



# Prediction of the in vitro developmental competence of early-cleavage-stage human embryos with time-lapse imaging and oxygen consumption rate measurement

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## Abstract

**Purpose:** To assess an embryo's ability to develop into a good-quality blastocyst during the early-cleavage stage using time-lapse imaging and the oxygen consumption rate.

**Methods:** In total, 942 zygotes had their oxygen consumption rates measured. In total, 282 zygotes were assessed by using time-lapse imaging. In total, 121 zygotes were examined by using both their oxygen consumption rate and time-lapse imaging.

**Results:** The embryos with moderate respiration rates of between 0.41 and 0.61 ( $\times 10^{14}/\text{mol s}^{-1}$ ) on day 3 had a 22.1% chance of becoming good-quality blastocysts; those outside that range had a 14.3% chance. With the time-lapse system, when the first division was within 24 hours, 22.3% of the embryos grew to good blastocysts. After 24 hours, the rate dropped to 8.6%. The intervals between two consecutive cleavages were calculated and the duration of the second cell cycle was defined. When the time was between nine hours and 13 hours, there was a higher rate of good blastocysts. Regarding both criteria, when the embryos had progressed in the optimal range, a high percentage of them had become good blastocysts; it was 8.0% outside of that range.

**Conclusion:** Individual embryos with the potential to develop into good-quality blastocysts could be selected at day 3 of culture using these systems.

## KEYWORDS

early stage, embryo quality, oxygen consumption rate, time-lapse, uninvase evaluation

## 1 | INTRODUCTION

In order to avoid multiple pregnancies, the method of selecting embryos for transfer is an essential issue for consideration for an elective single embryo transfer (e-SET). Presently, embryos are selected

for transfer by using grading systems that are based on morphology. Morphological evaluation has been used widely to evaluate embryo quality because it is uninvase and useful in predicting pregnancy rates.<sup>1-4</sup> However, morphological evaluations are subjective and the categorization standards often vary between investigators. As

morphological assessment has several limitations, more objective selection criteria are needed.

Among the different evaluated parameters, pronuclei (PN) scoring has been a subject of debate. Although some studies have shown a prognostic effect of PN scoring,<sup>5,6</sup> one study concluded that further refinement of embryo grading by PN scoring is not beneficial.<sup>7</sup> Over the last decade, an ET at the blastocyst stage has been used more frequently to select the best embryo and to decrease the number of transferred embryos. However, the transfer of embryos at the blastocyst stage is occasionally avoided and they are transferred at an earlier stage, especially when patients have few embryos or when embryos might not tolerate expanded culture conditions. Furthermore, prolonging the embryo culture time can increase the risk of epigenetic disorders, monozygotic twinning, preterm delivery, low birthweight, and other issues.<sup>8-12</sup> In the authors' previous report, the pregnancy rates of day 5 and day 3 ETs were equal, while the cancellation rate and the abortion rate of day 5 ETs were a little higher than those of day 3 ETs.<sup>13</sup> Thus, the reliable prediction of good blastocyst formation by day 3 is needed. The incidence of aneuploidy, which is prevalent in human oocytes and the resulting embryos, increases with maternal age.<sup>14</sup> Therefore, it is important to select the best embryo for e-SET. One study demonstrated that e-SET, coupled with enhanced embryo selection using preimplantation genetic screening (PGS), in woman older than 35 years reduced the multiple pregnancy rate while maintaining the cumulative success rate of the IVF program.<sup>15</sup> However, a number of randomized controlled trials had clearly demonstrated that routine PGS using fluorescence in situ hybridization (FISH) at the cleavage stage was not beneficial and a consensus was published by the European Society of Human Reproduction and Embryology PGD Consortium, stating that future studies with alternative biopsy timing and genetic testing were necessary in order to evaluate the clinical benefit of PGS.<sup>16</sup>

Respiration is a useful parameter for evaluating the embryo quality as it provides important information about metabolic activity. The scanning electrochemical microscopy (SECM) measuring system that was developed by Abe, Shiku, Aoyagi, and Hoshi provides an un-invasive, simple, accurate, and consistent measurement of the respiration activity of a single human embryo.<sup>17</sup> The oxygen consumption rate, using SECM, exactly expresses the mitochondrial activity in the embryo.<sup>18,19</sup> The authors previously reported that significant differences in oxygen consumption were registered, even among embryos with the same morphological classification.<sup>20</sup> Combining the measurement of embryo respiration with morphological evaluation can provide new information regarding the quality of human embryos, therefore improving the rate of selection of superior embryos before transfer in in vitro fertilization (IVF)-e-SET.

Recently, time-lapse investigation shows that time-lapse parameters can predict which embryos will develop to high-quality blastocysts.<sup>21</sup> Time-lapse monitoring enables a detailed evaluation of the morphology and cell division kinetics. The development of time-lapse incubators has enabled safe,<sup>22-24</sup> continuous monitoring of embryos that have been cultured for treatment purposes. This system could be useful for selecting high-potential embryos. Therefore, it

is proposed that additional time-lapse imaging, in conjunction with morphology and respiration examination, will allow the selection of the best-quality embryos.

In the present study, it was evaluated whether time-lapse parameters and oxygen consumption rates will be able to predict good-quality blastocysts at day 3.

## 2 | MATERIALS AND METHODS

### 2.1 | Evaluation of the oxygen consumption rates on day 3

In total, 942 zygotes were obtained from 403 couples who underwent IVF/intracytoplasmic sperm injection (ICSI) treatment at St. Luke's Clinic, Oita, Japan, between July, 2006 and June, 2014. Prior to the treatment, informed consent was obtained from all the patients. Ethical approval was given by the clinic's research ethics committee. The mean age of the patients was  $35.1 \pm 4.1$  years and the number of previous assisted reproductive technology (ART) cycles was  $3.5 \pm 3.0$ . The patients who were receiving treatment in the clinic's IVF-ET program have been described previously.<sup>13,20</sup> The embryos were cultured in Global medium (LifeGlobal, Guilford, CT, USA) and covered with mineral oil (Fuso Pharmaceutical Industries, Osaka, Japan) at 37°C in a 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> environment.

After morphological evaluation by Veeck's method,<sup>1</sup> the oxygen consumption rate of the individual embryos ( $\geq 6$  division by Veeck's method) was quantified with a modified SECM measuring system on day 3. The SECM has been marketed as "CRAS-1.0" (Clino, Ltd., Miyagi, Japan). For the measurement of oxygen consumption, a plate with cone-shaped microwells was filled with modified HFF99 medium (Fuso Pharmaceutical Industries) and the embryos were transferred onto each well. The medium was preheated and maintained at 37°C during the measurement. The air temperature of the oxygen consumption rate and gas composition in the atmospheric air was 26°C.

A microelectrode scanned in the z-direction from the outside of the embryo was located at the bottom of a microwell. The oxygen consumption rate of the embryo was calculated by software, using an algorithm based on spherical diffusion theory.<sup>25</sup> The measurement of the oxygen consumption rate of each embryo took ~30 seconds. After measurement of the oxygen consumption rate, these embryos were returned to the Global medium. The surplus embryos, after an ET on day 3, were cultured to freeze for future ET cycles until day 5. After the measurement, the developmental capacity of the surplus embryos was examined. The blastocysts were scored and graded according to Gardner's criteria on day 5.<sup>26</sup> "Good-quality blastocysts" were defined as having developed between scores 3 and 6 and not having grade C, according to Gardner's criteria.

In order to select the best-quality blastocyst, the threshold values of the oxygen consumption rates were examined. The threshold values of the oxygen consumption rates were determined based on the P-value of the developmental rates of the good-quality blastocysts.

## 2.2 | Evaluation of time-lapse imaging

In total, 282 zygotes were obtained from 72 couples who underwent ICSI fertility treatment between April, 2013 and June, 2014. The mean age of the patients was  $35.0 \pm 4.3$  years and the number of previous ART cycles was  $3.4 \pm 3.5$ . The embryos were cultured in the microwell dishes and monitored by automated time-lapse microscopes (Primo Vision; Cryo-innovation, Budapest, Hungary) inside of conventional water-jacketed multigas incubators. The outer dimensions of the microscope were 220 mm × 80 mm × 110 mm (length × width × height). The WOW dish (Cryo-innovation) consisted of a 35 mm diameter polystyrene Petri dish containing nine microwells, aligned in three rows and three columns, in the centre bottom of the dish. Global medium was transferred onto a WOW dish. The WOW dishes were placed on the top of glass window microscopes. The embryos were cultured at 37°C in a 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> environment. The microscope then was mechanically focused on the object. Illumination was provided by a reflected warm, white light. The system was set to take a picture every 10 minutes. The embryos were not moved or disturbed for the whole 5 day period of development, completely eliminating shear stress. The embryos were evaluated in the early-cleavage stage in order to determine the highest quality specimens. As the extrusion of the second polar body indicates the time of closing second meiosis, the exact timing for each embryo division was calculated, in hours, after extrusion of the second polar body. The timing of cleavage to the two-cell stage was recorded (t<sub>2</sub>), which was measured from the initiation of the second polar body extrusion and which finished at the first sign of cell division. The duration of the second cell cycle was defined as the time from division to a two-blastomere embryo, until division to a three blastomere embryo (t<sub>3</sub>-t<sub>2</sub>), which was measured

from the initiation of the second cytokinesis—the complete separation of the daughter cells—and finished at the first sign of daughter cell division. In order to select the best-quality blastocyst, the division threshold time was examined. The threshold times for the first division (t<sub>2</sub>) and (t<sub>3</sub>-t<sub>2</sub>) were determined based on a *P*-value of the developmental rates of the good-quality blastocysts.

## 2.3 | Combining the measurement of embryo respiration with the time-lapse evaluation

In order to improve the accuracy of the evaluation, additional time-lapse prediction (t<sub>2</sub> and t<sub>3</sub>-t<sub>2</sub>), in conjunction with respiration examination on day 3, was evaluated. In total, 121 zygotes were obtained from 36 patients who underwent ICSI fertility treatment between August, 2013 and September, 2014. The mean age of the patients was  $35.3 \pm 4.7$  years and the number of previous ART cycles was  $3.4 \pm 3.8$ . Blastocyst development was analyzed as an outcome (high-quality or low-quality/arrested development).

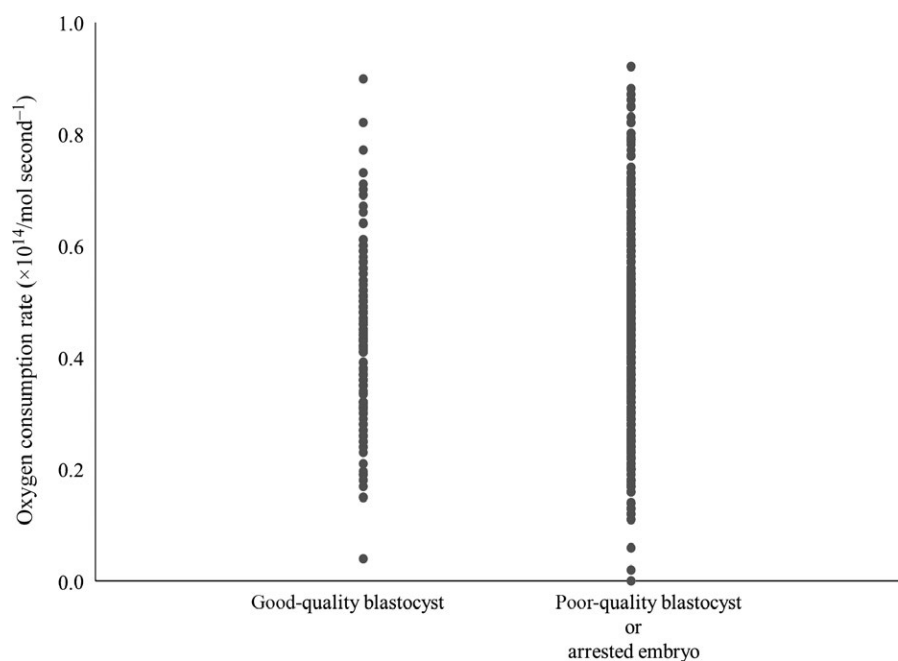
## 2.4 | Statistical analysis

The results were analyzed by using a  $\chi^2$ -test for comparison of the proportions. A *P*-value of <.05 was considered to be statistically significant.

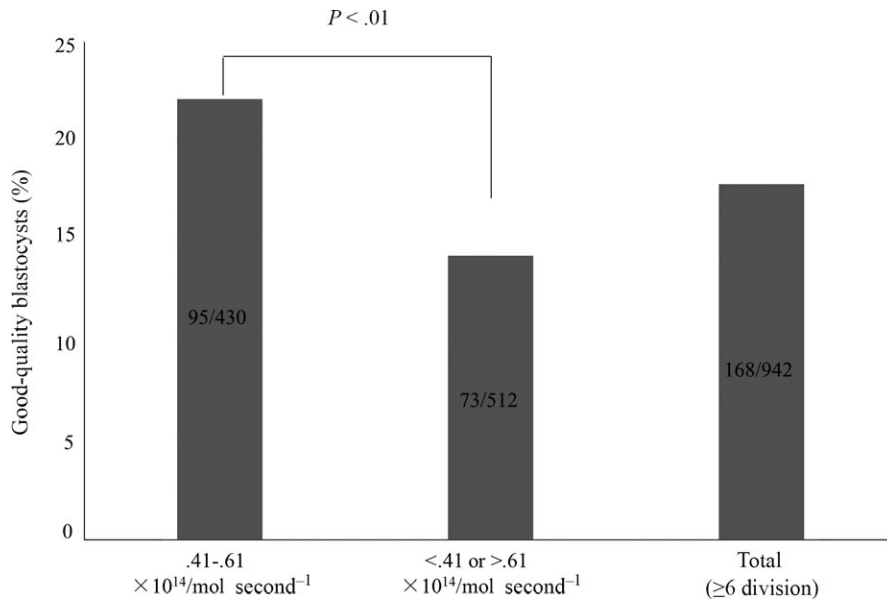
## 3 | RESULTS

### 3.1 | Evaluation of the oxygen consumption rates on day 3

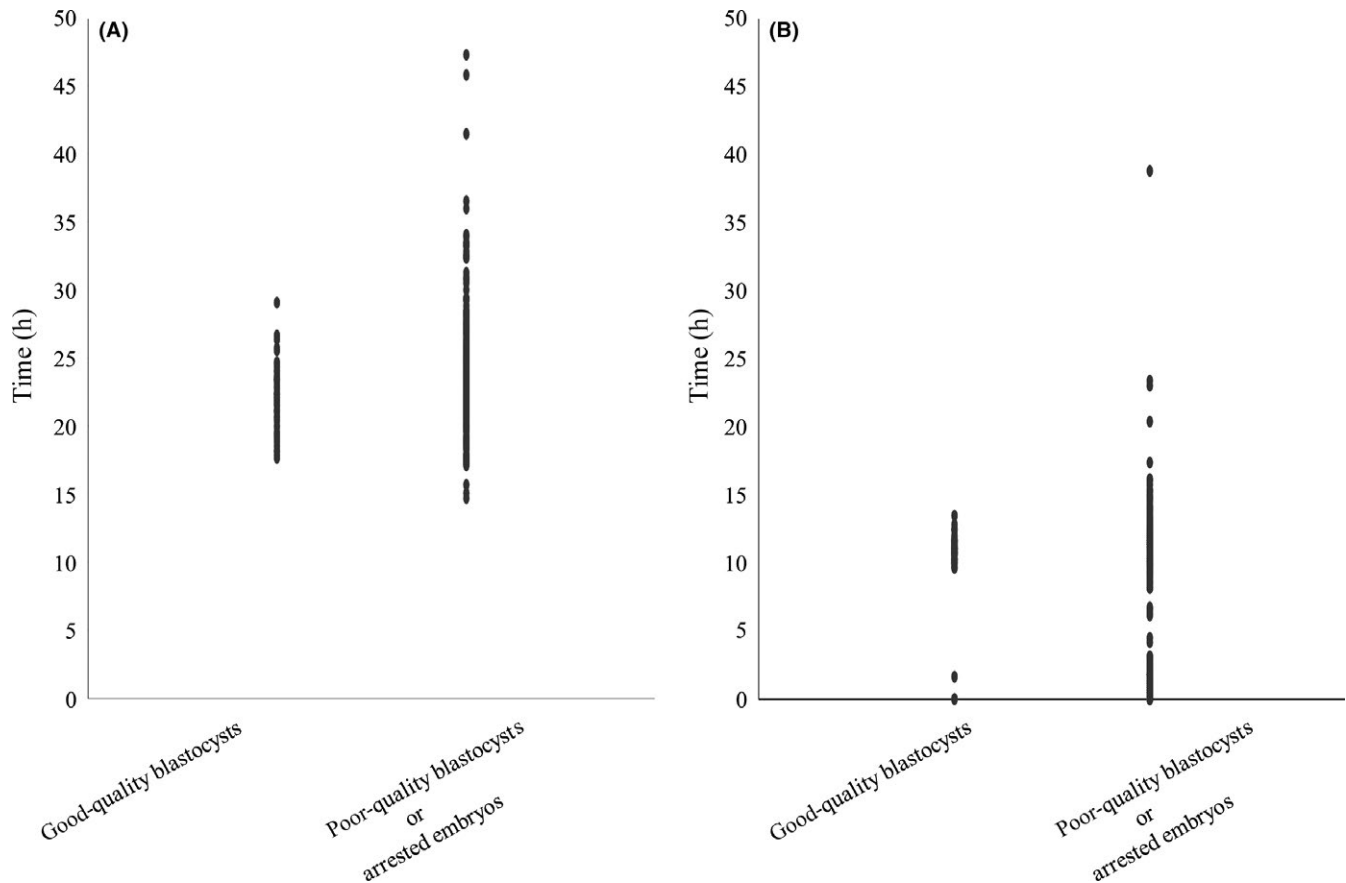
The mean age of the patients was  $35.1 \pm 4.1$  years and the number of previous ART cycles was  $3.5 \pm 3.0$ . After measuring their



**FIGURE 1** Relationship between the oxygen consumption rates and the quality of the blastocysts. The embryos with moderate respiration rates of  $>0.41 \times 10^{14}/\text{mol s}^{-1}$  and  $<0.61 \times 10^{14}/\text{mol s}^{-1}$  tended to develop good-quality blastocysts



**FIGURE 2** Comparison of the oxygen consumption rates and morphology rates to reach good-quality blastocysts. The embryos with moderate respiration rates showed a high developmental rate (22.1%) to the blastocyst. The developmental rate of the embryos with lower and higher respiration rates was 14.3% ( $P < .01$ ), while 17.8% of the embryos with the  $\geq 6$  division by Veeck's method reached the good-quality blastocyst stage

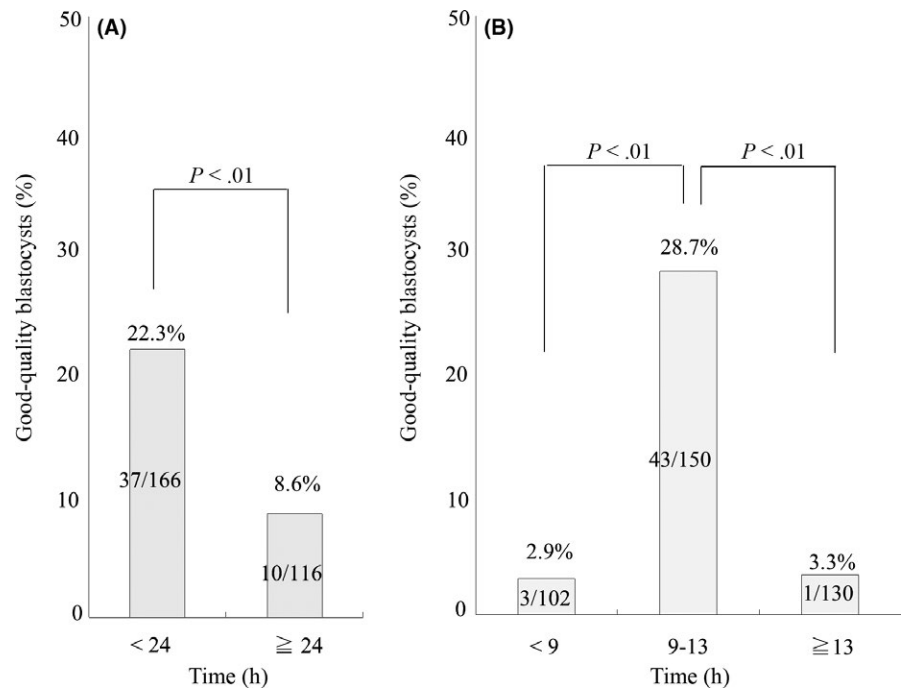


**FIGURE 3** Relationship between time and the quality of the blastocysts. A, The good-quality blastocysts developed in the first division before hour 24 (t2). B, The good-quality blastocysts developed in the division between hours 9 and 13 (t3-t2)

respiration rates with SECM, the embryos were cultured to examine their developmental capacity. Figure 1 shows a higher proportion of good-quality blastocysts within the moderate respiration rate range of  $>0.41 \times 10^{14}/\text{mol s}^{-1}$  and  $<0.61 \times 10^{14}/\text{mol s}^{-1}$ . In order to

examine the effectiveness of this threshold value, the rate of good-quality blastocysts was investigated. Embryos with moderate respiration rates ( $>0.41 \times 10^{14}/\text{mol s}^{-1}$  and  $<0.61 \times 10^{14}/\text{mol s}^{-1}$ ) had a 22.1% chance of reaching good-quality blastocysts. However, the

**FIGURE 4** Comparison of the division time to reach good-quality blastocysts. A, The developmental rates to the high-quality blastocysts with different first division times (t2). The rate of high-quality blastocysts to the first division (t2) within 24 hours was 22.3% and after 24 hours, it was 8.6% ( $P < .01$ ). B, The developmental rates to the high-quality blastocysts with a different duration of time from division into a two-blastomere embryo to division into a three-blastomere embryo (t3-t2). The time between 9 hours and 13 hours had a significantly higher rate of high-quality blastocysts (28.7%). In contrast, under 9 hours, the rate dropped to 2.9% ( $P < .01$ ) and above 13 hours, it was 3.3% ( $P < .01$ ).



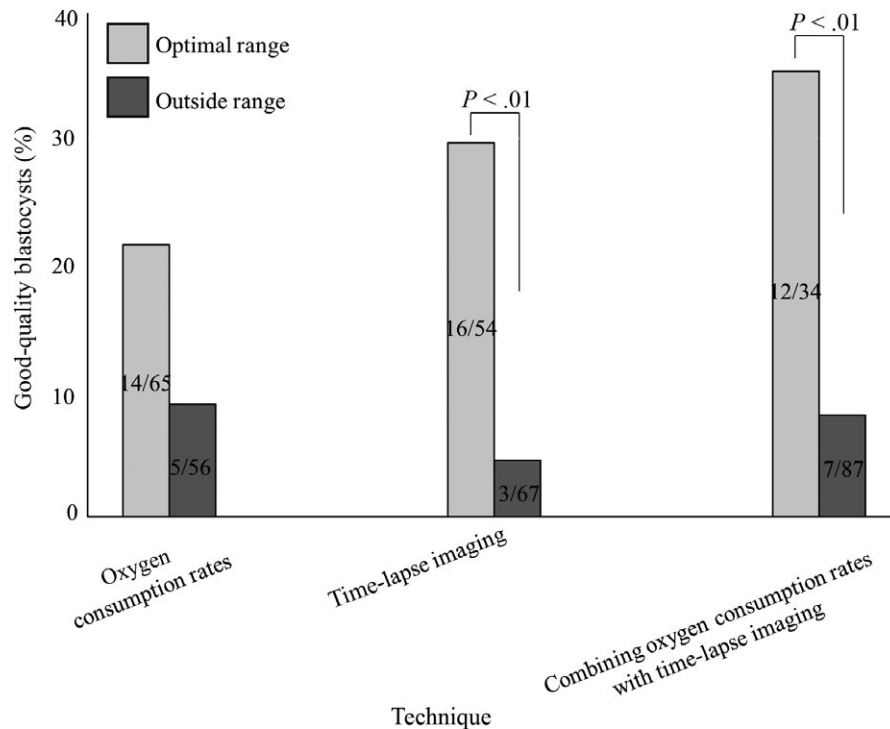
embryos with lower ( $< 0.41 \times 10^{14} / \text{mol s}^{-1}$ ) or higher ( $> 0.61 \times 10^{14} / \text{mol s}^{-1}$ ) respiration rates had only a 14.3% chance of reaching good-quality blastocysts ( $P < .01$ ). The number of total embryos with the  $\geq 6$  division by Veeck's method had a 17.8% (168/942) chance of reaching good-quality blastocysts (Figure 2). The respiration of  $0.41$ – $0.61 \times 10^{14} / \text{mol s}^{-1}$  on day 3 tended to develop good-quality blastocysts. These threshold values of the oxygen consumption rates were determined based on a  $P$ -value of the developmental rates of the good-quality blastocysts.

### 3.2 | Evaluation of time-lapse imaging

The mean age of the patients was  $35.0 \pm 4.3$  years and the number of previous ART cycles was  $3.4 \pm 3.5$ . The exact timing for each embryo division was calculated, in hours, after extrusion of the second polar body. In the first division (t2), Figure 3A shows a higher proportion of good-quality blastocysts under 24 hours. Figure 3B (t3-t2) shows a higher proportion of good-quality blastocysts between 9 hours and 13 hours. In order to examine the effectiveness of these threshold values, the rates of the good-quality blastocysts were investigated. When the first division was within 24 hours, 22.3% of the embryos grew to good-quality blastocysts. After 24 hours, the rate dropped to 8.6% ( $P < .01$ ) (Figure 4A). The intervals between two consecutive cleavages were calculated and the duration of the second cell cycle was defined as the time from division into a two-blastomere embryo to division into a three-blastomere embryo (t3-t2). The time between 9 hours and 13 hours had a significantly higher rate of high-quality blastocysts (28.7%). In contrast, under 9 hours, the rate dropped to 2.9% ( $P < .01$ ) and above 13 hours, the rate decreased to 3.3% ( $P < .01$ ) (Figure 4B). This threshold time for first division (t2) and (t3-t2) was determined based on the  $P$ -value of the developmental rates of the good-quality blastocysts.

### 3.3 | Combining oxygen consumption rates on day 3 with time-lapse imaging

The mean age of the patients was  $35.3 \pm 4.7$  years and the number of previous ART cycles was  $3.4 \pm 3.8$ . The embryos with moderate respiration rates ( $> 0.41 \times 10^{14} / \text{mol s}^{-1}$  and  $< 0.61 \times 10^{14} / \text{mol s}^{-1}$ ) on day 3 had a high chance of reaching good-quality blastocysts. The time-lapse prediction clearly showed a higher proportion of embryos within the optimal ranges defined for t2 and t3-t2. The embryos falling within the optimal ranges for t2 (<24 hours) and t3-t2 (9-13 hours) exhibited a significantly greater proportion of embryos than those falling outside these ranges. Additional time-lapse prediction (t2 and t3-t2), in conjunction with respiration examination on day 3, was evaluated. In the cohort, the embryos with moderate respiration rates had a 21.5% (14/65) chance of reaching good-quality blastocysts. However, the embryos with lower or higher respiration rates had only an 8.9% (5/56) chance of reaching good-quality blastocysts. Conversely, the embryos that had progressed in the optimal stages, t2 and t3-t2, had a 29.6% (16/54) chance of reaching good-quality blastocysts. However, in the outside ranges, they reached only 4.5% (3/67) ( $P < .01$ ). Combining oxygen consumption measurement rates on day 3 with time-lapse prediction, when the embryos had progressed in the optimal stage, meant a high percentage (35.3%) (12/34) of the embryos had become good-quality blastocysts. The outside ranges were 8.0% (7/87), which was statistically significant ( $P < .01$ ) (Figure 5). Although the good-quality blastocyst rate was the highest among the three groups in the combining method, there was no significant difference. Nevertheless, it was hypothesized that by combining the oxygen consumption measurement rates on day 3 with time-lapse prediction, it would be possible to select the best-quality embryo.



**FIGURE 5** Comparison of the oxygen consumption rates, time-lapse imaging, and combined oxygen consumption rates and time-lapse imaging to predict the percentage of good-quality blastocysts that would be reached. The embryos with moderate respiration rates had a 21.5% chance of reaching good-quality blastocysts. The outside ranges had only an 8.9% chance of reaching good-quality blastocysts. The embryos in the time-lapse imaging progressed to optimal stage t2 and, in t3-t2, had a 29.6% chance of reaching good-quality blastocysts. However, the outside ranges had only a 4.5% chance ( $P < .01$ ). When the oxygen consumption measurement rates and the time-lapse imaging were combined, the embryos progressed to good-quality blastocysts at a high percentage rate (35.3%) (12/34). The outside ranges were 8.0% (7/87) ( $P < .01$ ). The optimal range for moderate respiration rates on day 3 were  $>0.41 \times 10^{14}/\text{mol s}^{-1}$  and  $<0.61 \times 10^{14}/\text{mol s}^{-1}$  and t2 (<24 hours) and t3-t2 (9-13 hours)

## 4 | DISCUSSION

Currently, the most widely used embryo selection criteria rely only on static morphologic evaluation, which has a limited predictive value of embryo development. To obtain good results with ART, many techniques of selecting the best embryo have been reported.

This study combined the measurement of oxygen consumption rates and the time-lapse system to select the best-quality embryos for e-SET. This study demonstrated that the respiration of  $0.41\text{--}0.61 \times 10^{14}/\text{mol s}^{-1}$  on day 3 tends to develop a good-quality blastocyst. With the time-lapse system, the time of the first division of the embryos significantly influenced the probability of reaching the blastocyst stage. The shorter (<24 hours) the time of the cleavage to the two-cell stage and then from the two-cell to the three-cell stage (9-13 hours), the higher the chance of the embryo developing to a good-quality blastocyst. This study is the first to use combination trials, with the hope of determining the best methods of selecting good-quality embryos.

There have been many studies looking at the relationship between early kinetics and blastulation potential. One study developed an algorithm for selecting embryos that are likely to reach blastocysts, based on the duration of the first cytokinesis and the time between the first and second mitosis.<sup>27</sup> Another study found

that the development to high-quality blastocysts could be predicted within the first 48 hours of culture by a short duration of the first cytokinesis, duration of the three-cell stage, and the absence of direct cleavage to three cells (duration of the two-cell stage of <5 hours).<sup>21</sup> Furthermore, aneuploidy of human blastocysts was detectable by specific morphokinetic parameters in patients with an increased risk of aneuploidy due to advanced maternal age, history of unsuccessful IVF treatments, or both.<sup>28</sup> One study suggested that chromosomally normal and abnormal embryos have different kinetic behavior; thus, on the basis of these differences, the proposed algorithm served as a tool to classify embryos and to increase the probability of uninvatively selecting normal embryos. Yet another study suggested that morphokinetic characteristics cannot be used to select euploid blastocysts in poor-prognosis patients who are regarded as candidates for pre-implantation genetic screening. The FISH was the mainstay of PGS over the past two decades. Although initially promising, numerous authors failed to show an improvement in IVF outcomes with PGS by FISH. As opposed to FISH, comparative genomic hybridization or next generation sequencing work by analyzing all 24 chromosomes, allowing more accurate results when detecting for aneuploidy. These methods, whether at the cleavage or blastocyst stage, will lead to a far higher percentage of IVF patients undergoing a successful single ET. The time-lapse monitoring system seems to



be a valuable tool to identify all cases of multinucleation. One study concluded that the detection of multinucleation by time-lapse monitoring is associated with lower implantation and clinical pregnancy rates.<sup>29</sup> Many studies also have suggested that time-lapse systems were useful for selecting good-quality embryos.

The SECM measuring system provides an uninvase, simple, accurate, and consistent measurement of the respiration activity of human embryos. Mitochondria produce adenosine triphosphate (ATP), which is important for cell activity by oxidative phosphorylation. Oxygen consumption relates to the quantity of ATP production and mitochondrial respiration might be an effective index of embryo quality.<sup>30</sup> The authors reported that measurements of the oxygen consumption rate of individual blastocysts before freezing provides important information regarding their viability after warming from the view point of blastocoel reexpansion.<sup>31</sup> Another study demonstrated that oxygen consumption from individual embryos revealed significant differences, mainly close to the time of transfer, when the oxygen consumption pattern was associated with successful implantation.<sup>32</sup> In recent years, automated devices that measure embryo oxygen consumption have been manufactured and are reported to correlate with SECM functions. For example, like SECM, the chip-sensing embryo respiration monitoring system (CERM) measures the consumption rate, but does it automatically.<sup>33</sup> A device using a modified version of the nanorespirometer technology, called "EmbryoScope version C" (Unisense FertilTech, Aarhus, Denmark) has been marketed, albeit the function of measuring oxygen consumption has been removed in the updated version (version D). This device can use the combination of time-lapse observation and the oxygen consumption rate measurement. The current study reported that embryos with moderate respiration rates had a better potential for further development than those with lower or higher respiration rates.<sup>20</sup> Therefore, measuring the oxygen consumption pattern of human embryos in culture for  $\leq 72$  hours could be used to select the embryo with the best developmental potential. One of the previous studies concluded that the measurement of oxygen consumption rates for individual oocytes before fertilization provides an uninvase marker of oocyte quality and hence a quantitative assessment of the reproductive potential of the oocyte.<sup>32</sup> Combining embryo respiration activity measurement with morphological evaluation can provide new information regarding the quality of human embryos and improve the selection rate of superior embryos before transfer in IVF-e-SET.

This study's results suggest that the oxygen consumption rate at day 3 of the ET from individual embryos was associated with the development of good-quality embryos, leading to an increased pregnancy rate. The abnormality of the first cell division time after the second meiotic division might have an impact on the next cell division or cell cycle. This abnormality could be carried over into the next cell cycle. As there is the potential for harm to embryonic growth, it is useful to add confirmation by time, interval, and oxygen consumption rates at the ET to choose the best-quality embryos. Different culture conditions or ART might have different effects on embryonic development. However, these measurements will reflect the embryonic state. Future studies

should consider the effect of these parameters and confirm this study's prognosis using pregnancy and abortion rates.

In conclusion, the present study, using time-lapse imaging and the measurement of oxygen consumption rates, clearly has demonstrated that human embryos that will develop to good-quality blastocysts can be selected at day 3. Morphological evaluation has been the only method to evaluate embryo quality for the last 39 years. With the addition of time-lapse imaging, along with respiration rates, it now will be possible to use three methods to evaluate embryos. These methods will contribute to the growth in the field of ART, especially regarding e-SET.

## DISCLOSURES

**Conflict of interest:** The authors declare no conflict of interest. **Human rights statement and informed consent:** All the procedures were followed in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all the patients to be included in the study. **Animal studies:** This article does not contain any study with animal participants that were performed by any of the authors.

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