

—Mini Review—

Morphological Evaluation and Measurement of the Respiration Activity of Cumulus-oocyte Complexes to Assess Oocyte Quality

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Abstract: Scanning electrochemical microscopy (SECM) is a non-invasive and sensitive technique for measuring cellular respiration. In this paper, we review the SECM technique, to establish it as an accurate method for measuring the respiratory activity of single cumulus-oocyte complexes (COCs) and oocytes in animals as well as in humans. Oxygen consumption rates of COCs are influenced by the surrounding cumulus volume and the mitochondrial activity of the cumulus cells. An increase in the oxygen consumption rate was found in bovine oocytes, whereas the oxygen consumption of human oocytes tends to decrease during *in vitro* maturation (IVM). To analyze the metabolic activity of mitochondrial respiration, ATP content and mitochondrial distribution in bovine oocytes have been examined. An electron microscopic study confirmed mitochondrial reorganization in bovine oocytes during oocyte maturation. These results show that the respiratory activity of oocytes changes with maturation status during IVM and mitochondrial reorganization may partly influence respiratory activity. The SECM procedure is therefore a useful technique for evaluating the metabolic activity and quality of oocytes and cumulus cells in the IVM process.

Key words: Cumulus cells, Oocyte maturation, Mitochondria, Oxygen consumption, Electrochemical measurement

Introduction

The *in vitro* maturation (IVM) of human oocytes is an attractive technique that provides a patient-friendly approach to assisted reproductive technology. IVM is relatively simple with a shorter period of treatment and lower costs than conventional *in vivo* fertilization (IVF). For anovulatory patients with polycystic ovaries (PCO), a decrease in the dose of ovarian stimulating drugs lowers the risk of ovarian hyperstimulation syndrome. IVM has been successfully applied to animals [1, 2]. Cha *et al.* were the first group to show the success of IVM in human beings using immature donor oocytes retrieved from antral follicles [3]. Recent studies have demonstrated that the results from IVM are comparable to those achieved with contemporary IVF [4, 5]. The applicability and development of IVM technology is dependent on the improvement of *in vitro* culture systems. During *in vitro* culture, cumulus cells play an important role in oocyte maturation. If provided with the several factors that are essential for normal nuclear and cytoplasmic maturation, oocytes can mature and develop to an embryo after fertilization [6]. Therefore, an appropriate evaluation of cumulus-oocyte complexes (COCs) is indispensable for evaluating the quality of oocytes and improving of the results of IVM.

Over the years, several approaches have been used to evaluate COCs. Morphological evaluation is the main technique used to assess COC quality and to predict the subsequent maturation of oocytes in the IVM process. However, morphological evaluations are subjective and difficult, especially for COCs with

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intermediate morphological qualities. Therefore, more objective evaluation criteria are needed. Previous studies have suggested that a greater understanding of the metabolic respiration of cumulus cells might yield new strategies for evaluating the quality of bovine oocytes [7, 8]. In this paper we describe the morphological evaluation of COCs and the application of a novel cell respiration measuring system using scanning electrochemical microscopy (SECM) to the assessment of the metabolic activity of cumulus cells and oocytes in bovine and human specimens.

Morphological Evaluation of Cumulus-oocyte Complexes and Oocytes

An appropriate evaluation of COCs is indispensable for the improvement of the IVM system, because cumulus cells play an important role in oocyte quality. For a morphologically precise evaluation of human COCs, size of an oocyte is an important parameter. The precise evaluation of human COCs is needed to predict the competence of oocyte maturation. Prior research has indicated that the diameter of immature oocytes is one of most reliable parameters for predicting oocyte quality. Eppig and Schroeder reported that mice oocytes isolated from females younger than 13 days of age are capable of spontaneous break down of the germinal vesicle (GVBD) when the mean diameter is greater than 60 μm [9]. In a study of porcine immature oocyte, progression to metaphase II was observed in 40% of oocytes that were over 110 μm in diameter, whereas no oocyte less than 90 μm in diameter resumed meiosis [10]. In rhesus monkey oocytes, in which meiotic competence occurs late during oocyte development, oocyte diameters appear relatively constant as the competence to undergo GVBD increases. These phenomena suggest that there is no association between oocyte diameter and maturation [11].

In comparison to animal systems, little is known about humans. Based on data from unstimulated polycystic ovary syndrome (PCOS) patients, Cavilla *et al.* deduced that an oocyte diameter of 81 μm at the time of retrieval was the threshold for GVBD, whereas oocytes of more than 103 μm would mature to metaphase II [12]. They also noted that, during *in vitro* human oocyte maturation, an increase in the average diameter of only 3 μm (from 106 to 109) represents a large change in the cytoplasmic volume (increasing an astonishing 8% during culture), suggesting that oocyte diameter provided valuable information about oocyte potential

during IVM. In a proposal for the precise evaluation of human immature oocytes, the two-dimensional area of a depiction image was calculated using image analysis software "Image J".

Another important parameter of oocyte morphological quality is the volume of the human cumulus mass. During IVM, cumulus cells are known to maintain the oocyte nucleus at the GV stage [13]. Expanded human cumulus-oocyte complex patterns have a higher expression of LH receptor mRNA and are associated with more efficient oocyte maturation [14]. Early reports supported the idea that cumulus expansion during IVM improves the developmental capacity [15, 16]. In mice, the fertilization rate of IVM matured oocytes is correlated with the quantity and quality of the expanded cumulus mass [17]. The mechanical loss or spontaneous loss of cumulus cells from COCs has been shown to correlate with a loss of fertilizability [18]. Therefore, the quantity of cumulus mass is a factor influencing the success of IVM. For the morphological classification of cumulus mass, some researchers have separated the cumulus patterns into multilayered and expanded [19, 20].

In this review, we have estimated the multilayered and expanded cumulus mass as the consecutive change and made objective evaluations utilizing an image analysis software program. The COC area was calculated by tracing the edge of the cumulus mass. If the edge was not clear (usually observed in the expanded cumulus mass), the image was analyzed using an edge enhancement mode. The multiple layer formation of the cumulus mass was presented as the C-ratio (area of COC / area of immature oocyte). For the morphological evaluation of human COCs, forty-two human COCs, retrieved from eight women with the PCOS during an IVM program, were used. All COCs were aspirated 36 hours post-hCG between the 10th and 12th day of the menstrual cycle and cultured for 26 hours in TCM199 medium with 10% patient serum, 100 IU/L human chorionic gonadotropin and 75 IU/L follicle stimulating hormone under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. The parameters analyzed were (1) *Area*; area of the immature oocyte and (2) *Layer*; multiple layer formation of the cumulus mass, presented as the C-ratio. As shown in Fig. 1, these two morphological parameters were compared between the immature (germinal vesicle: GV or metaphase I: MI) and mature (metaphase II: MII) oocyte groups after *in vitro* culture. In the *Area* comparison, the mean level of *Area* was significantly higher in the mature group (8,886 \pm 184 vs. 9,806 \pm 161, Mean \pm SEM, $P < 0.05$). In the

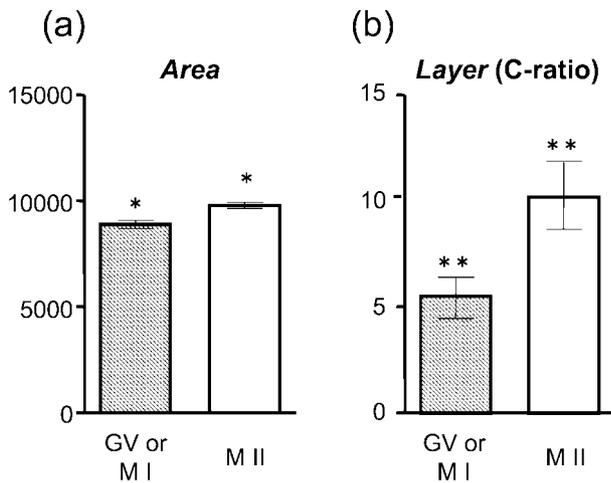


Fig. 1. Comparison of morphological parameters of human immature oocyte and COCs. a: *Area*, Area of immature oocyte; b: *Layer*, Multiple layer formation of cumulus mass was evaluated as C-ratio (Area of COC / Area of immature oocyte). Area and Layer were analyzed before *in vitro* culture and compared prospectively between mature (MII) and immature (MI or GV) group. *, **: significantly different ($P < 0.05$).

Layer comparison, the mean level of the C-ratio was significantly higher in the mature group (5.8 ± 0.8 vs. 10.3 ± 1.6 , Mean \pm SEM, $P < 0.05$). These results suggest the C-ratio is a useful parameter for predicting the maturation status of oocytes in the IVM process.

Subsequently, we examined the relationship between

the cumulus mass morphology and the oocyte quality. Human COCs were classified into five grades based on cumulus mass morphology as follows: Grade 1 (G1), cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; Grade 2 (G2), cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; Grade 3 (G3), regular round oocytes with cumulus cells covering half of the domain; Grade 4 (G4), naked oocytes without cumulus mass; Grade 5 (G5), naked and irregular shaped oocytes (Fig. 2). High maturation rates of immature oocytes were detected in G1 and G2 (70.0% and 64.3%, respectively) in contrast to the lower maturation rate of 17.7% (mean percentage from G3 to G5).

Cumulus cells are a production site of steroids, growth factor, proteins and other compounds that contribute to cytoplasmic maturation of oocytes. Beneficial effects of cumulus cells on microtubule dynamics and/or chromatin stability, oocyte maturation and early embryonic development have been reported in many species, including humans [21–23]. Cumulus cells are also known to play an important role in the regulation of the meiotic progression of oocytes. During the growth and development of meiotic competence of an oocyte, the cumulus cells are responsible for maintenance of nuclear arrest at the germinal vesicle (GV) stage by transfer of an inhibitory signal through gap junctions which elevates the intracellular cyclic adenosine monophosphate (cAMP) level in the oocytes

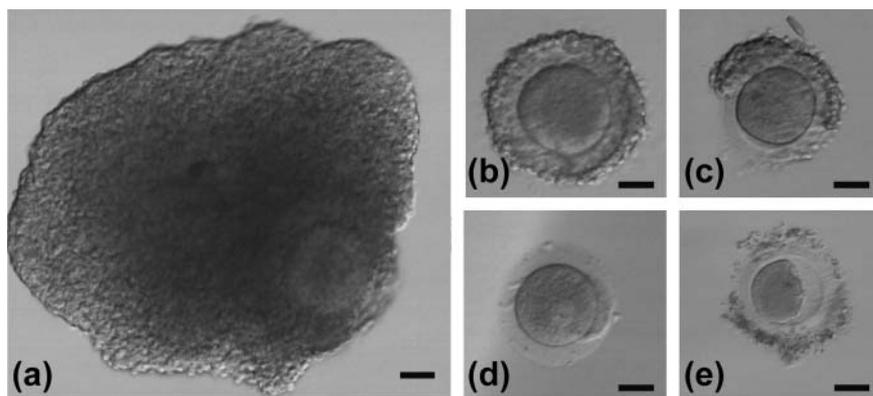


Fig. 2. Light micrographs of human COCs classified by morphological evaluation. (a) Grade 1, cumulus cells with multi-layers covering the whole oocyte, and a regular round oocyte; (b) Grade 2, cumulus cells with multi-layers (less than three layers), covering the whole oocyte and a regular round oocyte; (c) Grade 3, regular round oocytes with cumulus cells covering half of the domain; (d) Grade 4, naked oocytes without cumulus mass; (e) Grade 5, naked and irregular shaped oocytes. Bars = 20 μ m.

[24, 25]. Although the precise regulation mechanism of meiotic progression is still controversial [26], it has been suggested that well-developed cumulus cells have the capacity to regulate appropriate maturation and the development of immature oocytes.

Evaluating the Quality of Embryos and Oocytes with Measurement of Respiration Activity with an Electrochemical Measuring Technique

The metabolic activity of embryos and oocytes has been determined from the consumption of nutrients, such as glucose, pyruvate and amino acids [27–30]. Oxygen consumption is an idea indicator of overall metabolic activity because adenosine triphosphate (ATP) is predominantly generated by oxidative phosphorylation, a process in which oxygen plays an essential role [31–33]. Oxygen consumption by embryos and oocytes has been studied with various measuring techniques, such as the Cartesian diver [34, 35], spectrophotometry [36, 37], ultrafluorescence measurements [38, 39], and self-referencing microelectrodes [40–43].

Electrochemical measurement using scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species, such as oxygen near the sample surface [44]. This technique can measure the concentration profile of a metabolic product around a spherical sample, such as an embryo, with a probe microelectrode. We have employed the SECM technique to examine oxygen consumption by single embryos [45]. Using a modified SECM measuring procedure, we quantified the respiration activity of embryos in several animal species including humans [46]. SECM can non-invasively measure the respiration activity of single embryos from livestock, such as cattle and pigs, as well as those from small rodents, all with high reproducibility. We recently demonstrated that bovine embryos with high oxygen consumption are better candidates for further development into good quality embryos and yielded higher pregnancy rates after embryo transfer. The respiration activity correlates with the embryo quality. SECM is a highly sensitive and non-invasive method for measuring cellular respiration and may be a valuable tool for accurately assessing the quality of embryos, which could contribute to improved outcomes in assisted reproduction, including human IVF. On the other hand, an accurate method for evaluating the respiratory activity of oocytes remains to be developed.

Table 1. Oxygen consumption rates ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of bovine COCs and denuded oocytes in oocyte maturation cultures

Maturation status	COC (n)	Oocyte (n)
Immature	5.48 ± 0.82 (16) ^a	0.67 ± 0.02 (16) ^c
Mature	3.15 ± 0.42 (20) ^b	1.10 ± 0.05 (20) ^d

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

Oocyte quality could be the most important factor in determining successful fertilization and embryo development. Therefore, we attempted to establish an evaluation system for oocyte quality based on the respiratory activity of oocytes.

In previous studies, we evaluated the SECM technique, to establish an accurate method for measuring the respiratory activity of single bovine and porcine oocytes [8, 48]. With the SECM procedure, oxygen consumption of bovine COCs and denuded oocytes was monitored (Table 1). Oxygen consumption rates ($\times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of immature COCs and oocytes (immediately after recovery from an ovary) were 5.48 and 0.67, respectively. Although the respiration rate of denuded oocytes was lower than that of cumulus cells, the oxygen consumption rate by a single bovine oocyte was quantitatively measured by SECM.

Oxygen consumption has been monitored in COCs and oocytes cultured in serum-free medium for oocyte maturation. An increase in the oxygen consumption rate was found in oocytes [1, 10], whereas the oxygen consumption by COCs [3, 15] decreased during IVM. To analyze the metabolic activity of mitochondrial respiration, the ATP content and mitochondrial distribution in oocytes were examined. The ATP content of oocytes after maturation culture was significantly higher than that of immature oocytes (Fig. 3). In immature oocytes, staining with MitoTracker Orange revealed mitochondrial clumps with a strong signal in the periphery of the cytoplasm (Fig. 4). After IVM, the mitochondrial clumps were located more toward the center of the cytoplasm. An electron microscope study confirmed mitochondrial reorganization in bovine oocytes during oocyte maturation (Fig. 5). These results show that the respiratory activity of bovine oocytes increases during IVM and mitochondrial reorganization may thus be partly due to the respiratory activity. Therefore, we consider the SECM procedure is a useful technique for evaluating the metabolic activity and quality of single oocytes.

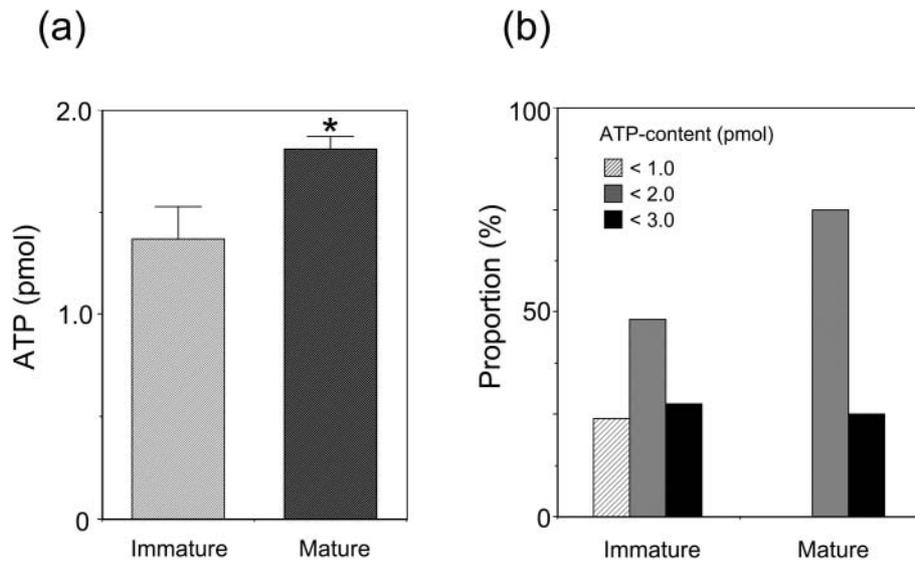


Fig. 3. (a) The ATP content and (b) proportion of oocytes categorized by ATP content: immature and mature bovine oocytes. *: significantly different ($P < 0.05$).

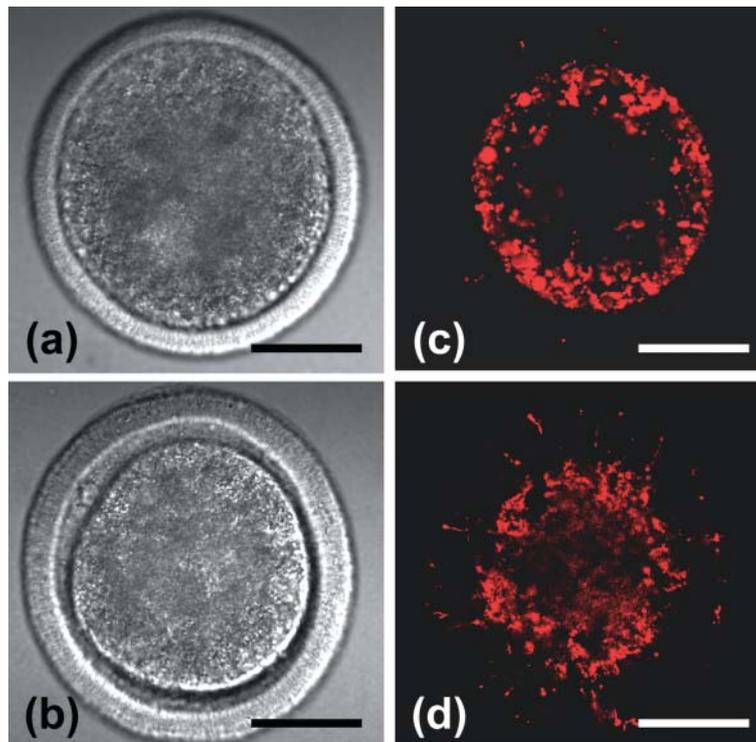


Fig. 4. Midline confocal sections of (a, b) immature and (c, d; cultured in IVMD101 medium) mature bovine oocytes stained by MitoTracker orange. Bars = 50 μ m.

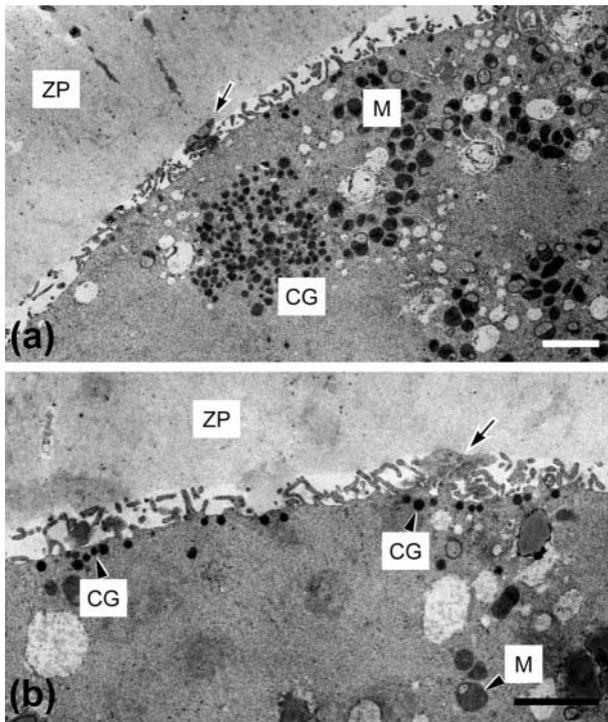


Fig. 5. Electron micrographs of (a) immature and (b) cultured in serum-free maturation medium mature oocytes. a: Many aggregates of mitochondria (M) and cortical granules (CG) were present in the cortex cytoplasm. b: Cortical granules were distributed in the periphery of the cytoplasm, but aggregates of mitochondria were not found. ZP, zona pellucida. Arrows: microvilli. Bars = 2 μm .

Measuring the Respiration Activity of Human COCs and Oocytes

In this part, we review the respiration activity of human COCs and oocytes. Eighty-five human COCs retrieved from eighteen women with the PCOS during an IVM program were examined. Informed consent for the use of the COCs in this study was obtained from all the patients. Thirteen of the eighteen women were administered a short course of follicular stimulating hormone (FSH) and five women accomplished an IVM program without the use of FSH before hCG administration. All COCs were aspirated 36 h post-hCG between the 10th and 12th day of the menstrual cycle and cultured 26 h in TCM199 medium with 10% patient serum, 100 IU/L human chorionic gonadotropin and 75 IU/L follicle stimulating hormone under an atmosphere of 5% CO_2 , 5% O_2 , and 90% N_2 . Cellular unevenness of the cumulus mass has an influence on SECM measurement results. Therefore,

Table 2. Oxygen consumption rates ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of human COCs classified by morphological evaluation

Category	Pre-culture (n)	Post-culture (n)
G1	7.79 ± 1.00 (50)	6.11 ± 0.74 (50)
G2	1.46 ± 0.15 (25)	1.63 ± 0.33 (25)
G3	1.26 ± 0.35 (8)	1.60 ± 0.55 (8)
G4	0.86 ± 0.30 (2)	0.79 ± 0.11 (2)
G5	0.77 (1)	0.35 (1)

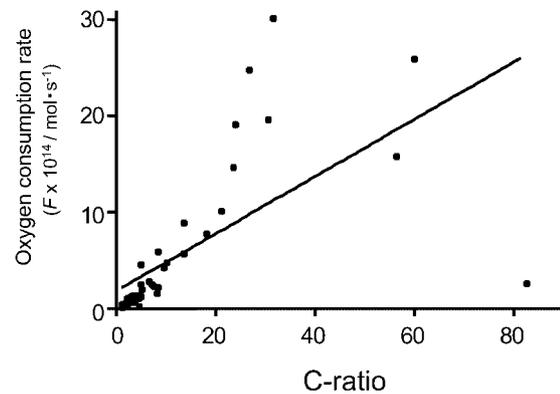


Fig. 6. Correlation between oxygen consumption rate and C-ratio.

the oxygen consumption rate was measured three times for each COC and the mean was used as the measured value.

Relationship between the oxygen consumption rates and morphological categories of human COCs in the pre-culture and post 26 hours-culture stages is shown in Table 2. A linear correlation between the oxygen consumption rate and the C-ratio was shown in Fig. 6 (correlation coefficient: $r^2 = 0.423$, $P < 0.01$). The respiration activity measured by SECM showed that the respiration activity of human COCs with multi-layer cumulus cells (G1) was higher than in the other categories (G2–G5). Ultrastructural studies revealed that the cumulus cells in G1 COCs, which showed high respiration activity, contained many well-developed mitochondria. In contrast, undeveloped mitochondria were scattered in the cumulus cells in G3 COCs (Fig. 7). These results suggest that respiration rates are directly influenced by the surrounding cumulus volume and mitochondrial activity in cumulus cells.

A comparison of the oxygen consumption fluctuation between the pre-culture stage and after 26 hours of culture is shown in Fig. 8. In the FSH administration group, the mean oxygen consumption rate tended to

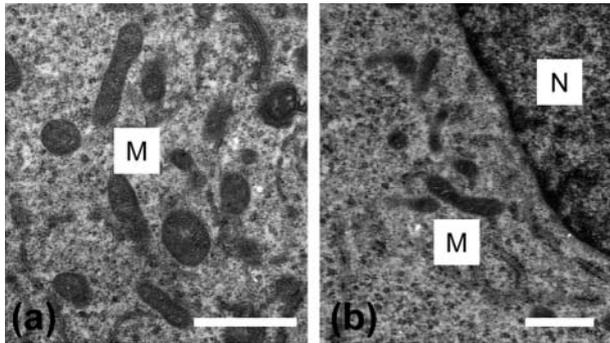


Fig. 7. Electron micrographs of human cumulus cells. Many well-developed mitochondria (M) are present in the cumulus cells of the Grade 1 COC (left image). In contrast, mitochondria showing small size are scattered in the cumulus cells of the Grade 3 COC. N: Nucleus. Bars = 1 μ m.

decrease after 26 h of culture (5.62 ± 0.83 vs. 4.25 ± 0.58). In contrast, the mean oxygen consumption rates were similar between the two stages in the non-FSH administration group (4.07 ± 1.19 vs. 4.17 ± 1.02). There was no clinical advantage gained by extending the FSH pre-treatment from 3 to 6 days to produce follicles more than 10 mm in diameter [49]. On the other hand, Wynn *et al.* demonstrated a higher maturation rate in a FSH treatment group [50]. In their study, the maturation rate to metaphase II was higher in the FSH

administration group (68.3% vs. 61.3%, in comparison to the non-FSH administration group, unpublished data). The benefit of FSH pre-treatment remains controversial and the development competence cannot be evaluated because of the limitations of the current study protocol.

The efficacy of the administration of hCG remains controversial. In hCG protocol, all patients are administered hCG before oocyte retrieval. After the LH surge, oocytes resume the first meiotic division and enter the second division [51]. At the same time, cumulus mass begins to change to the expanding form. Cumulus expansion may influence a variety of fundamental developmental changes which occur during fertilization. Regarding the use of hCG in bovine, the cumulus cells from antral follicles as small as 5 mm have mRNA transcripts for LH receptors and may respond to hCG stimulation [52]. This finding provides evidence of a mechanism by which hCG begins the maturation process of small antral oocytes *in vivo* and facilitates the completion of meiosis *in vitro*. Chian *et al.* demonstrated that the percentage of oocytes achieving maturation after 48 h *in vitro* culture was significantly higher in the hCG-primed group than in the non-hCG-primed group during human IVF-IVF [53].

Finally, the results of the respiration measurement of single human oocytes using a SECM system are listed in Table 3. The oxygen consumption rate of pre-cultured oocytes (GV stage) was $0.49 \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$,

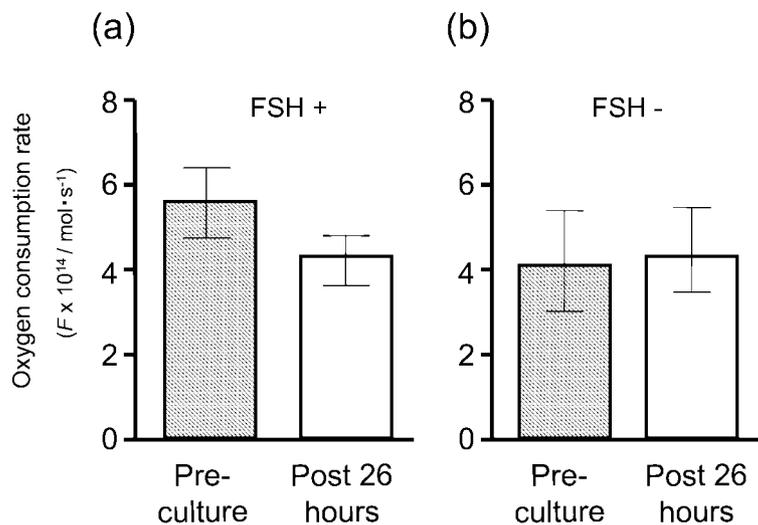


Fig. 8. Comparison of oxygen consumption with or without FSH administration at the pre-culture stage and after 26 h of culture. (a) oxygen consumption rate in the FSH administered group and (b) in the non-FSH administered group.

Table 3. Oxygen consumption rates ($F \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$) of human denuded oocytes in oocyte maturation cultures

Maturation status	Oxygen consumption rate (n)
GV (Pre-culture)	0.49 ± 0.07 (10)
GV or MI (Post-culture)	0.40 ± 0.21 (19)
MII (Post-culture)	0.41 ± 0.15 (30)

whereas the oxygen consumption rate tended to decrease in matured MII oocytes ($0.41 \times 10^{14}/\text{mol} \cdot \text{s}^{-1}$). These results suggest that the respiration activity of human oocytes changes with maturation status of oocytes, although the mechanism of this fluctuation needs to be confirmed with further studies.

Conclusions

The SECM technique is a non-invasive and sensitive method for measuring the oxygen consumption of individual COCs and oocytes in animal species including humans. The respiration activity of COCs is directly influenced by the surrounding cumulus cell volume and the mitochondrial activity of cumulus cells. Biochemical and cytological studies strongly suggest that oxygen consumption is an important parameter for evaluating the competence of oocyte maturation. It may be feasible to monitor the profile of an oocyte's mitochondrial activity by measuring its oxygen consumption, and select the oocytes that can sustain fertilization and the development of embryos. Therefore, the SECM technique may have a future in clinical application as a predictor of oocyte quality which could be used for determining to develop into good quality embryos.

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