore in the culture media may have toxic side effects.

Oxygen consumption of single zona-free mouse embryos has been measured with self-referencing microelectrodes [11, 17], a technique that has been applied to several biological systems [18, 19]. This technique can non-invasively measure the respiration rates of single embryos. However, as embryos are examined in the absence of the zona pellucida, these rates might not accurately reflect physiological oxygen. Recently, respiration rates of single bovine embryos also have been studied with a, high-resolution nanorespitometer system (Unisense A/S, Aarhus, Denmark) [20–22]. This system utilizes a high-sensitivity miniaturized Clark-type oxygen sensor. This technique can rapidly (8 min) measure the respiration rates of individual embryos at different developmental stages.

Application of an Electrochemical Measuring Technique for Quantifying Respiration of Embryos

Scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species near the sample surface [23]. SECM has been used to investigate numerous biological molecules including DNA [24], enzymes [25], and antigen-antibody interactions [26, 27]. This technique can non-invasively measure localized chemical reactions under physiological conditions. SECM examines the concentration profile of a metabolic product around a spherical sample, such as an embryo, with a probe microelectrode. We have employed SECM to examine oxygen consumption by single embryos. Previously, we examined the oxygen consumption of single, identical bovine embryos at the morula and blastocyst stages with this technique [28]. The oxygen consumption rates were quantified based on the spherical diffusion theory [29]. While SECM is useful for studying the respiration activity of embryos, this technique is difficult and

requires carefully positioning both the embryo and the microelectrode.

We designed a new SECM measuring system capable of examining many embryos in a short time [30]. This modified SECM system includes a measuring instrument mounted on the stage of an inverted optical microscope, a potentiostat, and a notebook computer for component control and analysis (Fig. 1a–c). Pt-microdisk electrodes (Fig. 1d) sealed in tapered soft-glass capillaries were fabricated according to published methods [31] and were used to monitor the reduction current of oxygen. The tip potential was held at -0.6 V versus Ag/AgCl with a potentiostat to monitor the local oxygen concentration in the solution. To measure oxygen consumption, human tubal fluid (HTF) medium [32] was employed. This medium contains salt electrolytes, glucose, sodium pyruvate, sodium lactate, HEPES and gentamycin sulfate. The Pt-microdisk electrode in HTF medium displayed a steady-state oxygen reduction wave. No responses from other electrochemically active species were observed near the embryo surface. The Pt-disk radius of the microelectrode was less than $3\text{ }\mu\text{m}$ and the oxygen reduction current of the electrode was less than 1.0 nA . To examine many embryos in a short period of time, we employed a plate with cone-shaped microwell (Fig. 1e) [30, 33]. As single bovine embryos were transferred to the microwell filled with HTF medium they fell to the bottom of the well and remained at the lowest point (Fig. 1f). The microelectrode was scanned along the z-axis from the side point of sample (“side-scanning”: Fig. 1g) [33]. The tip scanning rate was $20\text{--}30\text{ }\mu\text{m/sec}$. The motor-driven XYZ-stage and the potentiostat were controlled by a computer. The oxygen consumption rates were calculated with custom software based on spherical diffusion theory [28, 33]. Measurements of each embryo were performed very rapidly, with individual respiration rates being completed with 1 min.

Measuring Respiration Activity of Single Embryos with a Scanning Electrochemical Microscopy System

Using a modified SECM measuring procedure, we measured the oxygen consumption rates of single, identical bovine embryos cultured in a serum-free medium at several developmental stages (IVD101: Research Institute for the Functional Peptides, Yamagata, Japan) (Table 1). The oxygen consumption rates of single embryos were low from the 2-cell to 8-cell stages but increased by the morula stage.

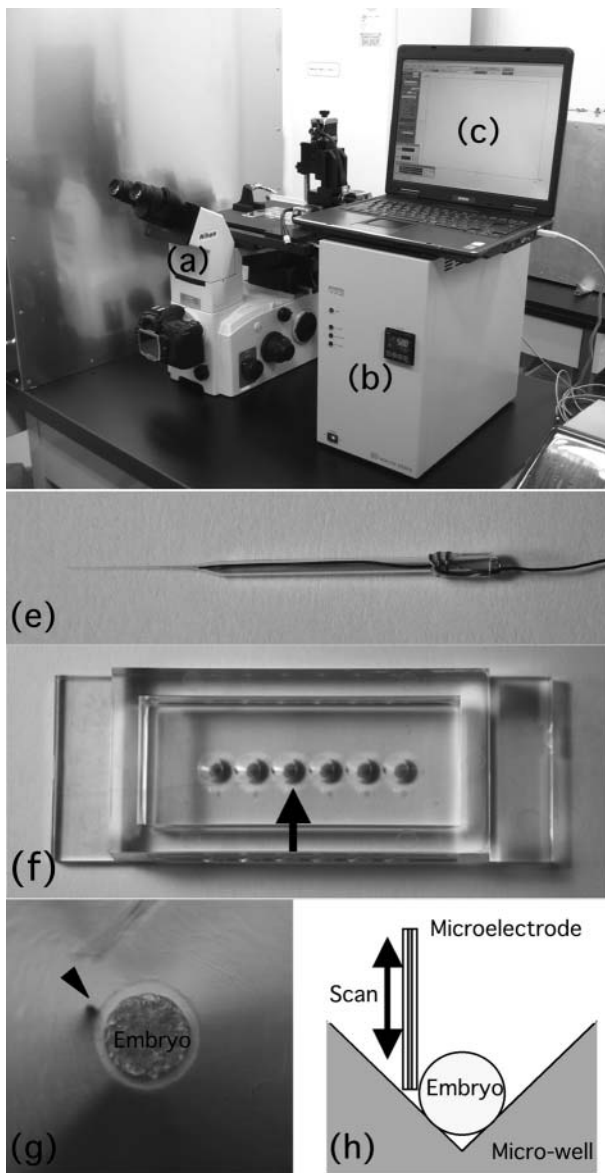


Fig. 1. A modified scanning electrochemical microscopy (SECM) system. SECM system includes a measuring instrument on the inverted optical microscope stage (a), potentiostat (b), notebook computer (c), microelectrode (e), and plate (f) for measuring respiration activity of embryos. The plate has six cone-shaped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

Blastocysts and hatched blastocysts exhibited even higher oxygen consumption rates. Ultrastructural studies revealed that most mitochondria in embryos up to the 8-cell stage are immature and have a spherical or

ovoid shape (Fig. 2a). However, by the morula stage, mitochondria have elongated (Fig. 2b). This morphology is even more pronounced in mitochondria present in blastocysts (Fig. 2c). The maturation of mitochondria correlates with an increase of oxygen consumption rates during the development of bovine embryos. Mitochondria exhibited specific morphological changes as the respiration activity increased, because the maturation of mitochondria is associated with increases in metabolism including oxygen consumption [13] and CO_2 production [34].

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 [35]. Morulae classified as low quality by morphological classification [36] 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.

Quality Evaluation of Embryos Based on Respiratory Activity

Although several methods have been employed to measure the oxygen consumption by embryos, most are incompatible with examining embryos that are candidates for assisted reproduction because of limited sensitivity or deleterious effects of the detection process. Recently, Lopes *et al.* [21] employed a nanorespirometer system that is non-invasive, sensitive, and capable of measuring respiration rates from individual embryos, to investigate the correlation between respiration rate and embryo morphology, diameter, and sex. Respiration rates were directly influenced by embryo diameter and only in partial agreement with the morphological quality of embryos. While there was a slight discrepancy between the morphological evaluation and the respiration assessment, the results did not differ with sex [20]. More recently, these authors have examined the correlation between the respiration of bovine embryos produced *in vivo* or *in vitro* and the pregnancy rates following transfer [22]. There was no statistically validated correlation between respiration rate and pregnancy status and the nanorespirometer

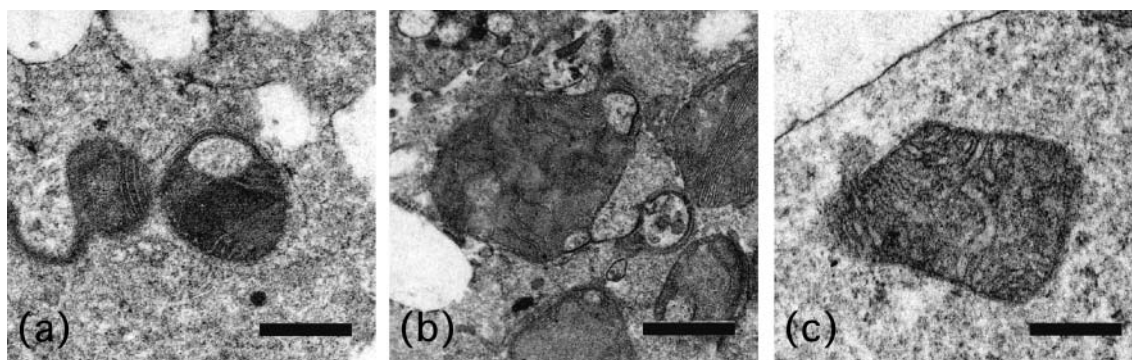


Fig. 2. Electron micrographs of bovine embryos derived from *in vitro*-matured and *in vitro*-fertilized oocytes at 2-cell (a), morula (b), and blastocyst (c) stages. a: Note immature mitochondria display ovoid structures with a hooded appearance and a dark electron-dense matrix. b, c: Note presence of elongated mitochondria with transverse cristae. Bars=0.5 μ m.

Table 1. Oxygen consumption rates of bovine embryos cultured in a serum-free medium (IVD101) at various developmental stages

Embryonic stage	No. of embryos measured	Oxygen consumption rate ($F \times 10^{14}/\text{mol s}^{-1}$)
2 cell	15	0.46 ± 0.05^a
4 cell	17	0.45 ± 0.03^a
8 cell	18	0.46 ± 0.02^a
Morula	48	1.03 ± 0.05^b
Blastocyst	55	1.86 ± 0.07^c
Hatched blastocyst	24	3.01 ± 0.07^d

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

Table 2. Oxygen consumption rates of bovine embryos classified by morphological evaluation (from Abe *et al.* [38])

Category of embryo	No. of embryos measured	Oxygen consumption rate ($F \times 10^{14}/\text{mol s}^{-1}$)
Excellent	48	1.03 ± 0.05^a
Good	48	1.02 ± 0.04^a
Fair	22	0.80 ± 0.04^b
Poor	16	0.43 ± 0.05^c

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

measurement did not influence the subsequent viability of embryos.

The SECM measuring system also has been employed to evaluate embryo quality. The oxygen consumption and quality of bovine embryos produced with an *in vitro* culture system using serum-free media were studied. Individual bovine morulae at day 6 after *in vitro* fertilization (IVF) were evaluated with a phase contrast microscope and categorized as excellent, good, fair or poor [35, 36]. Excellent and good quality morulae were spherical and symmetrical with cells of uniform size. Good quality morulae had minor

perturbations such as few extruded blastomeres. Fair quality morulae were characterized by the presence of many extruded blastomeres and vesiculation. Poor quality morulae had severe problems such as numerous vesicles but appeared viable. After morphological evaluation, the oxygen consumption of individual morulae was measured with the modified SECM system.

Respiration rates decreased with morphological quality (Table 2). Embryos classified as excellent and good quality had higher respiration rates that were significantly greater than those of embryos of lower

Table 3. Relationship between oxygen consumption and developmental potential of morulae cultured in serum-free IVD101 medium (from Abe *et al.* [30])

Oxygen consumption rate ($F \times 10^{14}/\text{mol s}^{-1}$)	No. of embryos* measured	No. of embryos developed to blastocyst after 96 h (%)	No. of blastocysts expanded or hatched after 96 h (%)
$F \geq 1.0$	56	50 (89.3)	35 (62.5)
$0.8 \leq F < 1.0$	44	34 (77.3)	20 (50.0)
$F < 0.8$	107	49 (45.8)	28 (26.2)

*Bovine embryos at the morula stage were selected at day 6 after *in vitro* fertilization. After measurement by modified SECM, embryos were incubated in IVD101 medium for 4 days in a humidified atmosphere of 5% CO₂/5% O₂/90% N₂ at 38.5°C.

quality (fair and poor). However, some excellent and good quality morulae had low respiration rates (under $0.5 \times 10^{14}/\text{mol s}^{-1}$) while a few fair quality morulae had high respiration rates (more than $1.0 \times 10^{14}/\text{mol s}^{-1}$). Thus, although embryos with a higher level of metabolic activity are more likely to also be of higher morphological quality, the correlation is not absolute. Similar findings have been reported by Lopes *et al.* [20]. Bovine blastocysts (day 7) produced by IVF were evaluated for morphology and given a quality score (quality 1, 2, 3 or 4) according to the definitions adopted by the International Embryo Transfer Society (IETS1998). The average oxygen consumption rate of quality 1 blastocysts (highest quality) was significantly higher than that of the other qualities and respiration activity decreased less with each quality step. However, there was considerable variation in respiration rates within blastocysts of the same quality. These studies support the hypothesis that measuring embryonic respiration provides additional and valuable information about embryo quality.

Correlation between Respiration Activity, Developmental Capacity and Pregnancy Rates of Embryos

After measuring their respiration rates with SECM, embryos were cultured to examine their developmental capacity. Morulae with higher oxygen consumption (more than $1.0 \times 10^{14}/\text{mol s}^{-1}$) were more likely to develop into good quality embryos, such as expanded and hatched blastocysts (Table 3). In contrast, morulae with lower respiration rates (under $1.0 \times 10^{14}/\text{mol s}^{-1}$) had lower developmental rates compared to morulae with higher or moderate (more than $0.8 \times 10^{14}/\text{mol s}^{-1}$ and under $1.0 \times 10^{14}/\text{mol s}^{-1}$) respiration rates.

To explore the relationship between respiration activity and pregnancy rates, the oxygen consumption of individual bovine embryos collected from

superovulated and artificial inseminated Japanese black cows was measured and they were then transferred to recipients [37, 38]. First, the sex of these embryos was determined by Loop-mediated isothermal amplification (LAMP) [39]. Sexed embryos were cultured in serum-free IVD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) for 24 h. They were then classified into morula, early blastocyst, and blastocyst by morphological evaluation and measured by SECM. Within 3 h thereafter, embryos were transferred to recipient cows and pregnancy status were examined by ultrasonography approximately 40 days after estrus. The average oxygen consumption rates of embryos classified as blastocyst, early blastocyst, and morula were $1.44 \times 10^{14}/\text{mol s}^{-1}$, $1.25 \times 10^{14}/\text{mol s}^{-1}$, and $0.72 \times 10^{14}/\text{mol s}^{-1}$, respectively. These rates are significantly different ($P < 0.05$) between each category. A total of 54 sexed-embryos resulted in pregnancies. The pregnancy rates of embryos classified as blastocyst, early blastocyst, and morula were 45.0% (18/40), 51.6% (16/31), and 50.0% (21/42), respectively.

A retrospective analysis indicated that the respiration activity of embryos that resulted in pregnancies was higher than those that did not. The pregnancy rates are summarized in Table 4. The highest pregnancy rates were obtained from embryos with the oxygen consumption rates of greater or equal to $1.0 \times 10^{14}/\text{mol s}^{-1}$ for blastocysts (58.3%), greater or equal to $0.8 \times 10^{14}/\text{mol s}^{-1}$ for early blastocysts (64.0%), and greater or equal to $0.5 \times 10^{14}/\text{mol s}^{-1}$ for morulas (60.7%), respectively. Pregnant animals were rarely obtained from embryos with the respiration rates less than $1.0 \times 10^{14}/\text{mol s}^{-1}$ for blastocysts, $0.8 \times 10^{14}/\text{mol s}^{-1}$ for early blastocysts, and $0.5 \times 10^{14}/\text{mol s}^{-1}$ for morulas. Similar findings have been obtained from cryopreserved *in vivo*-derived bovine embryos [40]. We conclude from these studies measuring respiration activity with SECM does not adversely influence the viability and development of

Table 4. Relationship between oxygen consumption and pregnancy rates of *in vivo*-derived bovine embryos* (from Moriyasu *et al.* [37] and Abe *et al.* [38])

Embryonic stage at transfer	Oxygen consumption rate ($F \times 10^{14}/\text{mol s}^{-1}$)	Pregnancy rate (%)
Blastocyst	$F \geq 1.0$	21 / 36 (58.3)
	$F < 1.0$	0 / 6 (0.0)
Early blastocyst	$F \geq 0.8$	16 / 25 (64.0)
	$F < 0.8$	0 / 6 (0.0)
Morula	$F \geq 0.5$	17 / 28 (60.7)
	$F < 0.5$	1 / 12 (8.3)

*: Embryos at the morula stage were collected from superovulated and artificial inseminated cows. After determining gender LAMP, embryos were incubated in IVD101 medium for 24 h in a humidified atmosphere of 5% CO₂ in air at 38.5°C.

embryos. Furthermore, the measurement of oxygen consumption prior to embryo transfer may be useful for estimating embryo quality and may contribute to improving the success rate for embryo transfer.

Application of SECM in Assisted Reproductive Technology

SECM has been employed to quantify the respiration activity of embryos in several animal species including humans (Fig. 3). 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. Here we demonstrate that SECM can detect differences in respiration activity of embryos under various developmental and culture conditions. Previous studies have demonstrated that developmental and culture conditions affect mitochondrial morphology, respiration activity, and embryo quality [41–48]. Measuring oxygen consumption with SECM may facilitate determining suitable culture conditions for embryos.

In addition, we successfully measured the respiration activity of single human embryos. These surplus embryos were unused upon completion of fertility treatment and their use in academic research was with consent of the patient. We have initiated a study of respiration activity and ultrastructural analysis of human embryos in cooperation of private fertility clinics. Preliminary studies performed with our modified SECM system have revealed that the respiration rates of individual human blastocysts (including hatched blastocysts) range between $0.80 \times 10^{14}/\text{mol s}^{-1}$ and $1.51 \times 10^{14}/\text{mol s}^{-1}$ (unpublished results). We are currently

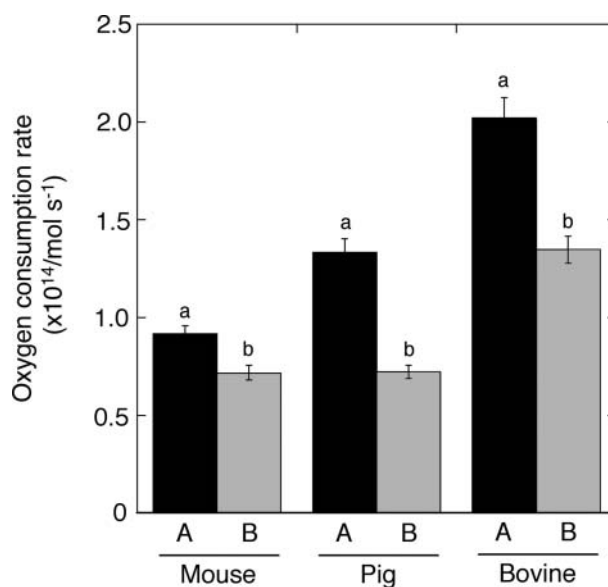


Fig. 3. Comparison of the oxygen consumption rates in mouse, porcine, and bovine blastocysts. Mouse (A): Blastocysts are collected from uterus of mated mice. Mouse (B): The 2-cell stage embryos are collected from the oviducts of mated mice and then cultured in KSOM medium until the blastocyst stage. Pig (A): Blastocysts are collected from the uterus of artificially inseminated pigs. Pig (B): Blastocysts are developed from *in vitro*-matured (IVM) and -fertilized (IVF) oocytes cultured in NCSU23. Bovine (A, B): Blastocysts are developed from IVM and IVF oocytes cultured in serum-free medium (A) and serum-containing (5% calf serum) medium (B), respectively. Values with different superscripts in each column are significantly different ($P < 0.05$).

collecting data to establish a diagnostic system for determining the quality of human embryos.

Conclusions

SECM can non-invasively measure oxygen consumption by single embryos. Respiration activity correlates with the quality of bovine embryos. This technique may be a valuable tool for accurately assessing the quality of embryos as this approach provides objective criteria for evaluating embryos that complements morphological evaluation. Together these methods offer an accurate method for embryo classification and subsequent selection. As SECM can measure the respiration activity of human embryos, this technique may contribute to improving the outcomes of assisted reproduction in *in vitro* fertilization clinics.

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