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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

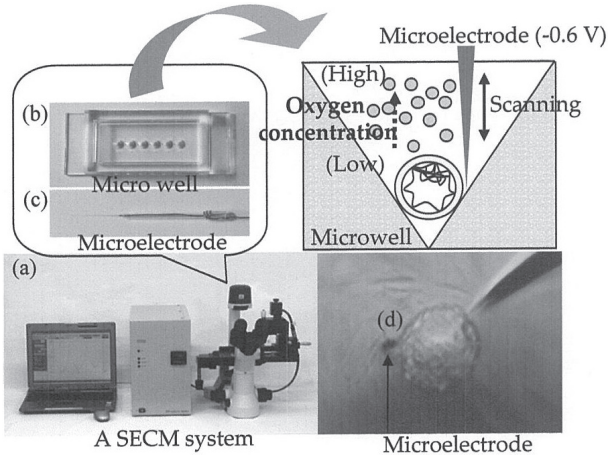

Fig. 1. (a) SECM system, (b) a plate, (c) a microelectrode for measuring the respiration activity of the embryos. The embryo is transferred into a microwell filled with medium, the microelectrode sinks down to the bottom of the microwell and remains at (d) the lowest point. Oxygen concentration profiles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

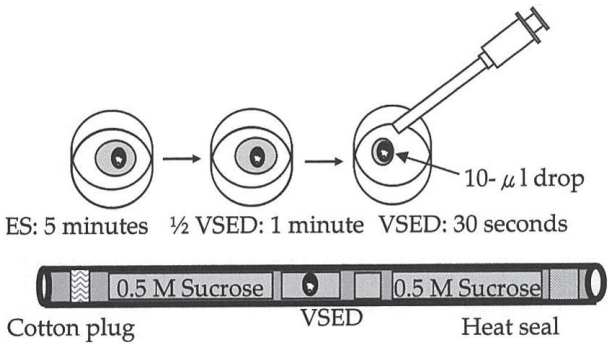

Fig. 2. Vitrification method using a 0.25-ml plastic straw. ES: 10% ethylene glycol. 1/2VSED: 12.5% ethylene glycol + 12.5% dimethyl sulfoxide. VSED: 25% ethylene glycol + 25% dimethyl sulfoxide.

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).

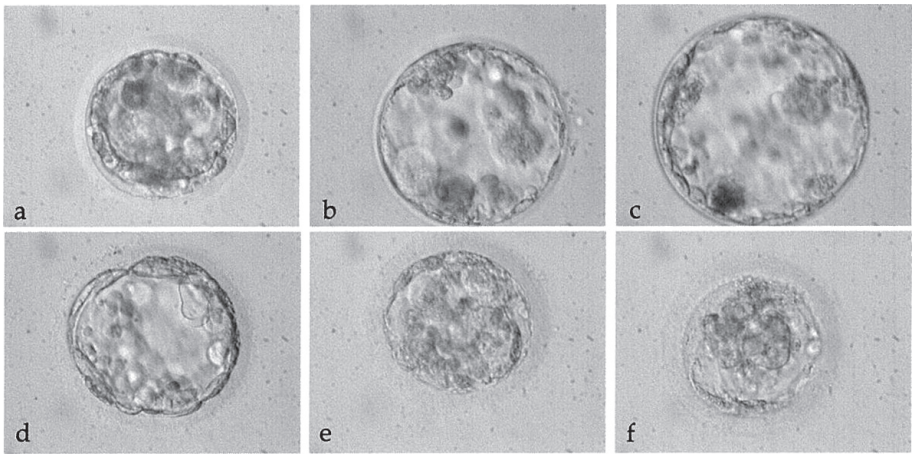

Fig. 3. Morphological changes of frozen blastocysts. (a, d) Same initial morphological level, before freezing; (b, e) After warming for 2 h, and (c, f) After warming for 24 h. (a–c): (a) Before freezing. (b) Two-hour culture after warming shows >50% of blastocoel re-expansion. (c) Following day (24 h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

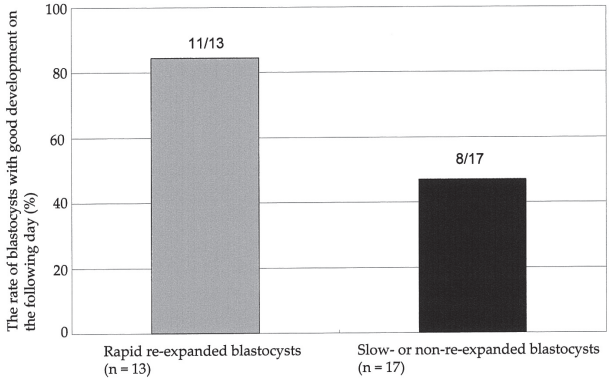

Fig. 4. Eleven out of 13 rapid re-expanded blastocysts with >50% blastocoel re-expansion exhibited good development on the following day. Eight out of 17 slow or no re-expansion blastocysts exhibited good development.

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).

× 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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