表2　ヒト胚（余剰胚）の発生過程における酸素消費量（呼吸量）変化

発生ステージ	胚数	酸素消費量 ($F \times 10^{14}$/mol・s^{-1})
2～8細胞	18	0.51 ± 0.05
桑実胚	5	0.61 ± 0.11
初期胚盤胞	13	0.72 ± 0.06
胚盤胞～孵化胚盤胞	5	1.05 ± 0.02

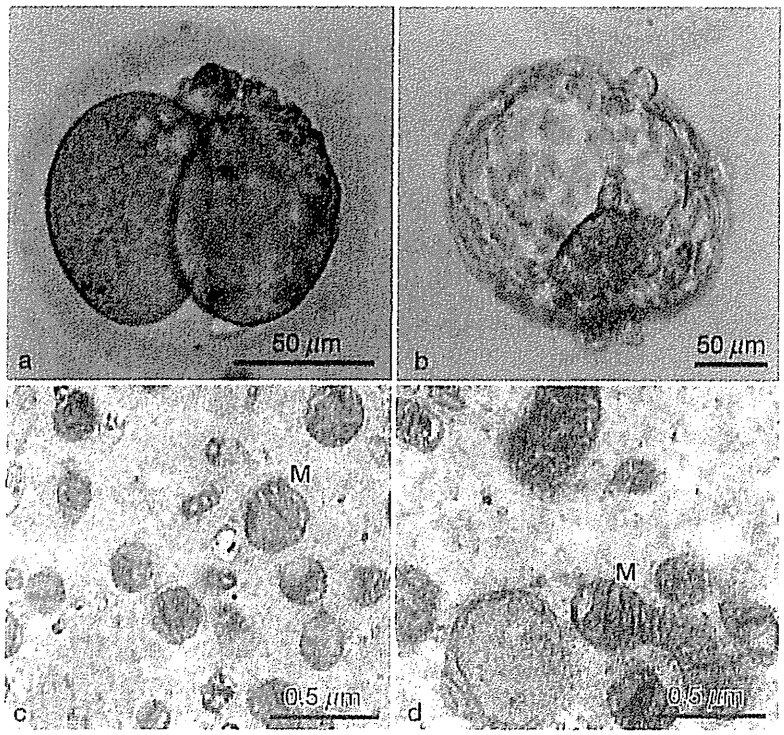

図5　ヒト体外受精胚の電顕像

a：2細胞期胚．未成熟な形態のミトコンドリア（M）．
b：胚盤胞期胚．拡張したクリステ構造をもつ発達したミトコンドリア（M）．

ヒト胚評価への応用

「受精卵呼吸測定装置」は，短時間で非侵襲的に胚の呼吸量を測定できることから，臨床応用可能な計測機器として有望である．不妊治療への臨床応用を実現するためには，呼吸測定によるヒト胚品質評価の有効性と装置の安全性の検証が不可欠である．

これまでに，試験的臨床研究として，ヒト余剰胚の酸素消費量測定を試みている．**表2**に，ヒト胚の発生過程における呼吸量変化を示す．ヒト胚ではウシ胚と同様に，発生に伴いミトコンドリアの発達とともに呼吸量が顕著に増加する（**図5**）．また，受精後3日目に比較的高い呼吸活性（$0.26 \sim 0.56 \times 10^{14}$/mol・s^{-1}）を有する胚は，$0.26 \times 10^{14}$

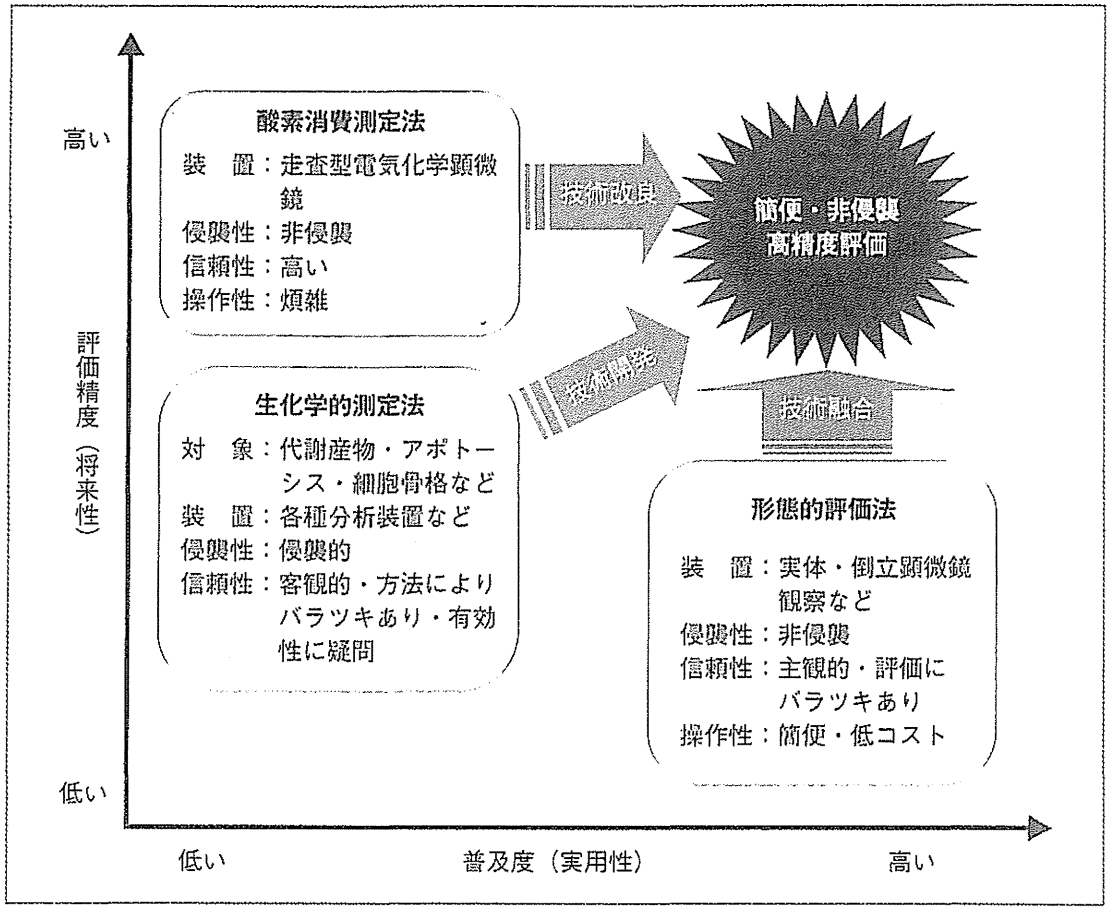

図6　胚評価の現状と将来像を示した模式図
それぞれの評価法の長所を融合した新しい胚評価法の開発が望まれる．

/mol・s^{-1}未満または0.56×10^{14}/mol・s^{-1}を上回る呼吸活性を示す胚と比べて有意に高い確率で胚盤胞へ発生することが示されている[21, 22]．また，形態的に同じクオリティと評価された胚の酸素消費量を測定し，呼吸活性が最も高い胚を移植した場合，形態観察のみで評価した胚を移植した症例と比べて妊娠率が高い傾向がある（未発表データ）．これらの結果は，ヒト胚においても呼吸活性を指標とする品質評価が可能であることを示唆している．

今後，多くの動物実験とヒト余剰胚を用いた前臨床研究を実施し，装置および測定技術の有効性と安全性を詳細に検証する．さらに，基礎研究の成果を踏まえ，所定の倫理承認を得たのち，不妊治療での臨床応用を目的に呼吸測定胚の移植を計画している．

おわりに

今後，生殖補助医療においては不妊治療技術の高度化や高齢不妊患者の増加に伴い，移植の対象となる胚もより厳密に評価する必要がある．このため，これまでに開発されているいくつかの品質評価法のメリットを有機的に融合した新しい胚評価システムの開

発が必要になってくる（**図 6**）．例えば，呼吸測定による胚品質評価は，形態的評価法との併用が可能であり，これによってより厳密に胚の品質評価が可能であると考えている．今後の詳細な研究によって細胞呼吸計測法および測定装置の安全性と有用性が確認され，新しいヒト胚評価システムとして実用化されることを期待している．

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—Original—

Respiratory Activity of Single Blastocysts Measured by Scanning Electrochemical Microscopy: the Relationship between Pre-freezing and Post-warming

Yoko Kumasako[1, 2*], Kaori Goto[1], Megumi Koike[1], Yasuhisa Araki[3], Hiroyuki Abe[2] and Takafumi Utsunomiya[1]

[1] *St. Luke Clinic, Oita 870-0823, Japan*
[2] *Graduate School of Science and Engineering, Yamagata University, Yamagata 992-0038, Japan*
[3] *The Institute for Advanced Reproductive Medical Technology, Gunma 371-0105, Japan*

Abstract: The aim of this retrospective study was to investigate the relationship between the oxygen consumption rate of blastocysts before freezing and their viability after warming with respect to their re-expansion and blastomere loss after warming. A total of 41 blastocysts from 29 *in vitro* fertilization (IVF) treatment cycles, that were not scheduled for cryopreservation for the next cycle, were examined. Good quality blastocysts were defined those having as less than 20% of blastomere loss, and rapid re-expanded blastocysts were defined those having as more than 50% blastocoel re-expansion during post-warming culture of 2 h. We evaluated the oxygen consumption rates before freezing and after warming as well as their relationship with the morphological features of good-quality and rapid re-expanded blastocysts during the post-warming culture. Good-quality blastocysts had a significantly higher oxygen consumption rate after warming than damaged blastocysts; furthermore, rapid re-expanded blastocysts had a significantly higher oxygen consumption rate before freezing than slow or no re-expansion blastocysts. These observations suggest that measurements of the oxygen consumption rate of individual blastocysts before freezing provides important information regarding viability after warming from the viewpoint of blastocoel re-expansion.

Key words: Oxygen consumption rate, Blastocyst, Cryopreservation, Blastocoel re-expansion

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*To whom correspondence should be addressed.
e-mail: lukelab@oct-net.ne.jp

Introduction

Success in assisted reproductive technology (ART) is related to the quality of oocytes and embryos prior to transfer. Embryo quality assessments include a number of invasive and non-invasive procedures. The invasive assessment methods are not useful for clinical application; therefore, in clinical practice, non-invasive assessments are used which merely observe the morphological features of embryos. In the great majority of *in vitro* fertilization (IVF) clinics, cleavage stage embryos are assessed by the method introduced by L. Veeck [1], which evaluates the degree of fragmentation, and blastocyst stage embryos are assessed by Gardner's scoring method [2], which assesses blastocoel development based on the density of the inner cell mass and the number of cells in the trophectoderm. Thus, embryo quality scoring methods are based on morphological evaluations, and better morphology correlates with higher pregnancy rates following transfer. Nevertheless, in some cases, transfer of embryos with low morphological quality results in successful pregnancy, and many embryos with good morphology fail to develop in the uterus. Therefore, it is desirable to employ an alternative embryo assessment in addition to morphological assessment.

Recently, new assessment procedures have been reported. These include the evaluation of the metabolic substances in the medium [3], embryo oxygen consumption in the medium [4, 5], and continuous observation with a time-lapse microscope [6, 7]. With advancements in ART, we have been able to produce comparatively better quality embryos. In addition, we now limit the number of embryos transferred to reduce the risk of multiple

gestations. Therefore, currently we must select a single, excellent-quality embryo for transfer. Oxygen consumption is a useful parameter for the evaluation of embryo quality, because it provides important information about metabolic activity. Shiku *et al.* [8] succeeded in non-invasively determining oxygen consumption of individual embryos with a scanning electrochemical microscopy (SECM) measuring system. This system enables embryo evaluation not only by morphological methods, but also by the mitochondrial activity in the cell [9–12]. In a previous study, we reported for the first time a method for estimating embryo suitability for IVF by measuring oxygen consumption with a SECM. That study suggested that measuring embryonic respiration provided additional and valuable information regarding embryo quality [13].

Currently, IVF centers and clinics are being asked to cryopreserve supernumerary embryos remaining after transfer. It is important to determine the embryos most suitable for cryopreservation; however, it is difficult to select the better-quality blastocysts, because we have no means of evaluating them before freezing. Therefore, the aim of the present study was to characterize morphological features and measure the oxygen consumption and respiratory activity of blastocysts both before freezing and after warming. In addition, we examined the relationship between blastocoel re-expansion after warming and the respiratory activity of each blastocyst.

Materials and Methods

Scanning respiration activity of a single blastocyst

From August 2010 through November 2011, the respiratory activities of 41 blastocysts retrieved from 29 patients were measured for oxygen consumption rate on day 5 (n=19) or day 6 (n=22) following insemination. In the present study, oxygen consumption was measured with SECM system [8]. The SECM system has a measuring instrument on an inverted optical microscope stage, a potentiostat (CRAS-1.1; Clino Ltd., Miyagi, Japan), as well as a notebook computer which acts as a controller and analyzer (Fig. 1). For the measurement of oxygen consumption, HFF 99 medium (Fuso Pharmaceutical Industries, Osaka, Japan) was transferred onto a plate with cone-shaped microwells. A microdisk electrode scanned in the z-direction from the outer edge of the blastocyst, which was located at the bottom of a microwell. The motor driven XYZ-stage was located on the microscope stage for electrode tip scanning. The XYZ stage and potentiostat were controlled by the computer. The oxygen consumption rate of the blastocyst was calculated by software, using an algorithm based on spherical diffusion

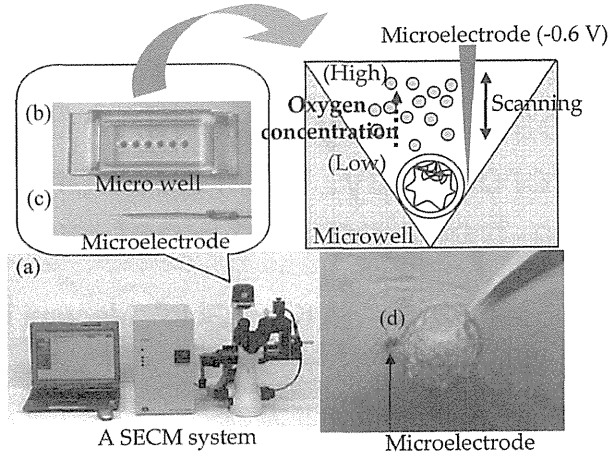

Fig. 1. (a) SECM system, (b) a plate, (c) a microelectrode for measuring the respiration activity of the embryos. The embryo is transferred into a microwell filled with medium, the microelectrode sinks down to the bottom of the microwell and remains at (d) the lowest point. Oxygen concentration profiles are calculated with customized algorithms based on the spherical diffusion theory.

theory [11]. The measurement of the oxygen consumption rate of each blastocyst took approximately 30 sec. It took less than two min to perform three measurements, which were used to calculate the average respiratory activity of each blastocyst.

The patients receiving treatment in our IVF-ET (embryo transfer) program were previously described [14, 15]. Following IVF-ET, surplus embryos that patients preferred not to preserve, or those embryos we deemed unsuitable for preservation due to their poor morphology were used study. Prior to the treatment, written informed consent was obtained from the patients. Ethical approval was given by our clinic's research ethics committee.

Vitrification and warming blastocysts with a closed system

A total of 41 early stage to expanded blastocysts (grade 3 to 4 by Gardner's blastocyst scoring method [2]) were frozen on day 5 (n=19) or day 6 (n=22). We vitrified them individually using Yokota's vitrification method [16]. Briefly, a blastocyst was exposed to 10% ethylene glycol for five min, then placed in a 50% vitrification solution [17] for one min. The vitrification solution contained modified-HFF with 20% Serum Substitute Supplement (Irvine Scientific, Santa Ana, CA, USA), ethylene glycol, and dimethyl sulphoxide at a 2:1:1 ratio. Finally (within 30 sec), the blastocyst was loaded into a 0.25-ml plastic straw containing the vitrification solution. Both sides of the straw were filled with a warming solution containing

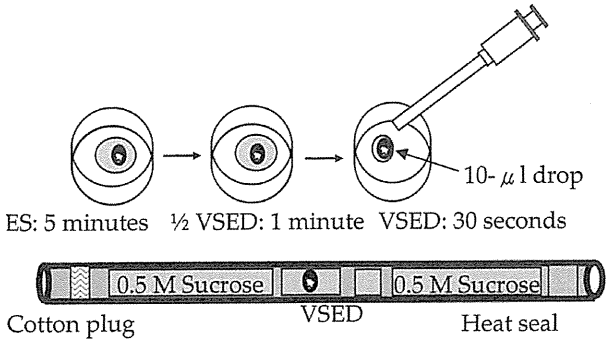

Fig. 2. Vitrification method using a 0.25-ml plastic straw. ES: 10% ethylene glycol. 1/2VSED: 12.5% ethylene glycol + 12.5% dimethyl sulfoxide. VSED: 25% ethylene glycol + 25% dimethyl sulfoxide.

0.5 mol/l sucrose. An air space was placed between the vitrification solution and the warming solution (Fig. 2). Following heat sealing, the straws were placed in liquid nitrogen vapor for 30 s, and then plunged immediately into liquid nitrogen. This method is a hermetically closed system with liquid nitrogen outside of the straw; thus, the embryos are not exposed to infection.

For blastocyst warming, the straw was warmed by plunging it into a 27 °C water bath. This was done after a one-step dilution of the cryoprotectant and was performed using the entire volume of sucrose solution within the straw. Five min after warming, the embryos were placed in the culture medium.

Evaluation of both morphological quality and respiratory activity of blastocysts

A week or more after freezing, the blastocysts were warmed and cultured for 2 h, then evaluated for the degree of blastomere loss and blastocoel re-expansion. We classified thawed blastocysts as "minimally damaged blastocysts" if they had less than 20% blastomere loss, and "severely damaged blastocysts" were those with more than 20% blastomere loss. From the viewpoint of blastocoel recovery, blastocysts were classified as rapid re-expanded blastocysts if >50% of the blastocoels were re-expanded after 2 h of culture following warming. At that time, the respiratory activity was measured again using the SECM. The reason why we measured respiratory activity after 2 h of culture was because, in the clinical setting, we transfer warmed embryos after 2 to 4 h of culture. Each blastocyst was recorded by a camera connected to the inverted microscope before freezing (at the first measurement of respiratory activity), and during the 2 h of culture after warming (at the second measurement of the respiratory activity). We evaluated the oxygen consumption rate and the morphological quality of each blastocyst by estimating their degree of blastomere loss and blastocoel changes both before freezing and after warming. The oxygen consumption rate is higher on the inner cell mass (ICM) side than it is on the trophoblast (TRP) side [8]; therefore, we located the blastocysts ICM and TRP concentrically at the bottom of a microwell.

Statistical analysis

The correlation of oxygen consumption rates before freezing and after warming was analyzed. The mean oxygen consumption rates were compared between groups using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Respiratory activity and morphological recovery of the good quality and damaged blastocysts after warming

We found no relationship between respiratory activity before freezing and after warming; we also found no significant relationship for the respiratory activities of the minimally damaged group and the severely damaged group before freezing. However, significantly different rates of oxygen consumption were found between the two groups after warming (minimally damaged group: 0.59×10^{14}/mol·s^{-1}; severely damaged group: 0.39×10^{14}/mol·s^{-1}; $P = 0.00508$; Table 1).

Respiratory activity and morphological recovery of the rapid re-expansion group, and the slow or no re-expansion group after warming

In a preliminary study, we extended the culture period and evaluated the blastocysts on the day after warming (n=30). The rapid re-expanded blastocysts with >50% blastocoel re-expansion (Fig. 3 a-c) during the two-hour culture after warming exhibited good development on the following day (11/13; 85%). In contrast, the slow or no re-expansion group (Fig. 3 d-f) exhibited poorer development (8/17; 47%) (Fig. 4).

Sixteen blastocysts, which exhibited a rapid re-expansion had significantly higher respiratory activity before freezing than the 25 blastocysts that exhibited slow or no re-expansion (rapid: 0.70×10^{14}/mol·s^{-1}; slow or no: 0.58×10^{14}/mol·s^{-1}; $P = 0.01482$). Similarly, the rapid re-expanded blastocysts had higher respiratory activity during the two-hour culture after warming (0.60×10^{14}/mol·s^{-1}) than the slow or no re-expansion blastocysts (0.49×10^{14}/mol·s^{-1}), but the difference was not statistically significant (Table 2).

Table 1. Comparison of the mean oxygen consumption rates before freezing and after warming between the minimally damaged group and the severely damaged group

	Oxygen consumption rate before freezing ($\times 10^{14}$ / mol·s^{-1})	Oxygen consumption rate after warming ($\times 10^{14}$ / mol·s^{-1})
Minimally damaged blastocysts with less than 20% blastomere loss (n = 28).	0.64 ± 0.20[a]	0.59 ± 0.22[b]
Severely damaged blastocysts with more than 20% blastomere loss (n = 13).	0.59 ± 0.10[a]	0.39 ± 0.20[b]

[a]: $P = 0.17166$ (not significantly different), [b]: $P = 0.00508$ (significantly different).

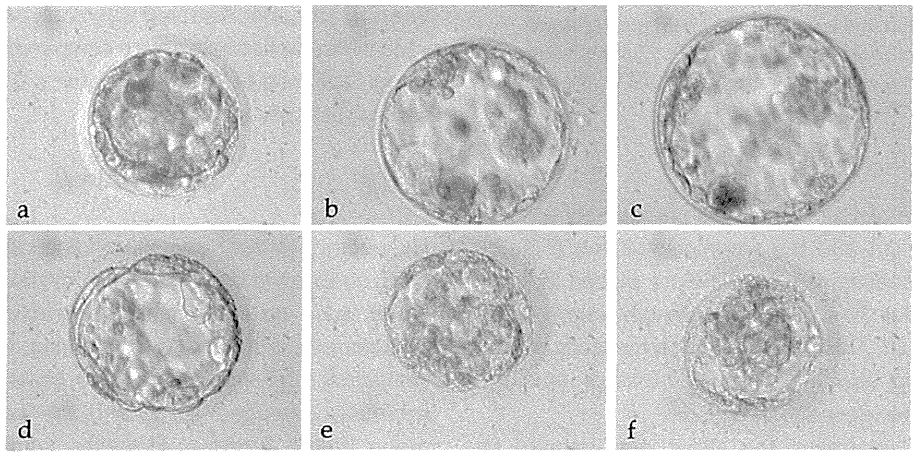

Fig. 3. Morphological changes of frozen blastocysts. (a, d) Same initial morphological level, before freezing; (b, e) After warming for 2 h, and (c, f) After warming for 24 h. (a–c): (a) Before freezing. (b) Two-hour culture after warming shows >50% of blastocoel re-expansion. (c) Following day (24 h of warming), blastocyst develops to fully expanded blastocyst. (d–f): (d) Before freezing. (e) Two-hour culture after warming shows no blastocoel re-expansion. (f) Following day arrested development (Original magnification ×400).

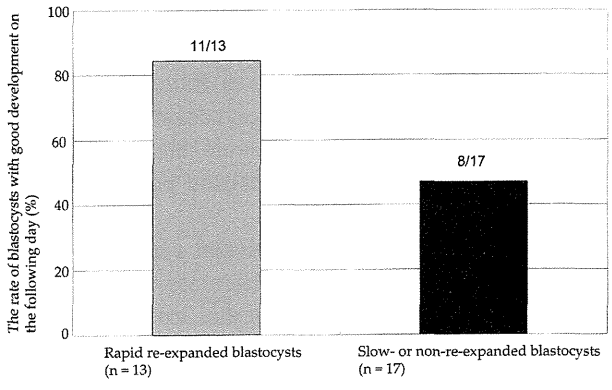

Fig. 4. Eleven out of 13 rapid re-expanded blastocysts with >50% blastocoel re-expansion exhibited good development on the following day. Eight out of 17 slow or no re-expansion blastocysts exhibited good development.

Discussion

We measured the oxygen consumption rate of human blastocysts with the SECM both before freezing and after warming. Furthermore, after warming, blastocysts that had minimal morphological damage showed higher respiratory activities (measured after warming) than severely damaged blastocysts. In addition, blastocysts with rapid blastocoel re-expansion after warming had higher respiratory activities (measured before freezing) than those that exhibited slow or no re-expansion. In this study, the ages of the embryos were day 5 (n=19) to day 6 (n=22) the embryonic stages ranged from the early (n=19) to expanded stages (n=22), and were grade 3 to 4 by Gardner's blastocyst scoring method. The oxygen consumption rates were not significantly different between embryo ages (day 5: 0.64 ± 0.18 × 10^{14}/mol·s^{-1}; day 6: 0.61 ± 0.17

Table 2. Comparison of the mean oxygen consumption rates before freezing and after warming between the rapid re-expansion group and the slow or no re-expansion group

	Oxygen consumption rate before freezing ($\times 10^{14}$ / mol·s^{-1})	Oxygen consumption rate after warming ($\times 10^{14}$ / mol·s^{-1})
Rapid re-expanded blastocysts after 2 h of culture following warming (n = 16).	0.70 ± 0.22^{c}	0.60 ± 0.24^{d}
Slow or no re-expansion blastocysts after 2 h of culture following warming (n = 25).	0.58 ± 0.12^{c}	0.49 ± 0.22^{d}

[c]: $P = 0.01482$ (significantly different), [d]: $P = 0.06213$ (not significantly different).

× 10^{14}/mol·s^{-1}) or blastocyst stages (grade 3: 0.64 ± 0.19 × 10^{14}/mol·s^{-1}; grade 4: 0.61 ± 0.16 × 10^{14}/mol·s^{-1}).

In IVF, patient safety and reassurance are of utmost importance. We must preserve embryos in liquid nitrogen to avoid infection or contamination. Therefore, we must freeze embryos using a closed container. To do this, we employ a plastic straw, the ends of which are closed by heat sealing and a cotton plug. Consequently, the accurate evaluation of the embryo quality without damage is possible. The quantitative prediction of mitochondrial activity was achieved in the embryos evaluated in this study. The SECM developed by Abe [9] is a non-invasive and useful system, which can evaluate oxygen consumption rate. The evaluation is based on the spherical diffusion theory and several novel findings have been reported for bovine embryos using this method [8]. We previously reported that embryos with the same morphological grade exhibited considerable variation in respiratory rate, and suggested that this system would be of benefit in IVF [13]. Vitrified blastocysts had significantly lower respiratory activities than non-vitrified blastocysts. Furthermore, well-developed blastocysts after warming were found to have higher respiration rates than arrested or degenerated blastocysts [18].

The aim of present study was to demonstrate the correlation between the morphological features and respiratory activity before freezing. There was no significant difference in respiratory activity before freezing between the minimally damaged group and the severely damaged group (Table 1). However, the blastocysts with higher respiratory activity before freezing exhibited a greater potential for recovery (Table 2). Thus, measuring respiratory activity before freezing is an effective method for the prediction of embryo viability after warming, in terms of re-expansion. The clinical efficacy of observing blastocoel re-expansion after warming was reported by Shu *et al.* [19]. They reported that rapid re-expanded blastocysts (>50% re-expansion) should be prioritized for transfer. They concluded that rapid re-expanded blastocysts need to be differentiated from slow and unexpanded blastocysts in post-thaw cultures. The literature contains a few reports of lower pregnancy rate in the absence of blastocoel re-expansion [20, 21]. The results of our present study also provide evidence in support of the efficacy of observing the degree of blastocyst re-expansion after warming, to predict the success of frozen-warmed embryo transfer, as assessed by oxygen consumption rate. Clinically, SECM technology makes it possible to select blastocysts which have grater survival ability before freezing, and next cycle, transfer one to a patient's uterus.

In conclusion, the SECM can non-invasively measure the oxygen consumption of a single human blastocyst. This technique contributes to the estimation of embryo viability by better evaluating embryos suitable for freezing. In the future, the cutoff value for the oxygen consumption rate will need to be investigated.

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Quality evaluation of IVM embryo and imprinting genes of IVM babies

Hiroaki Yoshida · Hiroyuki Abe · Takahiro Arima

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Abstract
Purpose Oxygen consumption rates of human embryos derived from in vitro matured (IVM) oocytes and controlled ovarian hyperstimulation (COH) were compared with scanning electrochemical microscopy (SECM) non-invasively in order to answer why embryos from IVM oocytes have lower developmental potential. We also analyzed the epigenetic disorders for IVM babies born in our clinic.
Methods The oxygen consumption rate was calculated with the SECM system for different maturation stages of human oocytes, IVM and COH embryos. Blood from umbilical cords of IVM babies was collected to examine the imprinting genes.
Results There were no significant differences in oxygen consumption of embryos at each cleavage stage between IVM and COH (range $0.26–0.56\times10^{14}/\mathrm{mol\,S^{-1}}$). There also was no abnormality found in expression of imprinting genes in IVM babies.
Conclusions There are no differences in terms of oxygen consumption between embryos derived from IVM and COH. There was no imprinting gene disorder founded from IVM babies.

Keywords IVM · Oocytes · Embryo · Oxygen · Epigenetics

Capsule There are no differences in oxygen consumption between embryos derived from IVM and COH, and there was no imprinting gene disorder founded from the IVM babies.

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H. Yoshida (✉)
Yoshida Ladies Clinic, Center for Reproductive Medicine, Sendai, Japan
e-mail: hiroaki@yoshida-lc.jp

H. Abe
Department of Biochemical Engineering, Graduate School of Science and Engineering, Yamagata University, Yamagata, Japan

T. Arima
Environment and Genome Research Center, Tohoku University School of Medicine, Sendai, Japan

Introduction

Since the first in vitro fertilization embryo transfer (IVF-ET) baby born [1], the field of assisted reproductive technology (ART) has progressed tremendously. A variety of ovulation inductions is developed to acquire more oocytes for IVF in order to improve the pregnancy rate. In late 1990s, in vitro maturation (IVM) of human oocytes has been applied clinically [2], and the first pregnancy and delivery of IVM baby from woman with polycystic ovary syndrome (PCOS) was reported in 1994 [3]. The advantage of IVM is to avoid side effect of ovarian stimulation, including ovarian hyperstimulation syndrome (OHSS), and to reduce the treatment cost and mental stress. However, IVM treatment showed poor embryo development and low pregnancy rate [4]. To evaluate oocytes and embryos accurately, several approaches have been made non-invasively [5, 6]. We reported that mitochondria are important cell organelle producing ATP through respiration, which is essential for cellular activity and closely related to oxygen consumption [7, 8]. When mitochondria developed normally in the oocyte, based on animal model studies, it indicated that the developmental potential of embryos was improved when mitochondria developed normally in oocytes [7, 9]. Therefore, an oocyte and embryo respiration measuring system has been developed using Scanning Electrochemical Microscopy (SECM).

The respirational measuring system was used with microelectrode as a sensor to analyze oxygen consumption by the oxygen reduction based on the spherical diffusion theory. This system can measure cell respiration with non-

invasively and high-sensitively. Moreover, with the respiration activity as an index, it is capable of securely examining embryos and oocytes quality in a short time.

The oxygen consumption of embryos from IVM and COH

Total of 253 embryos from IVM oocytes were measured for oxygen consumption rate individually. The average oxygen consumption of each early cleavage stage embryo are 0.34± 0.1 at 4-cell stage, 0.37±0.1 at 6-cell stage, 0.4±0.2 at 8-cell stage, and 0.50±0.2 $F\times10^{14}/molS^{-1}$ at 10cell stage at same grade by Veeck classification [10]. There was no significant difference between IVM and COH. Specifically, IVM is 0.44 $F\times10^{14}/molS^{-1}$, and COH is 0.34 $F\times10^{14}/molS^{-1}$ respectively by comparison on day 3 embryos. There was also no significant difference in blastocyst development between IVM and COH based on oxygen consumption (Fig. 1). It may suggest that pregnancy rate may be predicted by selecting embryos for transfer with combination of morphological and respiration rate evaluation [11].

Evaluation of physical and mental development of IVM babies as well as epigenetic expression

There are few reports about the evaluation of IVM babies. We were able to examine 7 babies out of 52 pregnancies for physical and mental disorders, such as mode of delivery, weight, height, and apgar score (Table 1). There was no abnormal disorder in babies from 11 months to 4.7 years of age.

Genome imprinting is a genetic phenomenon controls the allele-specific gene expression, which is an inheritance process that only a certain allele from a parent (father or mother) is expressed selectively. Since the increase of imprinting disorder with ART is reported recently [12], IVM-IVF may bring more imprinting disorders than normal IVF due to its necessity of mature culture process. DNA methylation has been considered the most important epigenetic modification in gametogenetic process for the establishment of imprint. Arima et al. [5, 13] identified the region of allele-specific human imprinted gene and analyzed DNA methylation. Among 8 imprinted genes, *H19, GTL2,* and *ZDBF2* are paternal, and *PEG1, PEG3, LIT1, ZAC,* and *SNRPN* are maternal. Any disorder of methylation pattern was not observed in regulatory region of analyzed 8 imprinted genes of born babies at our clinic (Fig. 2-a, b).

Discussions

The morphological examination has been applied for quality evaluation of oocyte and embryo for a long time. However, if it is only by morphological approach, it might be difficult to increase embryo development and pregnancy rate. Thus, the evaluation technique noted embryo metabolism is developed for quality evaluation of oocyte and cleavage stage embryo [13], and embryo metabolic activity focused on nutrient consumption, such as glucose, pyruvic acid, and amino acid, was also studied [1, 14]. Cell oxygen consumption products ATP in a process of oxidative phosphorylation, and it is regarded as one of the evaluation criteria of embryo metabolic activity [3].

As a new method for embryo evaluation, Tesarik et al. [15] reported the case which is improved the blastocyst developmental rate and implantation rate using the sequence of pronuclear of nuclear after 24-h culture. However, the recent report using time-lapse cinematography by Mio et al. [16] proved that the sequence of pronucleolus is just a part of certain evaluation at dynamic changed using his system observation because the sequence moves variously in individual cleavage stage embryo. That is, nucleolus transfers the sequence dramatically in a process of the development.

The oxygen consumption rate was measured using mouse and rabbit embryos at first [17]. This study measured mouse blastocyst, human oocyte and blastocyst [17], and oxygen consumption by transform of hemoglobin from oxygen hemoglobin indirectly. After that, mammal embryo oxygen consumption rate was measured with parameter of various

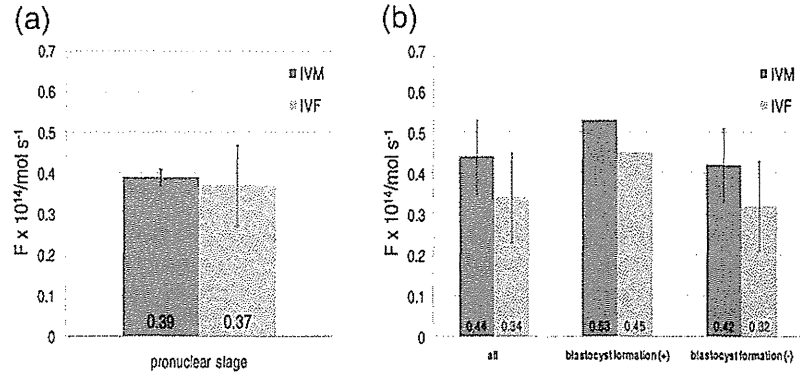

Fig. 1 Comparison of embryo oxygen consumption rate (pronuclear stage, Day 3). **a** shows oxygen comparison of embryo at pronuclear stage between IVM and COH; **b** shows oxygen comparison of embryo on day 3 between IVM and COH. Also it shows whether the embryos developed to blastocysts or not based on oxygen comparison

Table 1 Birth findings and prognosis of IVM babies born

Patient	Outcomes	Mode of delivery		Weight	Height	Apgar. score	Cord pH	Prognosis & others
M.I	Delivery (40 w–6 d)	Vaginal	M	3102 g	49.5 cm	9 / 9	–	1.8 y.o. N.P.
M.S	Delivery (twin, 36 w–1 d)	C/S	M/F	2678 g / 2320 g	46.2 cm / 44.5 cm	9 / 9	–	2 y.o. N.P.
H.K	Delivery (38 w–4 d)	Vaginal	M	2810 g	47 cm	9 / 9	7.438	4.7 y.o. N.P.
E.S	Delivery (38 w–5 d)	Vaginal	M	3070 g	48 cm	8 / 9	7.114	1.6 y.o. N.P.
M.S	Delivery (41 w–6 d)	C/S	M	3966 g	51 cm	9 / 10	7.29	1.4 y.o. N.P.
J.Y	Delivery (39 w–4 d)	C/S	M	2964 g	48.6 cm	9 / 9	7.23	–
M.K	Delivery (40 w–2 d)	Vaginal	M	3056 g	47.8 cm	8 / 8	7.032	11 month

metabolic process data, for example, the method using diver by Magnusson [6], use of spectrophotometrics [18], method using fluorescent dye [19], and electrochemical technique [20]. More accurate and simple oxygen respiration measuring was required, so embryo respiration measuring system, which can measure single embryo oxygen respiration, was developed [7]. This system enables to greatly shorten the measuring time and improve the operability by the use of measuring plate with a conic micro well at the bottom. Also, this system achieved high-sensitive measuring of single cell

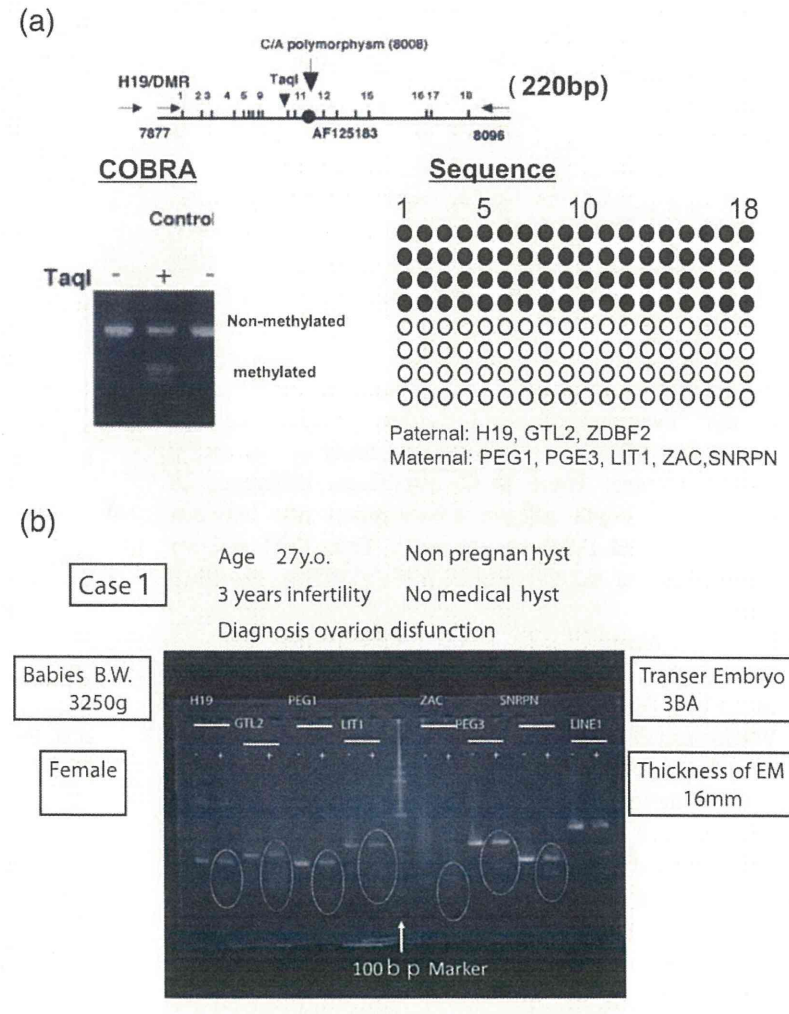

Fig. 2 **a** Analysis of methylation for imprinting genes from IVM babies; **b** Imprint gene Methylation assay with umbilical cord from IVM baby

respiration, so eventually it can evaluate individual embryo quality with high-precision [7].

According to Abe report et al. [7, 9], respiration increase corresponds with mitochondrial development from morula to blastocyst stage in most of animal embryos, and the embryo with high consumption rate has a good viability and freezing ability. In addition, it is proved that the embryo with respiration rate above standard value is also high in pregnancy rate at transplant experiment after respiration measuring. These results demonstrate that consumption measuring is effective to evaluate embryo quality [7]. As for human embryos, it is evident there is a difference of respiration rate between embryos at same grade by Veeck classification, and embryo development needs respiration more than constant rate. Recently, we also found when the respiration rate increases in a group of thawing embryo, developmental rate for blastocyst and freezing ability for cryopreservation will be high.

Moreover, measuring consumption rate of single animal embryo by SECM system enables much detailed examination of metabolic capacity in a process of oocyte development. Abe et al. [7] reported that mitochondria distribution manner, the ATP amount of oocyte, and respiration ability of oocyte and cumulus alter prominently at pre or post maturation, and the respiratory activity is influenced with condition of culture medium. They also proved the oocyte respiration ability varies with the condition of adherent cumulus, and COC with high respiration activity increase maturation rate after oocyte maturation culture. Likewise, human oocyte respiration rate is significantly high at Grade 1 and 2, which are adhered with more than three layers of cumulus, and it influences the maturation, fertility, and embryo developmental rate thereafter. These results suggested that SECM is effective for quality evaluation of human oocytes. There is no significant difference of subsequent oocyte oxygen consumption rate between from COH and IVM, or any ages. Thus IVM embryo established as oocyte has same functions as COH oocyte.

Imprint disorder in ART was originally rare, but it is increasing with the development of technique in recent years. In the U.S, there is a report that the frequency of Beckwith-Wiedemann syndrome is 0.76 % in a general group, whereas it is 4.1 % (about 6 times higher) after ART [12]. In addition, it is reported that about 25 % of male sperm under infertility treatment shows imprint disorder [13]. Since IVM requires maturation culture, the culture duration becomes longer than IVF, and it may bring an epigenetic transform; however, there is no epigenetic disorder of newborn babies from IVM process in our clinic. In any case, ART has still to be investigated to prove the safety in the future.

Conclusions

Embryo respiration measuring system based on SECM enables to measure the respiration of single human embryos. Combination of morphological evaluation and respiration rate may provide an effective barometer of embryo selection. IVM embryos have the same function as COH embryos after fertilization. None physical and mental disorders or defects in epigenetic genes were observed in IVM babies. It requires further study with more large numbers in the future.

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細胞呼吸計測技術を応用した胚品質評価システムの開発

阿部宏之

山形大学大学院理工学研究科・バイオ化学工学専攻

はじめに

近年、高性能受精卵培養液や体外受精・顕微授精などの先進生殖技術が開発され、移植可能胚の作製効率は飛躍的に向上している。しかし、生殖技術が高度化する一方で、胚移植における受胎率は伸び悩んでいる。この原因の一つとして、治療に供する卵子や受精卵の品質評価の精度に問題があると考えられている。体外受精・胚移植（IVF-ET）において、移植前に質的に最も良好な胚を選択することは、妊娠率の向上、多胎妊娠の回避、流産率の低下のために極めて重要である。

現在、胚の品質（クオリティー）は割球の形態や数などの形態的特徴を基準に評価されている。形態観察による品質評価は、簡単・迅速で非侵襲的な方法であることから、現状では最も有効な胚の品質評価法である。しかし一方で、評価の基準となる胚の形態的特徴は定量性に欠けるため、判定結果が観察者の主観に左右される可能性がある。そこで筆者は、胚の品質を客観的に評価するための指標としてミトコンドリアの呼吸機能に着目し、細胞の呼吸活性を指標とする新しい胚品質評価システムの開発に取り組んできた。本稿では、ウシとヒトを中心に、これまでに開発されてきた胚の品質評価法を解説するとともに、電気化学計測技術を応用した独自の胚品質評価法を紹介する。

1. 形態観察による胚の品質評価

一般に、胚の品質は実体顕微鏡または倒立顕微鏡を用いた形態観察により、割球の数や形態的特徴を基準に評価されている[1-3]。例えば、ウシの桑実胚は割球が集まった細胞塊（embryo mass）の形態（コンパクッションの程度）やフラグメンテーションなどを基準に4つのカテゴリーに分類される（図1）。また胚盤胞は、胞胚腔の状態や胚の拡張度、内部細胞塊（inner cell mass: ICM）や栄養膜（trophoblast）の状態を基準に3段階の品質に分類することができる。さらに、胚の色調、細胞の数と密度、細胞の輪郭や色調など多くの評価基準が加わることもある。これら形態観察によって質的に良好とされるグレードA（Excellent）とB（Good）に分類された桑実胚は、移植後の受胎率が40%と高い[1]。しかし一方で、形態不良とされるグレードC（Fair）とD（Poor）の胚でも受胎率が20%前後であることから、これら形態観察による品質評価の精度には課題が残されている。

ヒトの正常受精胚では、媒精16〜18時間後に雄性前核および雌性前核の2つの前核（2PN）、そして各前核中に核小体（nucleolar precursor bod-

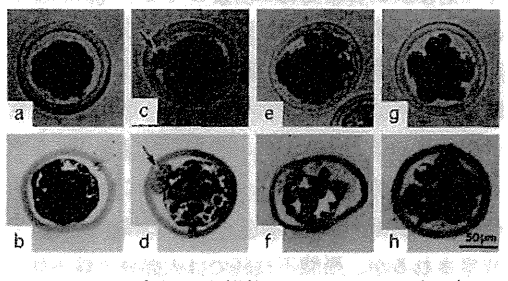

図1. ウシ桑実胚の形態的クオリティー評価（a, c, e, g　ノマルスキー微分干渉顕微鏡観察、b, d, f, h　準薄（semi-thin）切片（1 μm、トルイジンブルー染色）像）。細胞塊の形態とフラグメントの数を基準にそれぞれグレードA（a, b: Excellent）、グレードB（c, d: Good）、グレードC（e, f: Fair）、グレードD（g, h: Poor）の4つのクオリティーに分類される。（参考論文13を一部改変）

ies: NPB）が確認できる。前核の大きさ・接合の有無、核小体の数・大きさ・配列、さらに、細胞質の観察をもとに前核期胚のスコアリングを行い胚の品質を評価している[4,5]。ヒトの初期分割期胚は、割球の形態とフラグメンテーションの割合を指標として評価するVeeckの分類[6]が最も普及している。この評価法では、割球の形態が均等でフラグメンテーションが認められない胚をGrade 1と表記し、最も形態的に良好であると評価している。割球が不均等になりフラグメンテーションの出現が顕著になるに従いGradeの数値が上がり（Grade 2～Grade 5）形態不良と判定される。また、胚盤胞の品質評価にはGardnerらが提案した方法が広く用いられている[7]。この方法では、胚盤胞は胚盤胞腔の広がりと孵化（ハッチング）の程度によって1～6の6段階に分類され、さらに内部細胞塊（inner cell mass: ICM）と栄養膜細胞層（trophectoderm: Tr）の細胞数により、それぞれA、B、Cの3段階に評価される。

2. 胚の品質に関係する超微細形態

正常に受精した胚では、発生の進行に伴いダイナミックな微細形態変化が起こる。ウシの形態良好胚では、桑実胚および胚盤胞のステージにおいてギャップ結合やデスモゾームなどの細胞接着装置が良く発達しているが、形態不良胚ではデスモゾームや微絨毛が未発達である[8-11]。これは、胚の品質に細胞間接着装置が影響していることを示唆している。核小体は、核内にあってリボゾームRNA（rRNA）転写を行っており、その転写活性は核小体の微細形態によって把握できる[12]。正常に発生したウシ胚では、桑実胚期において最もrRNA転写活性の高いステージ4の核小体が多く観察されるが、形態不良胚では大部分の核小体はrRNA転写活性の低いステージ3であることから、低品質胚ではrRNAの発現活性が低いと考えられる[13]。また、高品質胚では桑実胚期から胚盤胞期にかけてミトコンドリアが顕著に発達するが、低品質胚ではほとんどのミトコンドリアは未成熟であり、過剰な脂肪滴の蓄積が観察される。このような脂肪滴蓄積は胚の耐凍能に大きく影響し、胚の品質低下の一因となっている[14,15]。

一方、ヒト胚において形態的品質と密接な関係にある超微細形態が明らかになっている。Gardner[7]らの方法で3BB以上を良好胚、それ以下のグレードを不良胚として分類し、それぞれの胚の微細構造を観察した。その結果、品質良好胚ではミトコンドリアや微絨毛は正常に発達しているが、品質不良胚ではミトコンドリアの多くは未発達であり、細胞内には多くの脂肪滴やアポトーシス像が観察された（図2）。これらの研究から、胚の品質にはミトコンドリアやアポトーシスなどが深く関与していることが明らかになった。

3. 代謝物質測定による胚の品質評価

前述した形態観察による胚の品質評価は、評価の基準となる形態的特徴が定量性に欠けるため、評価の精度に影響が出る可能性がある。そこでより客観的・定量的な指標として、胚によるグルコース、ピルビン酸、アミノ酸等の栄養素の消費に着目した品質評価が試みられてきた[16-20]。また、細胞の呼吸（酸素消費）を指標に胚の品質を評価する方法も行われている[21-23]。ミトコンドリアは

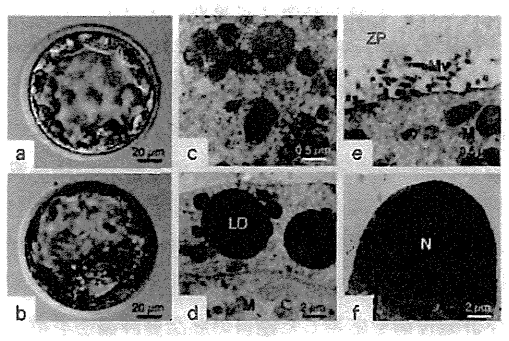

図2. ヒト体外受精胚の光学顕微鏡像（a, b ノマルスキー微分干渉顕微鏡観察）および電子顕微鏡像（c-f）。Gardner分類法によるクオリティー良好胚（a, c, e）と不良胚（b, d, f）。c, b クオリティー良好胚では発達した微絨毛（Mv）とミトコンドリア（M）が観察される。ZP 透明帯。d, f クオリティー不良胚では高電子密度で大型の脂肪滴（LD）と凝縮した核（N）が特徴のアポトーシス像が認められる。

酸化的リン酸化反応（呼吸）により細胞活動に必要なエネルギー（アデノシン三リン酸　ATP）を産生し、卵子や胚の代謝活動にも深く関与していることから、ミトコンドリア呼吸は胚の品質評価の有効な指標になると考えられている。

ミトコンドリアの呼吸機能に異常が生じると代謝異常や種々の疾患の原因となることから、これまでにいくつかの細胞呼吸計測技術が開発されてきた。代表的なものとして、蛍光発色法[24-26]や酸素センサー[27-30]を用いた細胞呼吸測定法が考案されている。しかし、その多くは測定感度や侵襲性などの面で課題があることから、胚の品質評価においては実用化されていない。初期胚や卵子ではミトコンドリアは十分に機能していないため、その呼吸活性は非常に低いと考えられる。また、移植に供する胚や卵子の品質評価において実用化するためには非侵襲計測であることが絶対条件である。したがって、非侵襲・超高精度・迅速計測という条件をクリアした技術でなければ、現在開発が望まれている精度の高い胚品質評価法として実用化することは難しい。

4. 電気化学計測法を応用した細胞呼吸測定装置の開発

高精度・非侵襲的という測定条件を満たす計測技術として、筆者らは電気化学計測技術に注目してきた。電気化学計測法はプローブ電極による酸化還元反応を利用し、局所領域における生物反応を電気化学的に検出する技術であり[31,32]、この技術の有効な装置としてマイクロ電極を探針とする走査型電気化学顕微鏡（scanning electrochemical microscopy: SECM）が注目されている。SECMの空間分解能は探針であるマイクロ電極径に依存するため原子や分子レベルの解析は困難であるが、局所空間での化学反応の評価やイメージング、生体材料を用いたリアルタイム解析や化学反応誘起が可能であることから、局所領域の電気化学センシングなど種々の系で用いられている[33-36]。例えば、酸素の還元電位を検出できるマイクロ電極を用いることで細胞の酸素消費量（呼吸）を高感度・非侵襲的にリアルタイムで測定することができる。

従来、SECMは微量な酸素消費を検出できることから、金属錆の検出装置として用いられてきた。筆者らは、従来型SECMを生物試料、特に受精卵の呼吸計測に応用するために、呼吸測定に関連した要素技術の開発を行ってきた。その結果、SECMをベースに受精卵や微小組織などの球状試料の酸素消費量を非侵襲的に測定できる「受精卵呼吸測定装置」の開発に成功した[37]。この呼吸測定システムは、倒立型顕微鏡、マイクロ電極の電位を一定に保持するポテンショスタット、酸素消費量算出のための専用解析ソフトを内蔵したノート型コンピューターにより構成されている（図3a）。倒立型顕微鏡のステージ上には、マイクロ電極の3次元走査を可能とするXYZステージが設置されており、生物試料の呼吸計測のために気相条件制御が可能な測定用チャンバーや保温プレートが設置できる。また、計測精度と操作性の向上を目的に、マイクロ電極の改良、専用の多検体測定プレートおよび測定液を開発している。従来型のSECMは、金属等の腐食部位を検出する装置として先端径10～20 μmのマイクロ電極を探針として用いているが、酸素消費量が非常に小さい細胞や受精卵の呼吸測定には電極の感度が不十分であり、より高感度のマイクロ電極が必要であった。マイクロ電極の計測感度は先端径が小さいほど高いため、受精卵や細胞の呼吸測定では先端径2～4 μmにエッチング加工した白金電極をガラスキャピラリー先端部に封止したディスク型マイクロ電極（図3b）を使用している。

5. 単一胚の呼吸量測定

「受精卵呼吸測定装置」を用いた呼吸測定には、専用の多検体測定プレートと測定液を用いる。多検体測定プレートは測定操作の簡易化を目的に開発され、プレートの底面には円錐形のマイクロウェルが施されている（図3c）。マイクロ電極が検出する微弱な電流は溶液の成分によって影響を受けるため、呼吸量測定にはマイクロ電極の

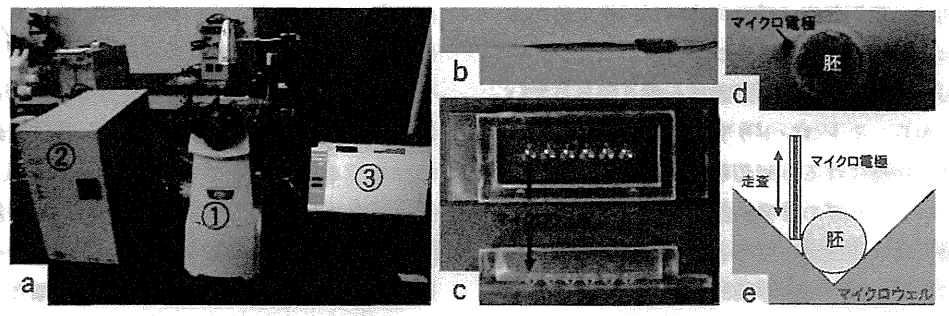

図3. (a) 走査型電気化学顕微鏡を改良した「受精卵呼吸測定装置　HV-405」。①倒立型顕微鏡、②ポテンショスタット、③ノートパソコン（呼吸能解析ソフトを内蔵）。(b) 呼吸測定用マイクロ電極　ディスク型白金マイクロ電極で、先端部が直径 2〜5 μm にエッチング加工された白金電極がガラスキャピラリーに熱封止されている。(c) 多検体測定プレート　プレート底面には円錐形のマイクロウェルが 6 穴施されている。(d) マイクロウェル底部に静置したウシ胚。(e) マイクロ電極は胚近傍を鉛直方向に走査することで、胚の酸素消費量を測定する。

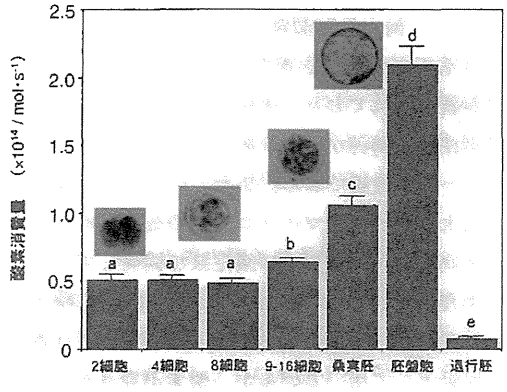

図4. ウシ体外受精胚の発生過程における呼吸量変化。桑実胚から胚盤胞期にかけて呼吸量が増加する。退行胚ではほとんど呼吸量は検出されない。異符号間で有意差（$P < 0.05$）がある。

測定感度に影響を与えず、胚に対して非侵襲な専用の測定液を用いる。測定液を満たしたマイクロウェル内に試料（胚）を導入した後、マイクロウェルの底部中心に静置させる（図 3d）。酸素が還元可能な −0.6 V *vs.* Ag/AgCl に電位を保持した後、マイクロ電極を移動速度 20〜30 μm/sec、走査距離 150〜300 μm の条件で透明帯近傍を鉛直方向に走査する（図 3e）。通常、1 回の呼吸測定ではマイクロ電極を 2〜3 回走査した後、胚の酸素消費量は球面拡散理論式[38,39] を基本に開発した解析ソフトを用いて算出する。

これまでに「受精卵呼吸測定装置」を用いて、

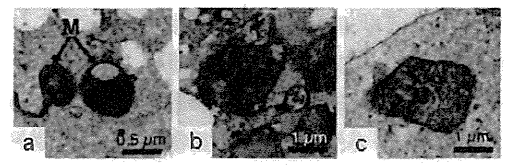

図5. ウシ体外受精胚の発生過程におけるミトコンドリアの微細形態変化。a　8 細胞期胚、b　桑実胚、c　胚盤胞。M　ミトコンドリア。

ウシ、ブタ、マウスの単一胚の呼吸量測定に成功している。ほとんどの動物胚では、8 細胞期までは酸素消費量は少なく、桑実胚から胚盤胞にかけて顕著に呼吸量が増加する（図 4）。呼吸測定の有効性を検証するために呼吸活性の変化とミトコンドリアの発達との関係を電子顕微鏡により調べた。その結果、呼吸活性の低い 8 細胞期まではほとんどのミトコンドリアは未成熟であり、呼吸量が急激に増加する桑実胚から胚盤胞にかけてミトコンドリアの顕著な発達（クリステの拡張）が認められた（図 5）[40]。このように、呼吸量の増加とミトコンドリアの発達は同じ発生ステージに起こることから、「受精卵呼吸測定装置」はミトコンドリアによる呼吸を高精度で検出していると考えられる。現在、ミトコンドリアの細胞内局在や膜電位活性の解析、ATP 産生の定量、呼吸鎖複合体（シトクローム c 酸化酵素　Cox）の遺伝子発現解析などのミトコンドリア呼吸機能に関連する

呼吸量が顕著に増加する。また、受精後3日目に比較的高い呼吸活性（0.26～0.56 × 10^{14} /mol・sec^{-1}）を有する胚は、0.26×10^{14}/mol・sec^{-1}未満または0.56×10^{14}/mol・sec^{-1}を上回る呼吸活性を示す胚と比べて有意に高い確率で胚盤胞へ発生することが示されている[4]。これらの結果は、ヒト胚においても呼吸活性を指標とする品質評価が可能であることを示唆している。今後は、多くの動物実験とヒト余剰胚を用いた前臨床研究を実施し、装置および測定技術の有効性と安全性を詳細に検証する予定である。さらに、基礎研究の成果を踏まえ、所定の倫理承認を得た後、不妊治療での臨床応用を目的とした呼吸測定胚の移植を目指してしている。

おわりに

本稿では、形態観察を基本とする従来の胚品質評価法と、電気化学計測技術を応用した細胞呼吸活性を指標とする新しい胚品質評価法を解説した。家畜繁殖領域で開発された技術は、不妊治療などの生殖補助医療に応用されるケースが多く、本稿で紹介した「受精卵呼吸測定装置」はヒト胚の品質評価など、医療分野への応用が十分に期待できる。今後、生殖補助医療においては不妊治療技術の高度化や高齢不妊患者の増加に伴い、移植の対象となる胚もより厳密に評価する必要があると思われる。このため、これまでに開発されているいくつかの品質評価法のメリットを有機的に融合した新しい評価システムの開発が必要になってくる（図6）。例えば、呼吸測定による胚品質評価は、形態的評価法との併用が可能であり、これによってより厳密に胚の品質評価が可能であると考えている。今後の詳細な研究により細胞呼吸計測法および測定装置の安全性と有用性が確認され、胚の新しい品質評価システムとして家畜繁殖現場や不妊治療領域において実用化されることを期待している。

「受精卵呼吸測定装置」は細胞呼吸を非侵襲的に測定できることから、胚の品質評価以外にも細胞の代謝機能解析や微小組織の活性評価への応用

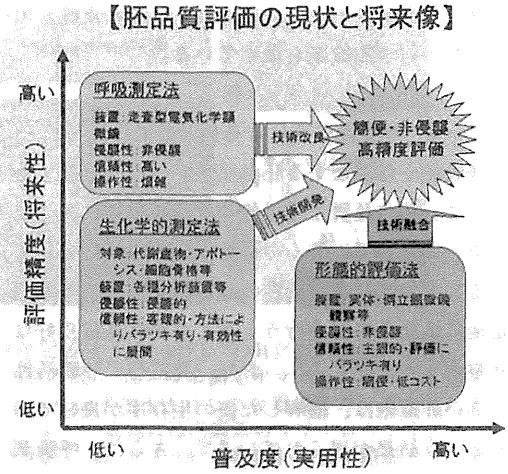

図6. 胚の品質評価の現状と将来像を示した模式図。それぞれの評価法の長所を融合した新しい品質評価法の開発が望まれる。

が可能である。この装置は、細胞呼吸の計測装置としては最も感度が高く、単一細胞レベルでの呼吸量計測も可能である。したがって、単一卵子の品質評価[5]、単一レベルでの細胞機能診断やミトコンドリア呼吸機能解析への展開も期待できる。細胞呼吸は動物・植物問わずほとんどの細胞に共通する生物現象であることから、電気化学的呼吸計測技術および装置の応用範囲は極めて広い。

近い将来、「受精卵呼吸測定装置」が基礎研究から先端医療に至る幅広い分野において実用化されることを期待している。

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