Oxidative Phosphorylation-linked Respiration in Individual Bovine Oocytes

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Abstract. Mitochondrial bioenergetics in mammalian oocytes has not been sufficiently characterized. In this study, the function of oxidative phosphorylation (OXPHOS), a major pathway in mitochondria, was investigated in individual bovine oocytes by monitoring oxygen consumption using modified scanning electrochemical microscopy (SECM). At the germinal vesicle (GV) stage, 65% of basal respiration was used for mitochondrial respiration, which was inhibited by complex IV inhibitor. Around 63% of mitochondrial respiration was coupled to ATP synthesis, as determined by sensitivity to an ATP synthase inhibitor, and the remaining 37% was attributed to proton leak. In contrast, 50% and 43% of mitochondrial respiration were used for ATP synthesis in in vivo- and in vitro-derived metaphase II (MII)-stage oocytes, respectively. ATP-linked respiration, in both in vivo- and in vitro-derived MII-stage oocytes, was significantly lower than in GV-stage oocytes, suggesting that OXPHOS in bovine oocytes is more active at the GV stage compared with the MII stage. Interestingly, basal respiration in in vitro-derived MII oocytes was significantly higher than for in vivo-derived oocytes, reflecting an increase in proton leak. Next, we assessed respiration in MII oocytes cultured for 8 h. The aged oocytes had a significantly reduced maximum respiratory capacity, which was stimulated by a mitochondrial uncoupler, and reduced ATP-linked respiration compared with non-aged oocytes. However, the aging-related phenomenon could be prevented by caffeine treatment. We conclude that OXPHOS in bovine oocytes varies in the transition from GV to MII stage, in vitro maturation and the aging process. This approach will be particularly useful for analyzing mitochondrial bioenergetics in individual mammalian oocytes.

Key words: Bovine, Mitochondrial function, Oocyte, Oxidative phosphorylation, Oxygen consumption

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Mitochondria play fundamental roles in the cell, and mitochondrial dysfunction has been linked with several pathologies, including infertility and developmental failure. Although they share general characteristics, mitochondria can have distinct features based on inner membrane invaginations and matrix structures. Depending on their cell type and functional status, mitochondria present an extensive range of morphologies, are functionally heterogeneous [1], and vary in number [2]. Oxidative phosphorylation (OXPHOS), the process that couples substrate oxidation to ATP synthesis, is the major and best-known metabolic function of mitochondria. During OXPHOS, electrons are transferred from nutrients to reducing equivalents (e.g., NADH), then to electron carriers, and finally to oxygen. Such electron transfer is mediated by oxi-do-reductive reactions of the tricarboxylic acid cycle in the mitochondrial matrix and by electron transport in the inner mitochondrial membrane. The energy harvested during these oxi-do-reductive reactions is stored in a proton gradient across the inner mitochondrial membrane that is dissipated during the production of ATP [3].

The health of mammalian oocytes appears to be highly regulated by mitochondrial metabolic pathways and functions. Oocyte mitochondrial dysfunction may contribute to diminished fertility and be a cause of developmental retardation and growth arrest in preimplantation embryos [4]. Mitochondrial dysfunction in oocytes may be induced by various factors such as conditions of in vitro maturation [5] and maternal and postovulatory aging [6]. In a previous study, it was shown that in vitro maturation (IVM) conditions may cause incomplete movement of the mitochondria to the inner cytoplasm and decreased ATP production [5, 7]. In addition, Marutino et al. observed that oocyte energy/oxidative status was affected by within/ between-subject, in vivo versus IVM and age-related variations [8]. Moreover, it was confirmed that oocytes aged in vitro exhibited decreased mitochondrial membrane potential and Ca2+ levels in the endoplasmic reticulum, and that addition of caffeine ameliorates most of these aging-associated changes [9].

Thus far, mitochondrial function in mammalian oocytes has been assessed by analyzing mitochondrial localization ATP content and mitochondrial membrane potential [7, 10, 11]. However, these techniques can be disruptive and are indirect methods for assessing physiological mitochondrial function. Meanwhile, oxygen consumption in embryos has been measured as a noninvasive and strong indicator
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of metabolic activity as well as mitochondrial function [12]. We also measured oxygen consumption in cloned and in vitro fertilized embryos with scanning electron microscopy (SECM) [13, 14], a method that appears to be reliable, noninvasive and highly sensitive for measuring oxygen consumption in individual embryos. However, this is not to say that the oxygen consumption under the basal condition (basal respiration) is the only readout of OXPHOS because respiration appears to be controlled by ATP turnover, non-mitochondrial reactions such as NADPH oxidase, and proton leak across the mitochondrial inner membrane [15]. Non-mitochondrial respiration accounts for approximately 10% of basal oxygen consumption in somatic cells [15]. At the end of cell respiratory control experiments, it has been common to inhibit mitochondrial electron transport. In addition, mitochondrial respiration and ATP turnover can be estimated by inhibiting ATP synthase with oligomycin. In this case, oligomycin-insensitive mitochondrial respiration can be attributed to proton leak [15]. Furthermore, the degree of electron transport can be estimated by the addition of a mitochondrial uncoupler at maximum respiratory capacity. A decrease in maximum respiratory capacity is then an indicator of mitochondrial dysfunction [15].

Although techniques assessing OXPHOS activity in intact somatic cells have been developed to quantify mitochondrial function [15, 16], quantifying OXPHOS activity of individual oocytes has been more challenging. Here, we show a technique for assessing OXPHOS activity in individual oocytes by using SECM and mitochondrial inhibitors such as a complex V inhibitor (oligomycin), a mitochondrial uncoupler (carboxyl cyanide m-chlorophenyl hydradzone; CCCP) and a complex IV inhibitor (sodium cyanide; NaCN). We have systematically investigated non-mitochondrion-, ATP turnover- and proton leak-linked respiration, maximum respiratory capacity and coupling efficiency in GV- and MII-stage bovine oocytes both in vitro and in vivo, as well as in in vitro-aged oocytes with and without caffeine treatment.

Materials and Methods

Animal care and use

The present study was approved by the Ethics Committee for the Care and Use of Experiments Animals, National Livestock Breeding Center. Moreover, all animals received humane care according to Law No. 105 and Notifications No. 6 and 22 of the Japanese Guidelines for Animal Care and Use.

IVM

Collection and IVM of bovine cumulus-oocyte complexes (COCs) were performed as described by Imai et al. [17]. Ovaries from Japanese Black heifers or Holstein heifers were collected at a slaughterhouse, transferred to the laboratory and then washed and stored in physiological saline supplemented with 50 µg/ml gentamicin (Siga-Aldrich, St. Louis, MO, USA) at 20 C for approximately 20 h. COCs were aspirated from small follicles (2–6 mm in diameter). The IVM medium was 25 mM Hepes-buffered tissue culture medium 199 (M199, Gibco BRL, Grand Island, NY, USA) supplemented with 5% calf serum (Gibco BRL). COCs were cultured in IVM medium covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan) at 38.5 C in a humidified atmosphere with 5% CO₂ for 22 h.

Collection of in vivo matured oocytes

In vivo-matured oocytes were collected from six Holstein cows. A controlled internal drug release (CIDR) device (CIDR® 1900; Pfizer Animal Health, Hamilton, New Zealand) was inserted at arbitrary days of the estrous cycle (day of CIDR insertion = day 0). Follicles with a diameter of ≥8 mm were aspirated on day 5 by using an ultrasound scanner (SSD-900; ALOKA, Tokyo, Japan) and 7.5 MHz convex-array transducer (UST-9109P-7.5; ALOKA) with a 17-gauge × 500 mm disposable OPU needle assembled 1,500 mm polyvinyl chloride tube (COVA Needle, Misawa Medical Industry Co., LTD., Tokyo, Japan). Cows then received 30 mg of follicle-stimulating hormone (ANTORIN®-R•10; Kyoritsu Seiyaku, Tokyo, Japan) twice a day from the evening of day 6 through the morning of day 10 in decreasing doses (6, 6, 4, 4, 3, 3, 2, and 2 mg) by intramuscular injection. Prostaglandin F₂α (0.225 mg; D-Cloprostenol; Dalmazin; Kyoritsu Seiyaku) was administered on the evening of day 8. The CIDR device was removed on the morning of day 9. Following the administration of 200 µg of gonadotropin-releasing hormone analogue (Fertirelin acetate; Spornen; Kyoritsu Seiyaku) on the morning of day 10, follicles ≥5 mm in diameter were aspirated 25–26 h later.

Measurement of oxygen consumption using SECM

Oxygen consumption by individual bovine oocytes was measured noninvasively with an SECM system (HV-405; Hokuto Denko, Tokyo, Japan). Prior to measurement of oxygen consumption, cumulus cells were removed by pipetting in 0.1% hyaluronidase (Sigma). Oocytes were transferred individually to a plate filled with 5 ml of embryo respiration assay medium-2 (ERAM-2; Research Institute for the Functional Peptides, Yamagata, Japan), and the oocytes were dropped individually to the bottom of the microwell. The temperature of the medium was maintained at 38.5 C by placing the plate on a warming plate on the microscope stage. Oxygen consumption was measured as described by Shikui et al. [18]. The XYZ-stage and potentiostat were controlled by an HV-405 ver. 2.04 SECM system (Hokuto Denko). Voltammetry of the Pt-microdisc electrode (Hokuto Denko) in ERAM-2 solution showed a steady-state oxygen reduction wave. No response from other electrochemically active species was observed near the oocyte surface. The oxygen consumption rate of embryos was calculated using software. The oxygen consumption difference between the bulk solution and the sample surface (ΔC) and the oxygen consumption rate (F) of a single sample were estimated according to spherical diffusion theories [18]. We repeatedly scanned the electrode back and forth to estimate the mean ΔC for each sample two times.

Respiration demand assay

Non-mitochondrion-, proton leak-, and ATP-related respiration, and maximum respiratory activity were calculated by SECM (Fig. 1). Following measurement of basal oxygen consumption, 2 µg/ml oligomycin (Sigma) was added to ERAM-2 to inhibit ATP synthase (complex V) prior to measurement. Subsequently, 2 µM carbonyl cyanide m-chlorophenyl hydradzone (CCCP, Sigma) was added for estimating “maximal respiration activity.” Finally, 1 mM sodium cyanide (NaCN, Sigma) was added to ERAM-2 with oligomycin and CCCP. As NaCN blocks mitochondrial respiration, NaCN-sensitive respiration (subtracting respiration after NaCN supplementation from basal respiration) represented mitochondrial respiration, and
the insensitive respiration (respiration continuing after NaCN treatment) was attributed to non-mitochondrial respiration. The dose of each inhibitor was based on the results of previous studies [16, 19, 20]. The value obtained by subtracting respiration after oligomycin addition from basal respiration was defined as ATP-linked respiration, and the value obtained by subtracting respiration after NaCN and oligomycin addition was defined as proton leak-linked respiration.

Coupling efficiency, an index of the coupling of electron transport to proton translocation and ATP synthesis mediated by the proton motive force, was indicated as a percentage of ATP-linked respiration relative to mitochondrial respiration. To determine the apparent respiratory state, the assumption was made that State 3 respiration was equivalent to the rate measured after addition of CCCP. Similarly, State 4 was assumed to be the rate measured after addition of oligomycin. The State$_{apparent}$ can then be calculated from the following equation: 

$$State_{apparent} = \frac{4(\text{Basal} - \text{Oligo})}{(\text{CCCP} - \text{Oligo})}$$

**Experimental studies**

First, mitochondrial respiration in GV-stage and in vitro- and in vivo-derived metaphase II (MII)-stage bovine oocytes was examined. Respiration of 35 GV-stage oocytes that were collected from slaughterhouse-derived ovaries was assessed by SECM. Thirty-four in vitro-derived and 22 in vivo-derived oocytes that contained the first polar body were assessed as MII-stage oocytes. Next, effects of aging and caffeine treatment on mitochondrial respiration were examined. Following in vitro maturation for 22 h, the bovine oocytes were cultured in 100 µl of IVM medium with or without 5 mM caffeine (Sigma) for 8 h. Twenty MII oocytes following in vitro maturation for 22 h as non-aged oocytes, 20 MII oocytes cultured without caffeine for an extra 8 h as aged oocytes and 20 MII oocytes cultured with caffeine for an extra 8 h as caffeine-treated aged oocytes were assessed for respiration by using SECM, respectively.

**Statistical analysis**

Data were analyzed using analysis of variance (ANOVA) followed by Fisher’s protected least significant difference test. For all data, P<0.05 was considered significant. All analyses were conducted using StatView (SAS Institute, Cary, NC, USA).

**Results**

**OXPHOS in bovine oocytes is more active in the GV stage compared with the MII stage**

Basal respiration in MII-stage in vivo-derived oocytes (0.30 ± 0.01 × 10⁻¹⁴ mol⁻¹) was significantly lower than in GV-stage oocytes (0.44 ± 0.02 × 10⁻¹⁴ mol⁻¹) (Fig. 2A). This decreased basal respiration appeared to be due to decreased ATP-linked respiration in in vivo-derived MII oocytes (GV vs. in vivo-derived MII: 0.18 ± 0.01 vs. 0.09 ± 0.01 × 10⁻¹⁴ mol⁻¹), but it did not affect proton leak or non-mitochondrial respiration (Fig. 2A and 2B). In GV and in vivo-derived MII oocytes, 39.8 ± 2.0% and 28.2 ± 3.1% of basal respiration were ATP-linked, 25.3 ± 2.2% and 29.8 ± 3.9% were proton leak-related, and 34.9 ± 2.5% and 42.0 ± 4.8% were non-mitochondrion-related, respectively, showing that the percentage of the ATP-linked respiration in MII-stage oocytes was significantly lower than that in GV-stage oocytes (Fig. 3). Furthermore, in vivo-derived MII oocytes (49.9 ± 4.4%) had significantly lower coupling efficiency than GV-stage oocytes (62.6 ± 2.8%). When mitochondrial workload was decreased, the State$_{apparent}$ approaches 4. In present study, the State$_{apparent}$ in in vivo-derived MII oocytes was significantly higher (3.80 ± 0.03) than in GV-stage oocytes (3.55 ± 0.04), suggesting that mitochondrial workload in GV oocytes is more active than in MII stage (Fig. 2D).

**In vitro maturation in bovine oocytes altered respiratory reserve capacity and proton leak**

In vitro-derived oocytes had significantly higher basal respiration (0.40 ± 0.01 × 10⁻¹⁴ mol⁻¹) compared with in vivo-derived oocytes (Fig. 2A), indicating increased proton leak-linked respiration (in vitro:
maximum respiration was significantly lower (0.55 ± 0.02 × 10^{-14} \text{ mol}^{-1}) than in non-aged oocytes (0.72 ± 0.02 × 10^{-14} \text{ mol}^{-1}) (Fig. 3A). On the other hand, ATP-linked respiration (0.15 ± 0.02 × 10^{-14} \text{ mol}^{-1}) and maximum respiration (0.15 ± 0.01 × 10^{-14} \text{ mol}^{-1}) but not increased ATP- or non-mitochondrion-linked respiration (Fig. 2A and 2B). In addition, the respiration stimulated by CCCP in in vitro-derived oocytes was significantly lower than in those derived in vivo, indicating that the maximum respiratory capacity of in vitro-matured oocytes is less compared with those derived in vivo (Fig. 2D). The State_{apparent} of in vitro-derived MII-stage oocytes (3.60 ± 0.04) was similar to that in GV-stage oocytes and was significantly lower than in in vivo-derived MII-stage oocytes.

**Maximum respiratory capacity and ATP-linked respiration in MII oocytes are decreased during in vitro aging, but these effects can be limited through caffeine treatment**

Basal respiration in aged oocytes was significantly lower (0.34 ± 0.01 × 10^{-14} \text{ mol}^{-1}) than in non-aged oocytes (0.44 ± 0.02 × 10^{-14} \text{ mol}^{-1}) (Fig. 4A). This decreased basal respiration was associated with decreased ATP-linked respiration (non-aged vs. aged: 0.16 ± 0.02 vs. 0.09 ± 0.01 × 10^{-14} \text{ mol}^{-1}) (Fig. 4B) but not with decreased proton leak- or non-mitochondrion-linked respiration (Fig. 4A and 4B). Furthermore, maximum respiratory activity was significantly lower in aged oocytes (0.55 ± 0.02 × 10^{-14} \text{ mol}^{-1}) than in non-aged oocytes (0.72 ± 0.02 × 10^{-14} \text{ mol}^{-1}) (Fig. 3A). On the other hand, ATP-linked respiration (0.15 ± 0.02 × 10^{-14} \text{ mol}^{-1}) and maximum respiratory...
capacity \((0.68 \pm 0.03 \times 10^{-14} \text{ mol}^{-1})\) in caffeine-supplemented aged oocytes were similar to the levels in non-aged oocytes (Fig. 4A and 4B). Coupling efficiency and State\text{apparent} were not significantly different among the experimental groups (Fig. 4C and 4D).

**Discussion**

In the present study, we examined mitochondrial respiration in bovine oocytes using SECM. Our results show that i) ATP-linked respiration, coupling of electron transport and ATP synthase, and mitochondrial workload in MII oocytes were lower compared with GV-stage oocytes; ii) in vitro-matured oocytes have a lower maximum respiratory capacity and higher proton leak-linked respiration compared with in vivo-matured oocytes; and iii) the maximum respiratory capacity and ATP-linked respiration of in vitro-matured oocytes were decreased by in vitro aging; however, addition of caffeine prevented these aging-related changes.

The regulatory mechanisms of OXPHOS in mammalian oocytes are unclear. In the present study, we observed in GV-stage and in vivo-derived MII oocytes that 62.6% and 49.9% of mitochondrial respiration was coupled to ATP turnover, respectively. However, the coupling efficiency in bovine oocytes seems to be lower than in somatic cells, as demonstrated in previous studies showing that 70–85% of mitochondrial respiration in skeletal muscle, hepatocytes, neural cells and carcinoma cells accounted for ATP turnover, which may be more sensitive to changes in proton conductance [15]. Although the physiological significance of the downregulation of proton conductance is unclear, it appears to be an interesting signature of oocytes.

Changes in mitochondrial distribution, mitochondrial membrane potential, and ATP content in the transition from GV to MII stage have been investigated for mitochondrial function [7, 10, 11]. However, these techniques cannot provide a comprehensive readout of OXPHOS activity. In the present study, we confirmed that OXPHOS activity in bovine oocytes decreases in the transition from the GV to MII stage by measuring OXPHOS-linked respiration. This result correlates with microarray data in mouse oocytes showing that OXPHOS-involved transcripts were dramatically degraded in the transition from the GV to MII stage, suggesting a low rate of energy production in MII oocytes [22]. Indeed, it was observed in *Xenopus* oocytes that Na\(^+/K^+\)-ATPases are completely downregulated by germinal vesicle breakdown following progesterone-induced maturation [23]. Moreover, a constant high level of cyclic-adenosine monophosphate (cAMP) maintains meiotic arrest in fully grown oocytes, and this high level of cAMP is produced from ATP by an oocyte-specific G-protein coupled adenyl cyclase [24, 25]. Therefore, the requirement for ATP in the maintenance of meiotic arrest and/or preparation of meiotic resumption may explain why OXPHOS activity was higher in GV oocytes.

Mitochondrial function in oocytes appears to be altered by in vitro maturation [5, 7]. The present study also demonstrates decreased maximum respiratory capacity and increased mitochondrial respiration, reflecting increased proton leak-related respiration in in vitro-matured oocytes. Stimulation of maximum respiratory capacity by CCCP may be due to substrate uptake, metabolism and electron transport activity [15]. The observed decrease in maximum respiratory capacity is in agreement with a previous study showing that oocytes matured in vitro under 20% oxygen tension have a lower membrane potential compared with those matured in vivo, as a result of increased reactive oxygen species (ROS) production during IVM [26]. Moreover, increased proton leak can also protect against mitochondrial damage induced by ROS such as superoxide [27]. Uncoupling proteins (UCPs) are activated by ROS, and UCP-mediated uncoupling leads to a reduction in ROS production by the respiratory chain in cell signaling [28]. Although the protective mechanisms against ROS in oocytes are unclear, increased proton leak-related respiration may be a sign of protection from oxidative damage during in vitro maturation.

Ovulated mammalian oocytes arrest at the MII stage and remain at that stage until fertilization. After fertilization and successful embryo development, time-dependent aging sets in, both in vivo and in vitro, that compromises development [6]. The aging-related impacts on oocytes include reduced levels of maturation promoting factor (MPF) and mitogen-activating protein kinase activity, increased ooplasmic microtubule levels, disturbed microfilaments, and increased histone acetylation and mitochondrial function [6]. In the present study, we confirmed impaired ATP-linked respiration and maximum respiratory capacity, which suggest mitochondrial dysfunction in aged oocytes. However, this aging-related aberration was prevented by caffeine treatment. Addition of caffeine during in vitro aging prevented aging-related changes, including decreased Ca\(^{2+}\) storage and mitochondrial membrane potential, both of which protect the mitochondria from the detrimental effects of ROS accumulation [9]. Therefore, protection against ROS by caffeine is a possible reason why ATP-linked respiration and maximum respiratory capacity were preserved in aged oocytes.

In conclusion, we demonstrated that OXPHOS in bovine oocytes changes in the transition from the GV to MII stage, as well as during in vitro maturation and the aging process. We believe that this approach using SECM and mitochondrial inhibitors will accelerate research for analyzing mitochondrial bioenergetics in individual mammalian oocytes.

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**References**

5. Zeng HT, Yeung WSB, Cheung MPL, Ho PC, Lee CKF, Zhuang GL, Liang XY. O ovulated matured rat oocytes have low mitochondrial deoxyribonucleic acid and
adeno~sine triphosphate contents and have abnormal mitochondrial redistribution. Fertil Steril 2009; 91: 900–907. [Medline] [CrossRef]