あります。

4　本課題の実施期間

登録は平成26年4月1日から1年間を予定しています。

5　本課題の実施体制（共同研究者等）

この臨床研究は東北大学産婦人科が中心となり、弘前大学産婦人科、秋田大学産婦人科、山形大学産婦人科、福島県立医科大学産婦人科と共同で行われます。

6　本課題の対象者（対象の受精卵）

既に生児獲得後や採卵後３年以上が経過し、不要となった余剰卵（廃棄卵）を所有している方が対象です。

7　本課題の実施方法

・測定方法

まず、受精卵を育てる培養器というものの中に開発した装置を設置します。次に受精卵を中央のチップと呼ばれる部分のくぼんだ場所に置きます（図2)。約５分間培養器内で静置し、微弱な電流を流すことにより受精卵の呼吸量を測定します。これは従来機器において不可能であった操作の単純化と培養器内環境下での測定を可能としたものです。

手順ですが、受精後３日目の卵を用いて呼吸量を測定し、通常の培養に戻します。さらに培養を継続しますと良好受精卵では分割を繰り返し、桑実胚（５日目頃）⇒胚盤胞（６日目頃）⇒孵化(7－8日目頃)、という形態の変化を起こします。一方、不良卵は途中で分割が停止してしまいます。

・評価方法

受精３日目に呼吸量測定と従来から行われている形態学評価をします。次に受精卵が孵化するまでの形態変化を確認し、呼吸量と比較検討します。また、従来からの顕微鏡による形態評価と本機器による呼吸量評価を比較検討します。さらに、呼吸量測定装置を用いた際の分割スピードの変化や夾雑物の増減を検討し、これまでの手法と比較した所見を調べます。

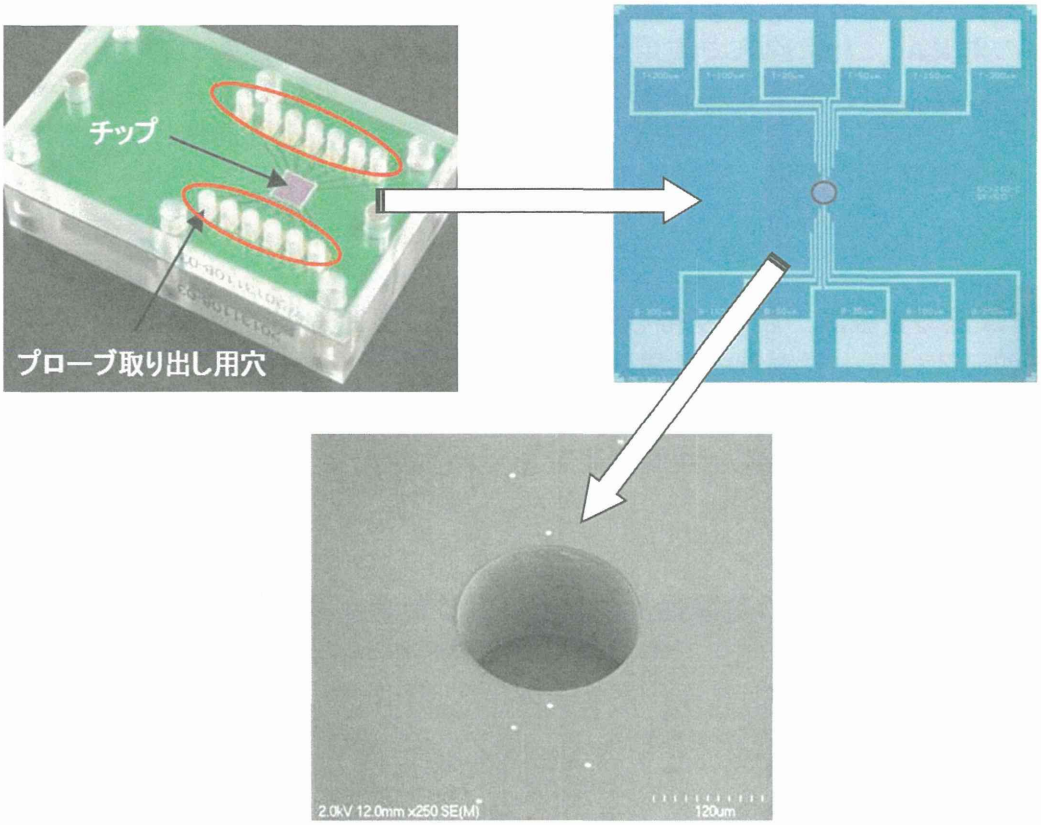

図 2. 開発した呼吸量測定装置（左上段　⇒　右上段　⇒　下段　の順に拡大像）
中央の穴に受精後 3 日目の卵を置き、培養器の中で測定します。

・本機器使用による有害事象発現の可能性

今回の研究では余剰卵（廃棄卵）を使用するため、受精卵をヒトやヒト以外の動物に戻すことはなく、研究使用後は直ちに破棄します。そのため、本研究により受精卵の所有者に有害事象が発生することはありません。ただし、将来的な研究に反映するため、本機器使用により起こりうる有害事象についても併せて検討します。具体的には、下記のような事象が想定されます。

a）微弱電流による受精卵への影響

今回開発した機器では、1 電極あたり 0.4-0.5nA（全体で 5-6nA で、従来機器の 1/5 程度）の非常に弱い電流を用いて約 5 分間計測を行います。従来機器では、ヒトおよび動物受精卵に明らかな異常所見（妊娠率、出生数、出生体重、奇形、染色体、生化学的検査など）は認められませんでした。本研究ではヒトやヒト以外の動物に戻すことはありませんので検討項目は限られますが、分割停止率や分割速度の変化、夾雑物の増減を検討します。

b）測定ウェルからの有害物質溶出の可能性

開発機器が培養液と接する部分はすべてシリコンで覆われており、電流

負荷などにより培養液に溶出することは想定できません。また、それ以外の器具は、日常臨床で使用している機材と同じ成分のものを使用しますので、新しい有害事象が発現する可能性は極めて低いと考えております。しかしながら、予想外の事態も想定し上記の微弱電流と同様に、分割停止率や分割速度の変化、夾雑物の増減を検討します。

8　本課題の資金源，起こり得る利害の衝突及び研究者等の関連組織との関わり

この研究は厚生労働省の科学研究費で行い、患者様の負担は一切ありません。科学研究費の研究代表者が所属する東北大学が中心となり、分担研究者の所属する４つの研究協力施設と共同で研究を行います。

9　本課題の実施に伴う危険性及び問題が生じた場合の対処

今回の試験では、既に生児獲得後や採卵後３年以上が経過し不要となった余剰卵（廃棄卵）を使用します。所有する患者様には危険や問題が生じることはありません。

10　資料の保存と廃棄

これらの研究用に使用された受精卵は、本研究以外の目的で使用されることはありません。また、これらのサンプルはヒトやヒト以外の動物に戻すことはなく、本研究が終了次第すぐに破棄されます。尚、研究データに関しては研究終了後も保存します。

11　個人情報の保護

この臨床研究に参加した場合、患者様とその検体が同定できないように連結不可能匿名化されます。あなたを特定できる個人情報を取り扱うことはありませんので、あなたのプライバシーは完全に守られます。

研究結果は学術雑誌や学会で発表される予定です。その際にあなたのお名前や個人を特定できるような情報が使われることはありません。そのため、ご希望がありましても、あなたにご自分の研究結果をお知らせすることはできません。

12　本課題に関する問い合わせ先

この研究について疑問や不安があるときや、何かご相談やご意見があればいつ

でもご連絡ください。相談窓口は以下の通りです。

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FAX 番号：022-717-7258
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13　経過中及び終了後の対象者からのクレームにつきまして

この研究の途中で協力を中止したいと思いましても、ご本人の検体を特定できませんので中断することは不可能です。また、調査中に不快な思いをされた場合などは、必ず担当医師にお伝えください。担当医師に言いにくい事は他のスタッフにお伝えいただいても結構です。いずれの場合でも、あなたの今後の治療で不利益を受けることはありません。

説明年月日　　平成　　年　　月　　日

説明者所属　＿＿＿＿＿＿＿＿＿＿＿＿

説明者氏名　＿＿＿＿＿＿＿＿＿＿＿＿

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kumasako Y., Goto K., Koike M., Araki Y., Abe H., Utsunomiya T.	Respiration activity of single blastocysts measured by scanning electrochemical microscopy: The relationship between pre-freezing and post-warming	Journal of Mammalian Ova Research	30	30-35	2013
Yoshida H., Abe H., Arima T.	Quality evaluation of IVM embryo and imprinting genes of IVM babies	Journal of Assisted Reproductive Genetics	30	221-225	2013
阿部宏之	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	日本胚移植学雑誌	35	7-14	2013
Hirobe T., Ito S., Wakamatsu K., Kawa Y. Abe H.	Mouse brown (*b/Tyrp1^{b}*) allele inhibits eumelanin but not pheomelanin synthesis	Zoological Science			In press
Miyano Y., Tahara S., Sakata I., Sakai T., Abe H., Kimura S., Kurotani R.	Regulation of LH/FSH expression by secretoglobin 3A2 in the mouse pituitary gland	Cell Tissue Research			In press
Hoshino S., Kurotani R., Miyano Y., Sakahara S., Koike K., Maruyama M., Ishikawa F., Sakata I., Abe H., Sakai T.	Macrophage colony-stimulating factor induces prolactin expression in rat pituitary glands	Zoological Science			In press
阿部宏之	ARTにおける新技術・酸素消費と胚評価	臨床婦人科産科	68	20-27	2014

阿部宏之	酸素消費測定による胚の品質評価　—超高感度細胞呼吸測定装置の開発と不妊治療における臨床応用—	医学のあゆみ			印刷中

IV．研究成果の刊行物・別刷

代表的な４本の文献の別刷を添付いたしました。

1) Kumasako Y., Abe H. et al. Respiration activity of single blastocysts measured by scanning electro chemical microscopy: The relationship between pre-freezing and post-warming. *Journal of Mammalian Ova Research.* 30:30-35 2013

2) Yoshida H., Abe H., Arima T. Quality evaluation of IVM embryo and imprinting genes of IVM b abies. *Journal of Assisted Reproductive Genetics* 30:221-225 2013

3) 阿部宏之. ARTにおける新技術・酸素消費と胚評価　臨床婦人科産科 68:20-27 2014

4) 阿部宏之.　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　日本胚移植学雑誌 35:7-14 2013

—Original—

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

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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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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

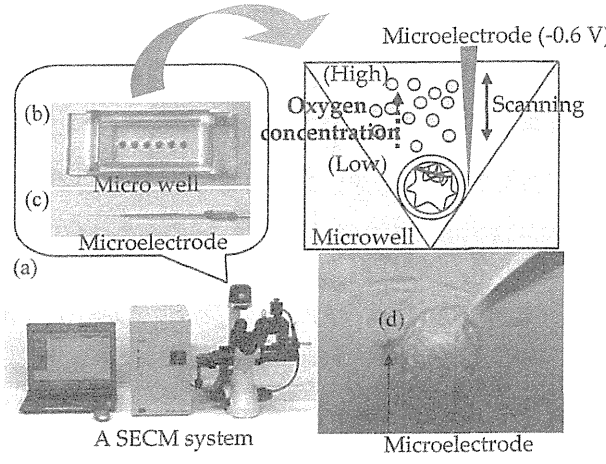

Fig. 1. (a) SECM system, (b) a plate, (c) a microelectrode for measuring the respiration activity of the embryos. The embryo is transferred into a microwell filled with medium, the microelectrode sinks down to the bottom of the microwell and remains at (d) the lowest point. Oxygen concentration profiles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

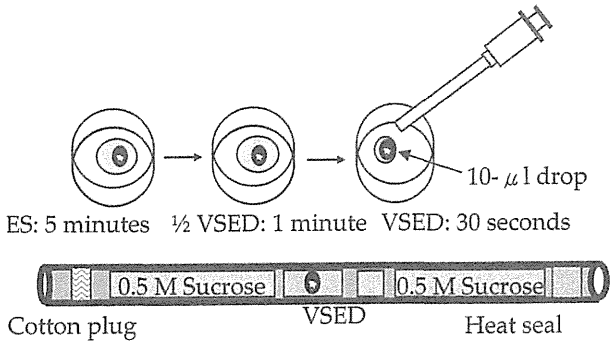

Fig. 2. Vitrification method using a 0.25-ml plastic straw. ES: 10% ethylene glycol. 1/2VSED: 12.5% ethylene glycol + 12.5% dimethyl sulfoxide. VSED: 25% ethylene glycol + 25% dimethyl sulfoxide.

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).

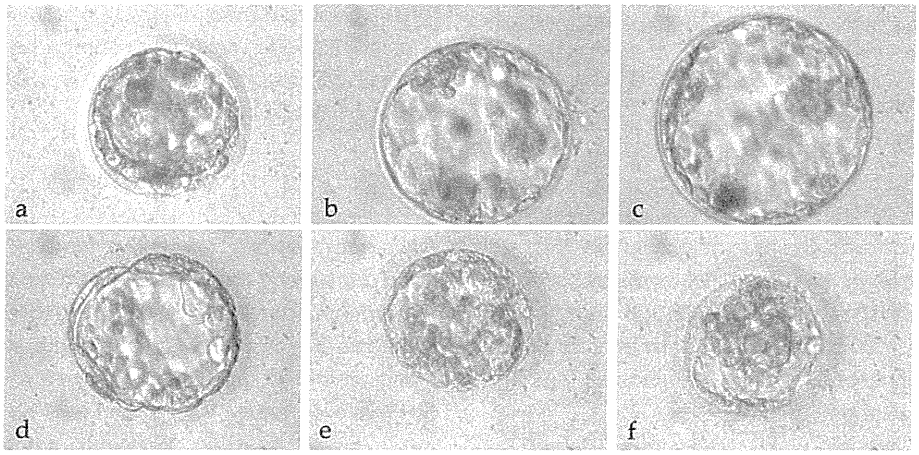

Fig. 3. Morphological changes of frozen blastocysts. (a, d) Same initial morphological level, before freezing; (b, e) After warming for 2 h, and (c, f) After warming for 24 h. (a–c): (a) Before freezing. (b) Two-hour culture after warming shows >50% of blastocoel re-expansion. (c) Following day (24 h 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).

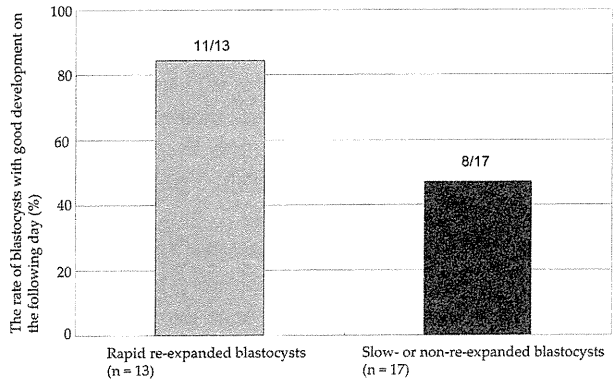

Fig. 4. Eleven out of 13 rapid re-expanded blastocysts with >50% blastocoel re-expansion exhibited good development on the following day. Eight out of 17 slow or no re-expansion blastocysts exhibited good development.

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: $0.64 \pm 0.18 \times 10^{14}$/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).

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

References

1) Veeck, L.L. (1991): Atras of the human oocyte and early conceptus. Willams & Wilkins. Baltimore.
2) Gardner, D.K., Lane, M. and Stevens, J. (2000): Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil. Steril., 73, 1155–1158.
3) Kaidi, S., Bernard, S., Lambert, P., Massip, A., Dessy, F. and Donnay, I. (2001): Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced in vitro. Biol. Reprod., 65, 1127–1134.
4) Lopes, A.S., Larsen, L.H., Ramsing, N., Løvendahl, P., Räty, M., Peippo, J., Greve, T. and Callesen, H. (2005): Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. Reproduction, 130, 669–679.
5) Tejera, A., Herrero, J., De Los Santos, M.J., Garrido, N., Ramsing, N. and Meseguer, M. (2011): Oxygen consumption is a quality marker for human oocyte competence conditioned by ovarian stimulation regiments. Fertil. Steril., 96, 618–623. e2.
6) Mio, Y. and Maeda, K. (2008): Time-lapse cinematography of dynamic changes occurring in vitro development of hu-

man embryos. Am. J. Obstet. Gynecol., 199, 660 e1-5.
7) Meseguer, M., Herrero, J., Tejera, A., Hilligsøe, K.M., Ramsing, N.B. and Remohí, J. (2011): The use of morphokinetics as a predictor of embryo implantation. Hum. Reprod., 26, 2658–2671.
8) Shiku, H., Shiraishi, T., Ohya, H., Matsue, T., Abe, H., Hoshi, H. and Kobayashi, M. (2001): Oxygen consumption of single bovine embryos probed with scanning electrochemical microscopy. Anal. Chem., 73, 3751–3758.
9) Abe, H., Shiku, H., Aoyagi, S. and Hoshi, H. (2004): In vitro culture and evaluation of embryos for production of high quality bovine embryos. J. Mamm. Ova. Res., 21, 22–30.
10) Shiku, H., Shiraishi, T., Aoyagi, S., Utsumi, Y., Matsudaira, M., Abe, H., Hoshi, H., Kasai, S., Ohya, H. and Matsue, T. (2004): Respiration activity of single bovine embryos entrapped in a cone-shaped microwell monitored by scanning electrochemical microscopy. Anal. Chim. Acta., 522, 51–58.
11) Shiku, H., Torisawa, Y., Takagi, A., Aoyagi, S., Abe, H., Hoshi, H., Yasukawa, T. and Matsue, T. (2005): Metabolic and enzymatic activities of individual cells, spheroids and embryos as a function of the sample size. Sens. Actuators, B 108, 597–602.
12) Agung, B., Otoi, T., Abe, H., Hoshi, H., Murakami, M., Karija, N.W., Murakami, M.K., Wongsrikeao, P., Watari, H. and Suzuki, T. (2005): Relationship between oxygen consumption and sex of bovine in vitro fertilized embryos. Reprod. Domest. Anim., 40, 51–56.
13) Utsunomiya, T., Goto, K., Nasu, M., Kumasako, Y., Araki, Y., Yokoo, M., Itoh-Sasaki, T. and Abe, H. (2008): Evaluating the quality of human embryos with a measuring of oxygen consumption by scanning electrochemical microscopy. J. Mamm. Ova. Res., 25, 2–7.
14) Utsunomiya, T., Naitou, T. and Nagaki, M. (2002): A prospective trial of blastocyst culture and transfer. Hum. Reprod., 17, 1846–1851.
15) Utsunomiya, T., Ito, H., Hirai, K., Otsu, E., Watanabe, H. and Mori, T. (2006): Developmentally retarded frozen blastocysts can be rescued by synchronizing culture prior to transfer. Reprod. Biomed. Online, 12, 622–629.
16) Yokota, Y., Sato, S., Yokota, M., Ishikawa, Y., Makita, M., Asada, T. and Araki, Y. (2000): Successful pregnancy following blastocyst vitrification. Hum. Reprod., 15, 1802–1803.
17) Ishimori, H., Saeki, K., Inai, M., Nagao, Y., Itasaka, J., Miki, Y., Seike, N. and Kainuma, H. (1993): Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxyde. Theriogenology, 40, 427–433.
18) Yamanaka, M., Hashimoto, S., Amo, A., Ito-Sasaki, T., Abe, H. and Morimoto, Y. (2011): Developmental assessment of human vitrified-warmed blastocysts based on oxygen consumption. Hum. Reprod., 26, 3366–3371.
19) Shu, Y., Watt, J., Gebhardt, J., Dasig, J., Appling, J. and Behr, B. (2009): The value of fast blastocoele re-expansion in the selection of a viable thawed blastocyst for transfer. Fertil. Steril., 91, 401–406.
20) Cohen, J., Simons, R.F., Edwards, R.G., Fehilly, C.B. and Fishel, S.B. (1985): Pregnancies following the frozen storage of expanding human blastocyst. J. In Vitro Fert. Embryo Transf., 2, 59–64.
21) Fehilly, C.B., Cohen, J., Simons, R.F., Fishel, S.B. and Edwards, R.G. (1985): Cryopreservation of cleaving embryos and expanded blastocysts in the human: a comparative study. Fertil. Steril., 44, 638–644.

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Quality evaluation of IVM embryo and imprinting genes of IVM babies

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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 $0.26–0.56 \times 10^{14}/\mathrm{molS}^{-1}$). 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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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

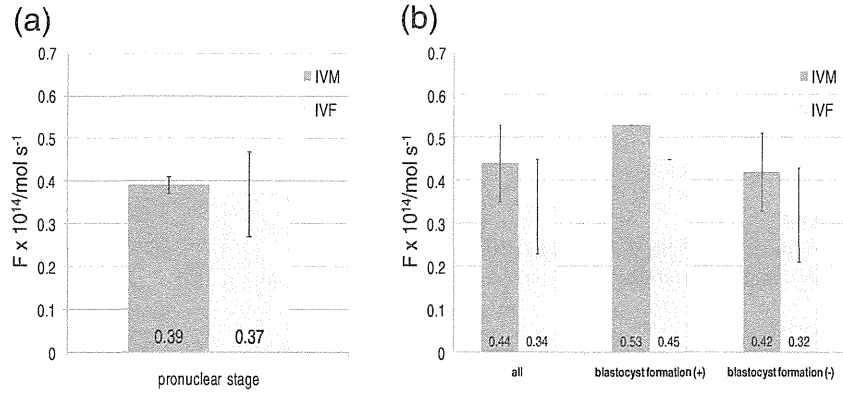

Fig. 1 Comparison of embryo oxygen consumption rate (pronuclear stage, Day 3). **a** shows oxygen comparison of embryo at pronuclear stage between IVM and COH; **b** shows oxygen comparison of embryo on day 3 between IVM and COH. Also it shows whether the embryos developed to blastocysts or not based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

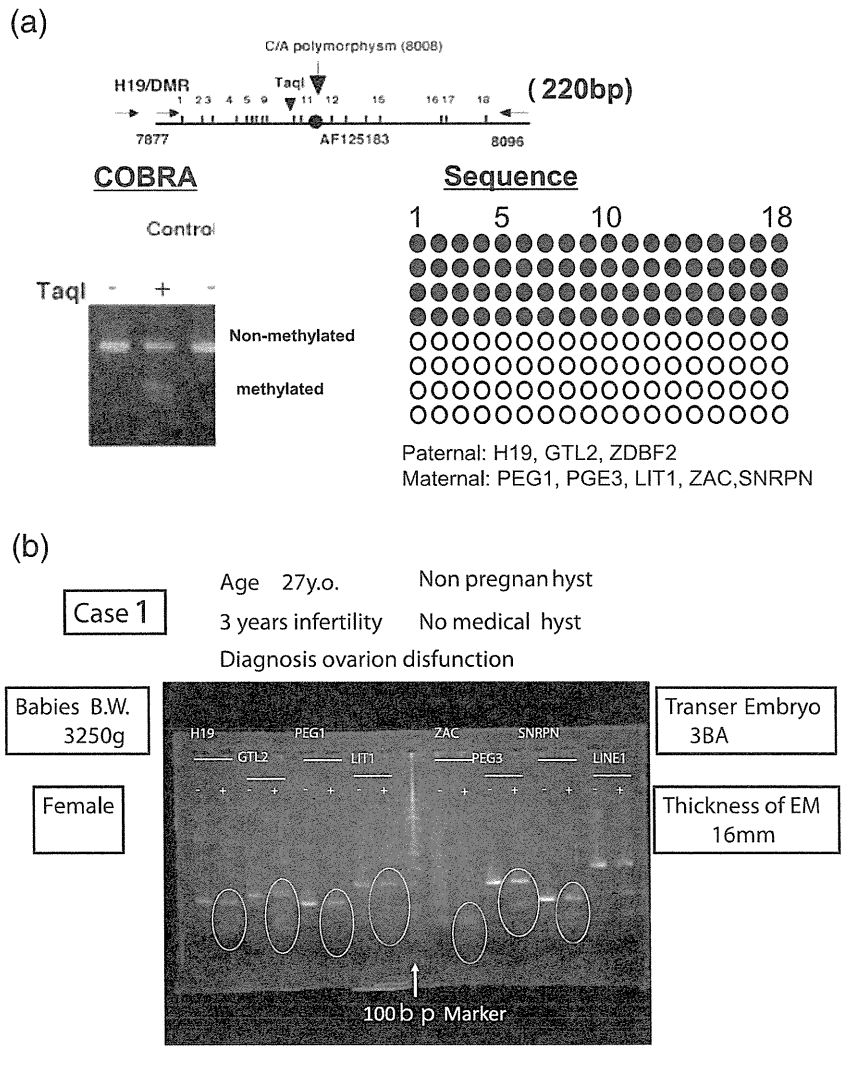

Fig. 2 **a** Analysis of methylation for imprinting genes from IVM babies; **b** Imprint gene Methylation assay with umbilical cord from IVM baby

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.

References

1. Rieger D, Loskutoff NM. Changes in the metabolism of glucose, puruvate, glutamine and Glycine and Glycine during maturation of cattle oocyte in vitro. J Reprod Fertil. 1994;100:257–62.
2. Boiso I, Veiga A, Edwards RG. Fundamentals of human embryonic for growth in vitro and the selection of high-quality embryos for transfer. Reprod Biomed Online. 2002;5:328–50.
3. Thompson JG, McNaughton C, Gasparrini B, McGowan LT, Tervit HR. Effect of inhibitors and uncuplers of oxidative phosphorylationduring compaction and blastulation of bovine embryos cultured in vitro. J Reprod Fertil. 2000;118:47–55.
4. Cha KY, Chian RC. Maturation in vitro of immature human oocytes for clinic use. Hum Reprod Update. 1991;4:103.
5. Takahiro A, Hisato K. Evaluation for epigenetic abnormality in infertile male sperm. Journal of Japan Society of Fertilization & Implantation. 2009;38:409 (in Japanese).
6. Nilsson BO, Magnusson C, Widéhn S, Hillensjö T. Correlation between blastocyst oxygen consumption and trophoblasts cytoclome oxidase reaction at initiation of implantation of delayed mouse blastocysts. J Embryol Exp Morphol. 1982;71:75–82.
7. Abe H. A non-invasive and sensitive method for measuring cellular respiration with a scanning electrochemical microscopy to evaluate embryo quality. J Mamm Ova Res. 2007;24:70–8.
8. Overstorm EW, Duby RT, Dobrinsky J, Roche JF, Boland MP. Viability and oxidative metabolism of the bovine blastocyst (Abstract). Theriogenology. 1992;37:269.
9. Abe H, Shiku H, Aoyagi S, Matsue T, Hoshi H. Respiration activity of bovine embryos cultured in serum-free and serum-containing media (Abstract). Reprod Fert Dev. 2005;17:215.
10. Veeck LL. Oocyte assessment and biological performance. Ann NY Acad Sci. 1988;541:259–95.
11. Utsunomiya T, Goto K, Nasu M, Kumasako Y, Araki Y, Yokoo T, et al. Evaluating the quality of human embryos with a measurement of oxygen consumption by scanning electrochemical microscopy. J Mamm Ova Res. 2008;25:2–7.
12. Manipalviratn S, DeCherney S, Segars J. Imprinting disorders and reproductive technology. Fert Steril. 2009;91:305–15.
13. Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, et al. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. Hum Mol Genet. 2007;16:2542–51.
14. Gopichandran N, Leese HJ. Metabolic characterization of the bovine blastocyst, inner cell mass, trophectoderm and blastocoel fluid. Reproduction. 2003;126:299–308.

15. Tesalik J, Junca AM, Hazout A, Aubriot FX, Nathan C, Cohen-Bacrie P, et al. Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of pronuclear morphology. Hum Reprod. 2000;6:1396–9.
16. Mio Y. Morphological analysis of human embryonic development using time lapse cinematography. J Mamm Ova Res. 2006;23:27–35.
17. Magnusson C, Hillensjö T, Hamberger L, Nilsson L. Oxygen consumption by human oocytes and blastocysts grown in vitro. Hum Reprod. 1986;1:183–4.
18. Donnay I, Leese HJ. Embryo metabolism during the expansion of the bovine blastocyst. Mol Reprod Dev. 1999;53:171–8.
19. Land SC, Porterfield DM, Sanger RH, Smith PJ. The self-referencing oxygen-selective microelectrode detection of transmembrane oxygen flux from single cells. J Exp Biol. 1999;202:211–8.
20. Lopes AS, Larsen LH, Rmsing N, Løvendahl P, Räty M, Peippo J, et al. Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive micro sensor system. Reproduction. 2005;130:669–79.

生殖医療の進歩と課題—安全性の検証から革新的知見まで

【ARTにおける新技術】

酸素消費と胚評価

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ARTにおける新技術

酸素消費と胚評価

阿部 宏之

- 細胞呼吸　細胞が外部から取り入れた酸素や酸素以外の酸化剤を用いて，養分を分解してエネルギーを発生させる生物現象．
- 電気化学計測　化学物質の性質を電気的に計測する方法．局所領域における生体反応を高感度・リアルタイムに計測できる．
- 走査型電気化学顕微鏡　マイクロ電極をプローブとして，目的試料の上部や近傍を走査し，プローブ電流を検出する装置．

はじめに

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きわめて重要である　現在，胚の品質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が，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，細胞呼吸活性を指標とする新しい胚評価システムの開発に取り組んできた．

本稿では，電気化学計測技術を基盤とする酸素消費測定装置と　この装置を応用した新しい胚評価法を解説する

形態観察および代謝物質測定による胚評価

形態的評価は，簡単　迅速で無侵襲的な方法であることから　最も有効な胚の品質評

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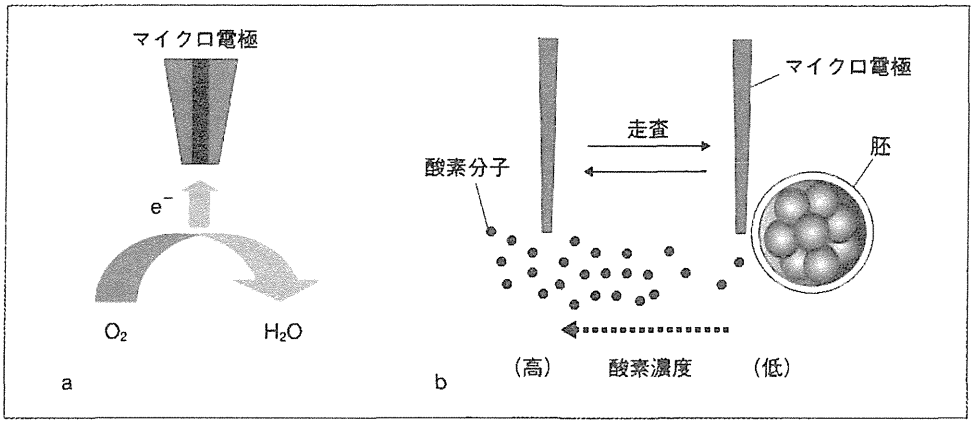

図1　マイクロ電極を用いた受精卵呼吸測定法

a　マイクロ電極は酸素の還元電位を検出する．

b　走査型電気化学顕微鏡による呼吸測定．呼吸により胚近傍の溶存酸素が減少するため，沖合との間に溶存酸素の濃度勾配が生じる．その酸素濃度差（電流値の差）から胚の酸素消費量を算出する．

価法として広く普及している　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 の分類法[1]や　Gardner らが提案した胚盤胞の評価法[2]が広く用いられている　これら形態観察による胚評価は，簡便で非侵襲的な方法であり　その有効性も認められているが，評価の指標となる形態的特徴が定量性に欠けるという課題も指摘されている

このため，客観的　定量的な指標として　胚の代謝産物や酸素消費に着目した胚評価が試みられている[3~7]　特に，ミトコンドリアは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として注目されている　これまでに，蛍光発色法[8,9]や酸素センサー[10,11]を用いた細胞呼吸測定法が考案され，胚評価への応用が試みられているが，多くは測定感度や侵襲性などの面で課題があり　実用化には至っていない．

電気化学計測を応用した細胞呼吸測定装置の開発

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[12]　この技術の有効な装置としてマイクロ電極をプローブ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 の空間分解能はプローブ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[13,14]　SECM は，酸素の還元電位を検出できるマイクロ電極をプローブとして用いることで　細胞の酸素消費量（呼吸）を高感度　非侵襲的に測定することができる（**図1**）