examined in comparison with wild-type mice (Table I). When oocytes from wild-type mice were fertilized with sperm from wild-type mice, the fertilized oocytes developed into blastocysts with normal frequency in 4 days. On the other hand, development of the fertilized oocytes from SOD1-deficient mice was totally arrested at the 2-cell stage. The zygotes/embryos from SOD1-deficient mice were examined by DNA labeling with Hoechst33342 from 5 to 42 h after IVF. No abnormality was observed in the timing of pro-nucleus formation and the first cleavage of SOD1-deficient zygotes/embryos, compared with wild-type zygotes/embryos (Fig. 2). Because SOD1 protein rescues the 2-cell arrest that occurs in embryos of some mouse strains (Nonogaki *et al.*, 1992), the effects of antioxidants, human SOD1 protein and β-mercaptoethanol on IVF and further development were examined, but 2-cell arrest was not negated in any of the embryos from SOD-deficient oocytes. Thus, oxidative stress caused by SOD1 deficiency appeared to be different from that in previously reported cases.

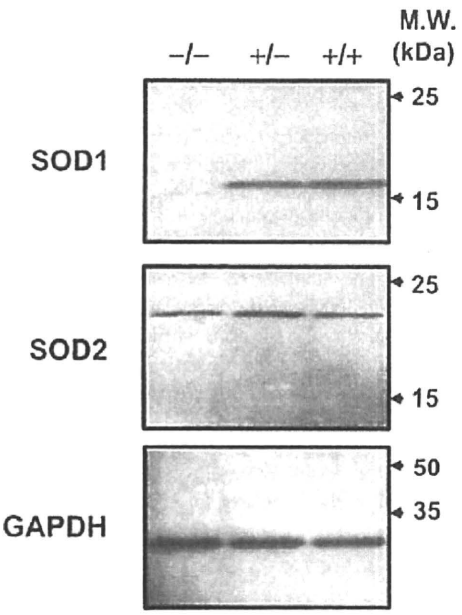

Figure 1 Western blot analysis of SOD1 and SOD2 in oocytes. Sixty superovulated MII oocytes from each genotypic female mouse were subjected to SDS–PAGE followed by immunoblot analysis. Antibodies against SOD1 (upper panel), SOD2 (middle panel) and GAPDH (lower panel, protein loading control) were used as primary antibodies.

Since oxygen content is ~2% in the body, which is one order lower than the conventional culture conditions with atmospheric oxygen (20% O_2, 150 mmHg), culture was attempted under hypoxic conditions with 1% O_2 (7 mmHg). As expected, embryos from SOD1-deficient mice developed beyond the 2-cell stage to blastocyst with efficacy comparable to those from wild-type mice (Table I). Thus, SOD1-deficient oocytes were found to be vulnerable to exposure to atmospheric oxygen conditions, but developed normally under hypoxic culture.

Higher concentration of superoxide in 2-cell arrest embryos

We estimated the oxidative stress in 2-cell embryos from wild-type and SOD1-deficient oocytes at 36 h after IVF using dihydroethidium, which is a fluorescent probe frequently used to measure intracellular superoxide levels (Wilhelm *et al.*, 2009). The resultant fluorescence was detected spottily in the cytoplasm of both wild-type and SOD1-deficient embryos under 20% O_2 (Fig. 3A). Then we quantified fluorescence intensity of all four groups of embryos. The data indicated that superoxide levels in SOD1-deficient embryos were significantly higher than that in the embryos from wild-type embryo under 20% O_2 (Fig. 3B). However, there was no significant difference between wild-type and SOD1-deficient embryos under 1% O_2 culture.

Embryos gradually changed to permanent 2-cell arrest under atmospheric oxygen

To determine the period required for the arrest, the embryos were incubated under atmospheric oxygen conditions for 12, 24 and 36 h after IVF, transferred to 1% O_2 and incubated up to 4 days (Fig. 4). There was no important effect of atmospheric culture on the

Table 1 Developmental arrest at 2-cell stage in embryos derived from SOD1-deficient (KO) mouse oocytes under 20% or 1% oxygen culture and effects of antioxidant supplementations.

Oocyte genotype and treatments	Number of oocytes cultured	Number of embryos (%)		
		2-cell ≤ at Day 1	4-cell ≤ at Day 2	Blastocyst at Day 4
Under 20% O_2				
Wild	89	85 (95.5)[a]	83 (93.3)[a]	75 (84.3)[a]
KO	76	72 (94.7)[a]	0 (0)[b]	0 (0)[c]
KO + 100 μM β-mercapotethanol	95	61 (64.2)[c]	0 (0)[c]	0 (0)[c]
KO + 500 μg/ml hSOD1	91	73 (80.2)[b]	0 (0)[c]	0 (0)[c]
Under 1% O_2				
Wild	79	77 (97.5)[a]	68 (86.1)[a]	53 (67.1)[b]
KO	96	93 (96.9)[a]	81 (84.4)[a]	59 (61.5)[b]

WT, wild type. Values with difference superscript letters within each day are significantly different ($P < 0.05$).

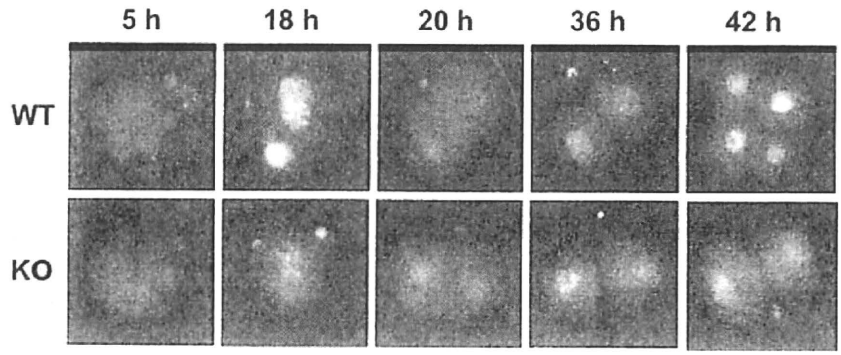

Figure 2 Chromosome labeling of zygotes/embryos. Superovulated COCs from wild-type (WT) or SOD1-deficient (KO) mice were subjected to IVF and incubated under atmospheric oxygen conditions. The zygotes/embryos were stained with Hoechst33342 at 5, 18, 20, 36 and 42 h after IVF and observed under a fluorescent microscope.

development of embryos from SOD1-deficient oocytes by 12 h. However, the developmental potential of the 4-cell and blastocyst stages in SOD1-deficient embryos decreased gradually during the 2-day incubation under atmospheric oxygen. The hypoxic culture did not rescue most embryos with 2-cell arrest after 36 h incubation under atmospheric oxygen (4-cell: 12.7%, blastocyst: 3.6%). This was regarded as permanent embryo arrest, with characteristics similar to the 2-cell arrest observed in embryos from ordinary mice (Betts and Madan, 2008).

No changes in mitochondrial membrane potential and mitochondrial function in 2-cell arrested embryos

Mitochondrial function was also examined by measuring the respiration and ATP content of individual 2-cell embryos. Respiratory activity, as judged by oxygen consumption in a single embryo using the SECM measuring system, did not differ significantly between the embryos from wild-type and SOD1-deficient mouse oocytes at 36 h after IVF (Fig. 5A). Measurement of ATP content again showed no significant difference between embryos from the two genotypes under 1% and 20% O_2 cultures (Fig. 5B). Taken together, these data indicate that mitochondria were normal in embryos that were derived from the SOD1-deficient mouse oocytes and arrested at the 2-cell stage by incubation under 20% O_2. Since mitochondria are major organelles that produce ROS, and their malfunction is a proposed cause of 2-cell arrest (Liu *et al.*, 2000; Thouas *et al.*, 2004), we assessed $\Delta\Psi$m using cationic dye, JC-1, which is an important parameter of mitochondria function and has been used as an indicator of cell activity. At 36 h after IVF, putative J-aggregate fluorescence was thickly detected in the peri-cortical cytoplasm of the 2-cell stage embryos, and J-monomer fluorescence was distinctly detected in the peri-nuclear region. There was no difference in the distribution of both J-aggregate and J-monomer fluorescence between the two groups (Fig. 6A). The $\Delta\Psi$m ratio of J-aggregate to J-monomer in SOD1-deficient embryos seemed to be slightly higher than that in the embryos from wild-type, but no prominent difference existed between the two groups (Fig. 6B).

Differential injury of zygotes versus 4-cell embryos from SOD1-deficient mouse oocytes by atmospheric culture

The question of whether oxygen toxicity was found only at the 2-cell stage was then examined. First the embryos were developed to the 4-cell stage by incubation under hypoxic conditions for 42 h after IVF, then transferred to atmospheric oxygen conditions and incubated further. The 4-cell embryos from SOD1-deficient mouse oocytes developed to the morula stage, but could not develop into blastocysts (Table II). Since cellular degeneration was evident in embryos from SOD1-deficient mouse oocytes, the impaired embryos were further characterized by reaction with FITC-annexin V, Hoechst33 342 and PI. FITC-annexin V strongly stained the embryos from SOD1-deficient mouse oocytes (Fig. 7, 2a–2f), but only minimally stained the embryos from wild-type mouse oocytes (Fig. 7, 1a–1f). Staining with Hoechst33 342, which detects nuclear DNA in any cell, and PI, which detects only the nuclear DNA of cells with leaky membranes, indicated nuclear fragmentation in degenerated embryos from SOD1-deficient mouse oocytes. Some FITC-annexin V positive cells were stained with Hoechst33 342 but not with PI, which is consistent with apoptotic cell death. Thus, the 4-cell embryos from SOD1-deficient mouse oocytes that overcame 2-cell arrest were more severely damaged than 1-cell embryos (zygotes) by oxidative stress.

Discussion

Developmental arrest in *in vitro*-produced embryos is commonly observed in several strains of mice and other species (Betts and Madan, 2008). Among a variety of factors that cause such arrest, oxygen deteriorates the development of the 2-cell mouse embryo (Auerbach and Brinster, 1968). Oxidative stress due to elevated ROS is a likely cause of 2-cell arrest and has been extensively studied in relation to the quality of oocytes (Noda *et al.*, 1991; Tarin, 1996). In the case of developing human embryos, EDTA effectively supports embryonic development (Suzuki *et al.*, 1988; Jinno *et al.*, 1989). It plays the role of an antioxidant by chelating transition metal ions, which cause production of a hydroxyl radical via the

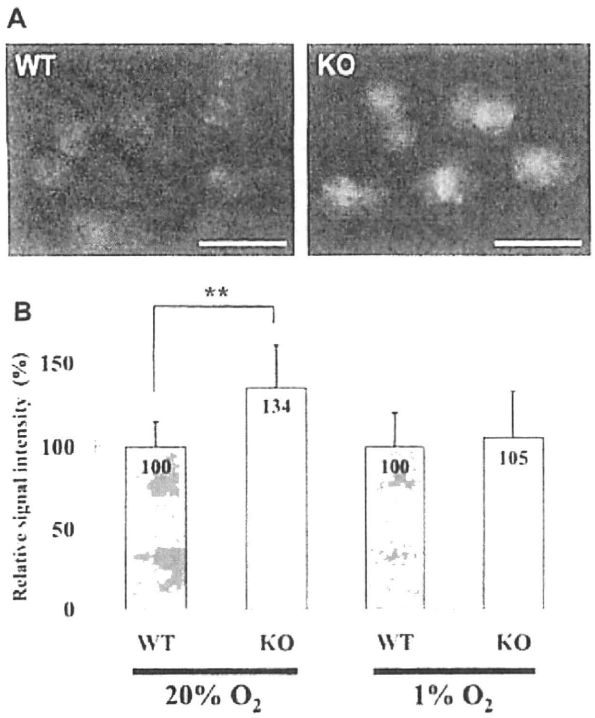

Figure 3 Fluorescent images and signal intensity of 2-cell embryos stained with dihydroethidium. (**A**) Two-cell embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes under 20% O_2 after dihydroethidium staining. Scale bars, 100 μm. (**B**) Embryos from wild-type (WT) and SOD1-deficient (KO) oocytes were cultured under 1% or 20% O_2 for 36 h after IVF. Then, superoxide in the individual 2-cell embryos was detected by dihydroethidium staining ($n = 14–17$). Signal intensity was shown as a relative value of averaged WT value into individual WT value or KO value in the same culture condition. Bars represent the mean ± SD of individual embryos. Differences were considered to be significant when $^{**}P < 0.01$. The numbers inside bars indicate the mean value.

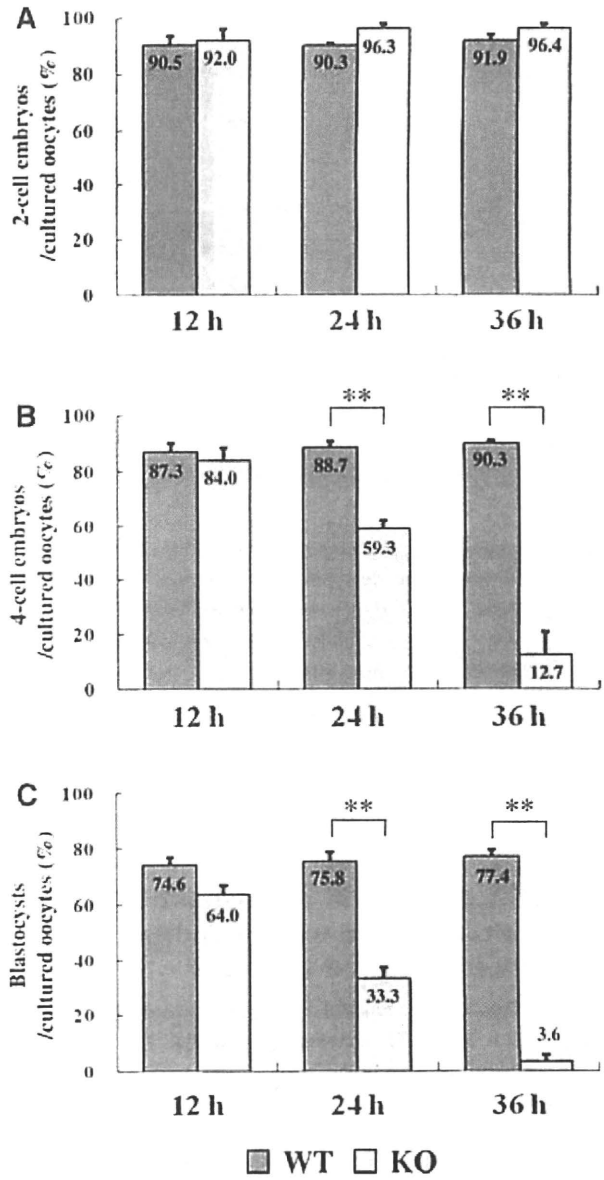

Figure 4 Time required for 2-cell arrest in oocytes from SOD1-deficient mice. Superovulated COCs from wild-type or SOD1-deficient mice were subjected to *in vitro* fertilization (IVF) and incubated under atmospheric oxygen conditions, and then transferred to 1% O_2 at 12, 24 and 36 h after IVF. (**A**) Fertilized oocytes were assessed as 2-cell embryos at 24 h after IVF. (**B**) The number of 4-cell embryos was counted at 48 h after IVF. (**C**) The number of blastocysts was counted after 4 days culture. Within each graph, the numbers inside the bars indicate the mean value. Numbers of each group embryo examined were 12 h-WT, $n = 63$; 12 h-KO, $n = 50$; 24 h-WT, $n = 62$; 24 h-KO, $n = 54$; 36 h-WT, $n = 62$; 12 h-KO, $n = 55$. Differences were considered to be significant when $^{**}P < 0.01$.

Fenton reaction. Experimental results provide direct evidence that supplementation of SOD protein and thioredoxin in the culture medium negates the arrest (Goto *et al.*, 1992; Nonogaki *et al.*, 1992). In this study, total 2-cell arrest was observed in embryos from SOD1-deficient mice oocytes under culture with atmospheric oxygen, but supplementation of SOD1 protein or β-mercaptoethanol was found to be ineffective (Table I).

In most studies regarding oxidative stress on embryonic development, hydrogen peroxides and other (pro) oxidants have been used to trigger oxidative stress extrinsically to embryos, and mitochondrial impairment has been observed (Liu *et al.*, 2000; Thouas *et al.*, 2004). In our study, a higher level of production for superoxide was detected in the prospective 2-cell arrest embryos from SOD1-deficient oocytes at 36 h after IVF, by dihydroethidium staining, suggesting that the 2-cell arrest was attributed to intrinsic oxidative stress caused by elevated superoxide due to SOD1 deficiency. Oocyte mitochondria consume ~50–70% of respired oxygen (Benos and Balaban, 1980), which is much less than most somatic cells, which consume more than 90%. Oxygenase present in oocytes consumes a portion of the remaining 20–30% oxygen (Balling *et al.*, 1985) and may be involved in superoxide production in the 2-cell embryos. Transient elevation of hydrogen peroxide, a

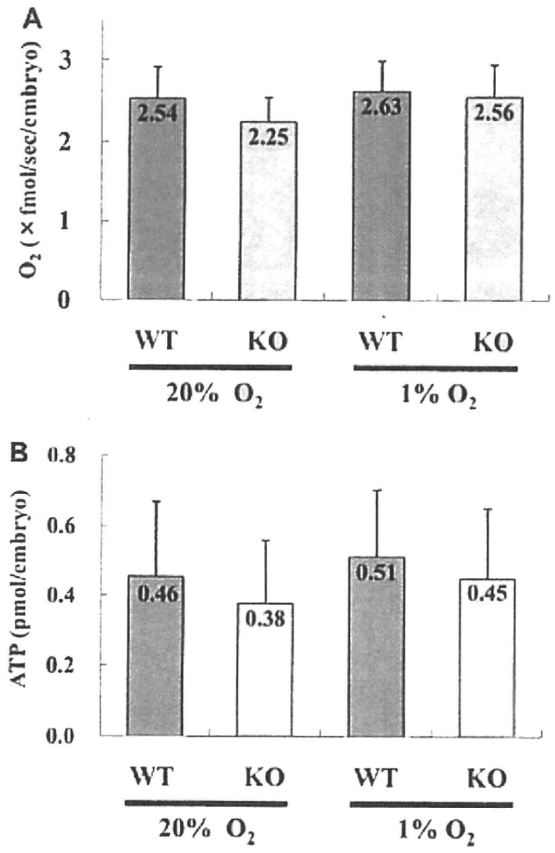

Figure 5 Respiratory activity and ATP levels of 2-cell embryos. (**A**) Embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes were cultured under 1% or 20% O_2 for 36 h after IVF, and oxygen consumption in the individual embryos was measured by an SECM measuring system ($n = 10-13$). (**B**) ATP content in the individual embryos treated under the same conditions as (A) was measured ($n = 22-25$). Within each graph, bars represent the mean ± SD of individual embryos. The numbers inside the bars indicate the mean value.

dismutation product of superoxide, is shown in mouse 2-cell stage embryos when developmental arrest occurs (Nasr-Esfahani *et al.*, 1990). In addition, immaturity of the mitochondrial electron transport system may also be responsible for ROS production. Although oxygen consumption remains relatively constant from the zygotes to the morula before increasing dramatically at the blastocyst stage (Houghton *et al.*, 1996; Thompson *et al.*, 1996), mitochondria remain immature in oocytes and early embryos (Van Blerkom, 2004). This immaturity of the mitochondrial electron transfer system would cause inappropriate electron transfer to molecular oxygen instead of cytochrome c oxidase (complex IV), and result in the production of superoxide. Superoxide originating from these sources would remain high in SOD1-deficient embryos, resulting in oxidative stress that consequently causes 2-cell arrest. Because superoxide itself is not very reactive, how the elevated superoxide causes 2-cell arrest is unclear. Although nitric oxide (NO) plays a pivotal role in oocyte activation at fertilization and also in embryogenesis (Kuo *et al.*, 2000; Nishikimi *et al.*, 2001; Tranguch *et al.*, 2003), superoxide reacts rapidly with NO to generate peroxynitrite, which is a highly reactive molecule and oxidizes lipids, proteins and nucleic acids. Thus, elevation of superoxide due to SOD1 deficiency would eliminate the important signaling molecule NO and convert it to the harmful oxidant peroxynitrite.

Mitochondria lack histone and possess weak DNA-repairing ability, which makes mitochondrial DNA vulnerable to oxidative modification. In *in vivo* studies, aging increases oxidative damage in mitochondria, and the incidence of mitochondrial DNA mutations increases in human ovarian tissues after the age of 45 (Kitagawa *et al.*,1993) and in fertilized oocytes collected from IVF patients at ages above 38 (Keefe *et al.*,1995). Morphological abnormalities in oocyte mitochondria from old mice were also observed (Tarin *et al.*, 2001); thus, mitochondrial damage by ROS produced over long periods may be a mechanism leading to age-related decline in oocyte quality and chromosome aneuploidy (Tarin, 1996; Tarin *et al.*, 1998, 2000), as further supported by *in vitro* studies. Treatment of mouse zygotes with 200 μM hydrogen peroxide for 15 min causes a decline in mitochondrial membrane potential and mitochondrial malfunction (Liu *et al.*, 2000). Since developmental arrest occurs after this treatment, it can be concluded that mitochondrial malfunction contributes to cell-cycle arrest. Similar results are obtained when oxidation of mitochondria is induced by photosensitization using mitochondria-specific sensitizing dye (Thouas *et al.*, 2004). In many studies in which oxidative stress is induced by extracellularly administered ROS or prooxidants, the abnormal development of embryos is attributed to energy depletion by mitochondrial malfunction or induction of apoptosis by cytochrome c released from mitochondria. Low mitochondrial ATP content has been correlated with reduced developmental competence and post-implantation outcomes (Van Blerkom *et al.*, 1995). In the present study, respiratory activity, ATP content and $\Delta\Psi m$ of 2-cell embryos were examined at 36 h after IVF because embryos from wild-type mouse oocytes, regarded as the control embryos, developed further after 36 h. Most of the SOD1-deficient embryos incubated under 20% O_2 for 36 h were unable to develop further (Fig. 2), which indicated that this developmental arrest arose during 36 h incubation under 20% O_2. However, there was no evident difference in mitochondrial function between the wild-type embryos and the 2-cell arrested embryos from SOD1-deficient mouse oocytes at 36 h after IVF (Figs 5 and 6). Acton *et al.* (2004) showed that $\Delta\Psi m$ tend to increase slightly in the complete 2-cell arrest embryos at 68 h post-hCG compared with that in normal 2-cell embryos at 44 h post-hCG, when ICR zygotes were cultured in HTF. Taken together, these data on SOD1-deficient embryos suggest that mitochondrial malfunction may not play a major role in the developmental arrest of SOD1-deficient embryos. Trimarchi *et al.* (2000) have examined oxygen consumption of individual embryos and have shown that healthy embryos consume more oxygen than those undergoing cell death. On the basis of their criteria, the 2-cell embryos obtained from SOD1-deficient mice under hypoxic and atmospheric oxygen culture can be considered healthy. Therefore, we concluded that the 2-cell arrest observed in SOD1-deficient embryos is not due to either a defective energy supply or to cell death by mitochondrial malfunction.

The mechanism that causes 2-cell arrest of developing embryos from SOD1-deficient mice is yet unclear. It was observed that

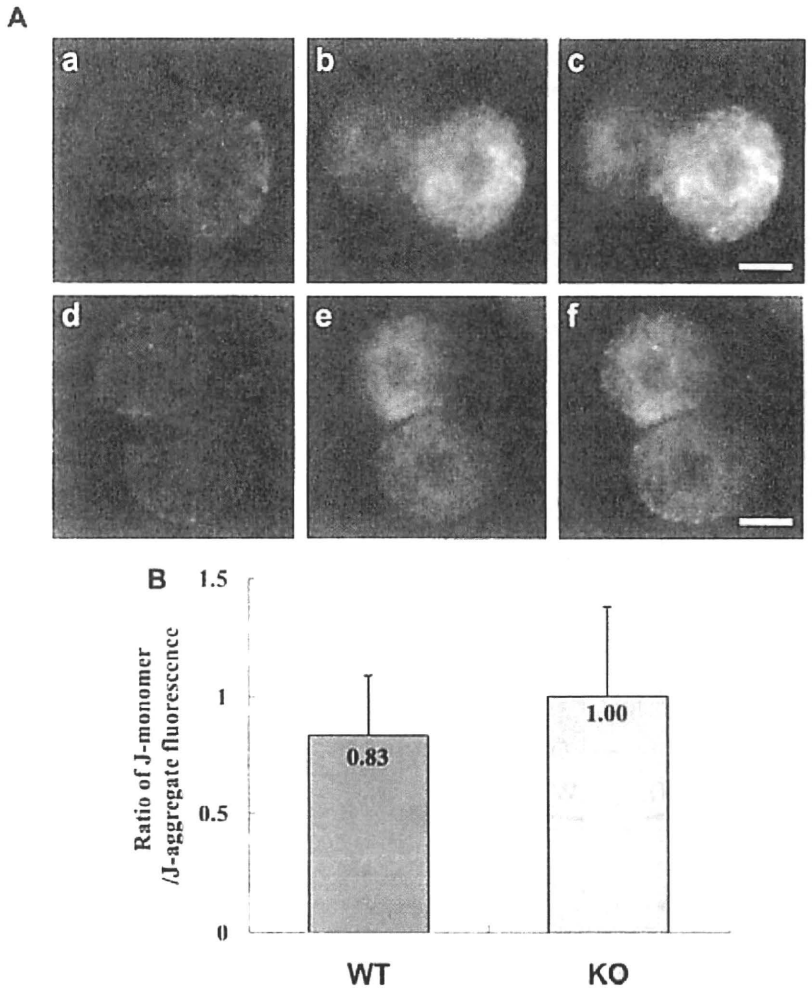

Figure 6 Fluorescent images and mitochondrial membrane potential of 2-cell embryos stained with JC-1. (**A**) Fluorescent images of JC-1-stained 2-cell embryos from wild-type (a–c) or SOD1-deficient (d–f) mouse oocytes under 20% O_2. (a and d) J-aggregate; (b and e) J-monomer; (c and f) Merge. Scale bars, 20 μm. (**B**) The ratio of J-aggregate to J-monomer fluorescence for individual embryo using JC-1 staining in embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes cultured under 20% O_2 for 36 h after IVF ($n = 15–20$). Bars represent the mean ± SD of individual embryos. The numbers inside the bars indicate the mean value.

Table II Effect of converted culture from 1% to 20% oxygen on the development of 4-cell embryos from SOD1-deficient (KO) mouse oocytes.

Oocyte genotype	Number of oocytes cultured	Number of embryos (%)			
		2-cell ≤ at Day 1	4-cell ≤ at Day 2	Morula ≤ at Day 3	Blastocyst at Day 4
WT	54	50 (92.6)	48 (88.9)	44 (81.5)	39 (72.2)
KO	59	56 (94.9)	55 (93.2)	39 (66.1)	0 (0)**

WT, wild-type. Differences within each day were significant when **$P < 0.01$.

embryonic cells were alive but permanently arrested, resembling cellular senescence. Knockdown of SOD1 by employing small interfering RNA actually induced senescence in human fibroblasts (Blander *et al.*, 2003). Regarding mouse embryos, defective $p34^{cdc2}$ kinase, a key regulator of the cell cycle, in 2-cell arrest and its restoration by SOD and thioredoxin, has been reported (Natsuyama *et al.*, 1993). Moreover, M-phase promoting factor and mitogen-activated protein kinase, whose activation constitutes a mitotic signal pathway, are

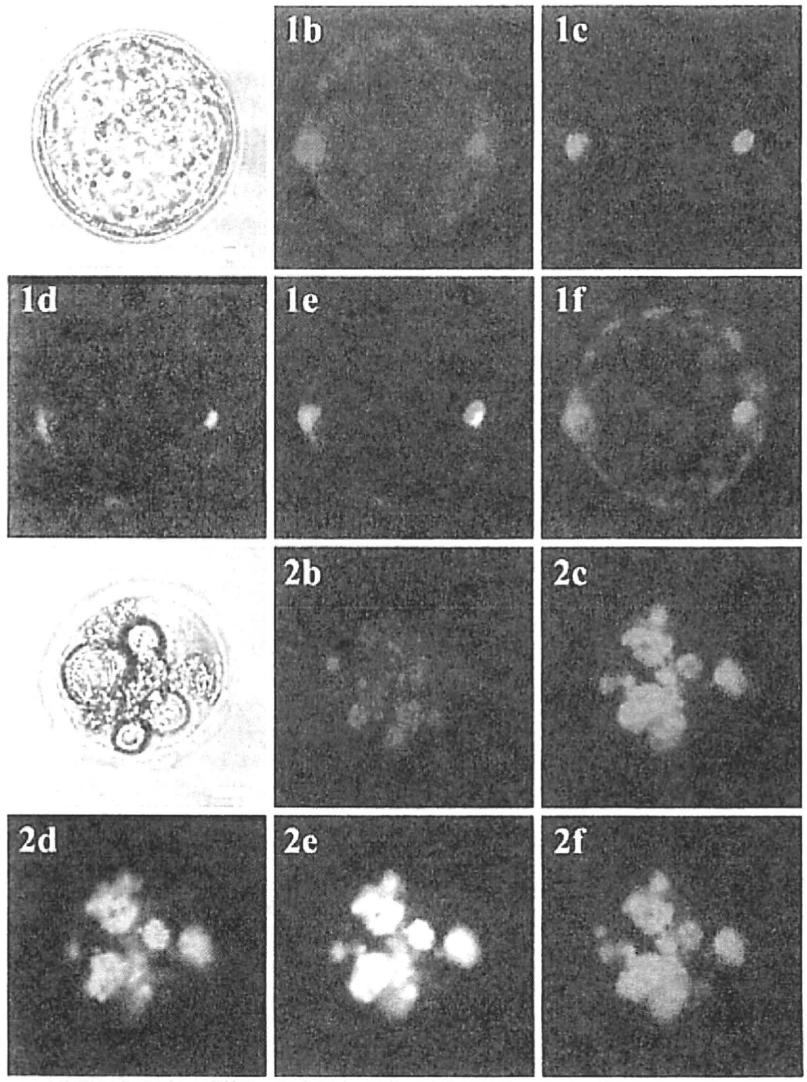

Figure 7 Cytological examination of embryos with abnormality. Superovulated COCs from wild-type mice (1a–1f) or SOD1-deficient mice (2a–2f) were subjected to IVF and incubated for 42 h after IVF under 1% O_2. Four-cell embryos were then transferred to atmospheric oxygen conditions and incubated further. At 4 days after IVF, embryos were stained with Hoechst 33342 (1b and 2b), PI (1c and 2c) and FITC-annexin V (1d and 2d) and were observed under a fluorescent microscope. Merged pictures between PI + FITC-annexin V (1e and 2e) and Hoechst33342 + PI (1f and 2f) are also shown. Scale bars, 20 μm.

down-regulated in oocytes ovulated from aged mice (Tatone *et al.*, 2006). On the other hand, the involvement of $p66^{Shc}$, a protein belonging to the Shc family of adaptors for signal transduction in mitogenic and apoptotic responses, in permanent embryo arrest in bovines has been proposed as a cause of oxidative stress (Favetta *et al.*, 2007; Betts and Madan, 2008). If this is the case, expression of a responsible gene(s) is essential to arrest the cell cycle. From this viewpoint, the stage of developmental arrest corresponds to the point when zygotic gene activation occurs in mouse embryos at the late 1- to 2-cell stages (Telford *et al.*, 1990; Schultz, 1993). When embryos from SOD1-deficient oocytes were developed to four cells under 1% O_2 and transferred to 20% O_2, embryos developed to the morula stage but degenerated thereafter (Table II, Fig. 7). In our preliminary experiment, when *in vivo*-developed 2-cell embryos were collected from SOD1-deficient oocytes and cultured for 3 days under 20% O_2, most of the embryos (24 of 26 embryos) arrested at the 3- to 4-cell stage. These results indicate that (i) the developmental arrest of SOD1-deficient embryos was caused by oxidative stress around the 2-cell stage regardless of IVF or *in vivo* fertilization and (ii) the oxygen exerted deteriorating effects on embryos in a different manner, depending on the developmental stage. Impaired regulation of the cell cycle would be responsible for 2-cell arrest by oxidative stress, while a mechanism for apoptosis appears to be involved in the degeneration of SOD1-deficient embryos at a later stage. Since mitochondria play pivotal roles in the initiation of apoptosis, the mitochondrial maturation process may be involved in the

differential damage to the oocytes/zygotes and 4-cell embryos. One of the special features in SOD1-deficient mice is female infertility (Ho *et al.*, 1998; Matzuk *et al.*, 1998). In this study, 30 oocytes per female were recovered from SOD1-deficient mice after superovulation, and 29.5 oocytes were recovered from wild-type, which implies no difference in the ovulation rate between the two genotypes. We also observed a number of *in vivo*-developed 2-cell embryos from SOD1-deficient mice at 36 h after superovulation and *in vivo*-developed morula and blastocyst 6 embryos at 84–96 h after superovulation (data not shown). At the least, this evidence could ensure *in vivo*-developmental ability of preimplantation embryos in SOD1-deficient female.

In conclusion, we found total 2-cell arrest of embryos from SOD1-deficient mice oocytes in culture under atmospheric oxygen conditions and the negation of the arrest by incubation under hypoxic conditions. Since the mitochondrial function of ATP production via oxidative phosphorylation was almost normal in the embryos, the mechanism of cell cycle regulation might be a target of elevated ROS. After the 4-cell stage, however, embryos suffering from oxidative stress undergo degeneration, most likely by apoptosis. Thus, investigation of embryos developed from SOD1-deficient oocytes would provide a useful clue to an understanding of the mechanisms involved in 2-cell arrest and cellular degeneration at the later developmental stage by intrinsic oxidative stress.

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References

Abe H. A non-invasive and sensitive method for measuring cellular respiration with a scanning electrochemical microscopy to evaluate embryo quality. *J Mamm Ova Res* 2007;**24**:70–78.

Acton BM, Jurisicova A, Jurisica I, Casper RF. Alterations in mitochondrial membrane potential during preimplantation stages of mouse and human embryo development. *Mol Hum Reprod* 2004;**10**:23–32.

Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. *Antioxid Redox Signal* 2008; **10**:1375–1403.

Aitken RJ, Baker MA. Oxidative stress, sperm survival and fertility control. *Mol Cell Endocrinol* 2006;**250**:66–69.

Auerbach S, Brinster RL. Effect of oxygen concentration on the development of two-cell mouse embryos. *Nature* 1968;**217**: 465–466.

Balling R, Haaf H, Maydl R, Metzler M, Beier HM. Oxidative and conjugative metabolism of diethylstilbestrol by rabbit preimplantation embryos. *Dev Biol* 1985;**109**:370–374.

Benos DJ, Balaban RS. Energy requirements of the developing mammalian blastocyst for active ion transport. *Biol Reprod* 1980;**23**:941–947.

Betts DH, Madan P. Permanent embryo arrest: molecular and cellular concepts. *Mol Hum Reprod* 2008;**14**:445–453.

Blander G, de Oliveira RM, Conboy CM, Haigis M, Guarente L. Superoxide dismutase 1 knock-down induces senescence in human fibroblasts. *J Biol Chem* 2003;**278**:38966–38969.

Chatot CL, Lewis JL, Torres I, Ziomek CA. Development of 1-cell embryos from different strains of mice in CZB medium. *Biol Reprod* 1990;**42**:432–440.

Erbach GT, Lawitts JA, Papaioannou VE, Biggers JD. Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol Reprod* 1994;**50**:1027–1033.

Favetta LA, St John EJ, King WA, Betts DH. High levels of p66shc and intracellular ROS in permanently arrested early embryos. *Free Radic Biol Med* 2007;**42**:1201–1210.

Feugang JM, de Roover R, Moens A, Léonard S, Dessy F, Donnay I. Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. *Theriogenology* 2004;**61**:71–90.

Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 1993;**99**:673–679.

Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 1995;**64**:97–112.

Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reprod Biol Endocrinol* 2005;**3**:43.

Goto Y, Noda Y, Narimoto K, Umaoka Y, Mori T. Oxidative stress on mouse embryo development in vitro. *Free Radic Biol Med* 1992;**13**:47–53.

Halliwell B, Gutteridge JMC. *Free Radical in Biology and Medicine*, 3rd edn. Oxford: Clarendon Press, 1999, 53–55.

Ho YS, Gargano M, Cao J, Bronson RT, Heimler I, Hutz RJ. Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *J Biol Chem* 1998;**273**:7765–7769.

Houghton FD, Thompson JG, Kennedy CJ, Leese HJ. Oxygen consumption and energy metabolism of the early mouse embryo. *Mol Reprod Dev* 1996;**44**:476–485.

Ishii T, Matsuki S, Iuchi Y, Okada F, Toyosaki S, Tomita Y, Ikeda Y, Fujii J. Accelerated impairment of spermatogenic cells in SOD1-knockout mice under heat stress. *Free Radic Res* 2005;**39**:697–705.

Iuchi Y, Okada F, Onuma K, Onoda T, Asao H, Kobayashi M, Fujii J. Elevated oxidative stress in erythrocytes due to an SOD1 deficiency causes anemia and triggers autoantibody production. *Biochem J* 2007; **402**:219–227.

Jinno M, Sandow BA, Hodgen GD. Enhancement of the developmental potential of mouse oocytes matured in vitro by gonadotropins and ethylenediaminetetraacetic acid (EDTA). *J In Vitro Fert Embryo Transf* 1989;**6**:36–40.

Jurisicova A, Acton BM. Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. *Reproduction* 2004;**128**:281–291.

Keefe DL, Niven-Fairchild T, Powell S, Buradagunta S. Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil Steril* 1995;**64**:577–583.

Kitagawa T, Suganuma N, Nawa A, Kikkawa F, Tanaka M, Ozawa T, Tomoda Y. Rapid accumulation of deleted mitochondrial deoxyribonucleic acid in postmenopausal ovaries. *Biol Reprod* 1993; **49**:730–736.

Kuo RC, Baxter GT, Thompson SH, Stricker SA, Patton C, Bonaventura J, Epel D. NO is necessary and sufficient for egg activation at fertilization. *Nature* 2000;**406**:633–636.

Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH *et al.* Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 1995;**11**:376–381.

Liu L, Trimarchi JR, Keefe DL. Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes. *Biol Reprod* 2000; **62**:1745–1753.

Loutradis D, Drakakis P, Kallianidis K, Sofikitis N, Kaltipolitis G, Milingos S, Makris N, Michalas S. Biological factors in culture media affecting in vitro fertilization, preimplantation embryo development, and implantation. *Ann N Y Acad Sci* 2000;**900**:325–335.

Matzuk MM, Dionne L, Guo Q, Kumar TR, Lebovitz RM. Ovarian function in superoxide dismutase 1 and 2 knockout mice. *Endocrinology* 1998; **139**:4008–4011.

Nasr-Esfahani MH, Aitken JR, Johnson MH. Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. *Development* 1990;**109**:501–507.

Natsuyama S, Noda Y, Yamashita M, Nagahama Y, Mori T. Superoxide dismutase and thioredoxin restore defective p34cdc2 kinase activation in mouse two-cell block. *Biochim Biophys Acta* 1993;**1176**: 90–94.

Nishikimi A, Matsukawa T, Hoshino K, Ikeda S, Kira Y, Sato EF, Inoue M, Yamada M. Localization of nitric oxide synthase activity in unfertilized oocytes and fertilized embryos during preimplantation development in mice. *Reproduction* 2001;**122**:957–963.

Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T. Involvement of superoxide radicals in the mouse two-cell block. *Mol Reprod Dev* 1991;**28**:356–360.

Nonogaki T, Noda Y, Narimoto K, Umaoka Y, Mori T. Effects of superoxide dismutase on mouse in vitro fertilization and embryo culture system. *J Assist Reprod Genet* 1992;**9**:274–280.

Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril* 1985;**44**:493–498.

Ruder EH, Hartman TJ, Blumberg J, Goldman MB. Oxidative stress and antioxidants: exposure and impact on female fertility. *Hum Reprod Update* 2008;**14**:345–357.

Schultz RM. Regulation of zygotic gene activation in the mouse. *BioEssays* 1993;**15**:531–538.

Shiku H, Shiraishi T, Ohya H, Matsue T, Abe H, Hoshi H, Kobayashi M. Oxygen consumption of single bovine embryos probed by scanning electrochemical microscopy. *Anal Chem* 2001;**73**: 3751–3758.

Shiku H, Shiraishi T, Aoyagi S, Utsumi Y, Matsudaira M, Abe H, Hoshi H, Kasai S, Ohya H, Matsue T. Respiration activity of single bovine embryos entrapped in a cone-shaped microwell monitored by scanning electrochemical microscopy. *Anal Biochim Acta* 2004;**522**: 51–58.

Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Hum Reprod Update* 2003;**9**:557–582.

Suzuki S, Komatsu S, Kitai H, Endo Y, Iizuka R, Fukasawa T. Analysis of cytoplasmic factors in developmental cleavage of mouse embryo. *Cell Differ* 1988;**24**:133–138.

Suzuki K, Tatsumi H, Satoh S, Senda T, Nakata T, Fujii J, Taniguchi N. Manganese-superoxide dismutase in endothelial cells: localization and mechanism of induction. *Am J Physiol* 1993;**265**:H1173–H1178.

Takahashi M, Keicho K, Takahashi H, Ogawa H, Schultz RM, Okano A. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology* 2000;**54**:137–145.

Tarin JJ. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol Hum Reprod* 1996;**2**:717–724.

Tarin JJ, Vendrell FJ, Ten J, Cano A. Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. *Mol Hum Reprod* 1998;**4**:281–288.

Tarin JJ, Perez-Albala S, Cano A. Consequences on offspring of abnormal function in ageing gametes. *Hum Reprod Update* 2000;**6**:532–549.

Tarin JJ, Perez-Albala S, Cano A. Cellular and morphological traits of oocytes retrieved from aging mice after exogenous ovarian stimulation. *Biol Reprod* 2001;**65**:141–150.

Tatone C, Carbone MC, Gallo R, Delle Monache S, Di Cola M, Alesse E, Amicarelli F. Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. *Biol Reprod* 2006;**74**:395–402.

Telford NA, Watson AJ, Schultz GA. Transcription from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* 1990;**26**:90–100.

Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil* 1996;**106**:299–306.

Thouas GA, Trounson AO, Wolvetang EJ, Jones GM. Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest in vitro. *Biol Reprod* 2004;**71**:1936–1942.

Tranguch S, Steuerwald N, Huet-Hudson YM. Nitric oxide synthase production and nitric oxide regulation of preimplantation embryo development. *Biol Reprod* 2003;**68**:1538–1544.

Trimarchi JR, Liu L, Porterfield DM, Smith PJ, Keefe DL. Oxidative phosphorylation-dependent and independent oxygen consumption by individual preimplantation mouse embryos. *Biol Reprod* 2000;**62**:1866–1874.

Valentine JS, Doucette PA, Potter SZ. Copper–zinc superoxide dismutase and amyotrophic lateral sclerosis. *Annu Rev Biochem* 2005;**74**:563–593.

Van Blerkom J. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. *Reproduction* 2004;**128**:269–280.

Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod* 1995;**10**:415–424.

Wilhelm J, Vytásek R, Ostádalová I, Vajner L. Evaluation of different methods detecting intracellular generation of free radicals. *Mol Cell Biochem* 2009;**328**:167–176.

電気化学計測技術を応用したシングルセル呼吸機能解析と応用

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5　電気化学計測技術を応用したシングルセル呼吸機能解析と応用

阿部宏之*

5.1　はじめに

ミトコンドリアは生命活動に必要なエネルギーの生産やアポトーシスなど重要な生物現象に関与しており，その機能が障害されると代謝異常や種々の疾患の原因となる。また，ミトコンドリアの呼吸活性は細胞の代謝活動や機能評価の有効な指標となることから，シングルセルレベルでの高精度呼吸計測技術は，細胞のクオリティー評価や疾患の診断などに極めて有効な手段となる。本節では，局所領域における生体反応をリアルタイムで追跡できる電気化学計測技術を応用した高精度・非侵襲細胞呼吸計測システムを解説するとともに，この技術を応用したシングルセルレベルでの呼吸機能解析や細胞間ネットワーク解析，受精卵クオリティー評価への応用研究を紹介する。

5.2　マイクロ電極を用いた細胞呼吸測定装置

電気化学計測法はプローブ電極による酸化還元反応を利用し，局所領域における生物反応を電気化学的に検出する技術であり[1,2]，この技術の有効な装置としてマイクロ電極を探針とする走査型電気化学顕微鏡（Scanning electrochemical microscopy：SECM）が注目されている。SECMの空間分解能は探針であるマイクロ電極径に依存するため原子や分子レベルの解析は困難であるが，局所空間での化学反応の評価やイメージング，生体材料を用いたリアルタイム解析や化学反応誘起が可能であることから，局所領域の電気化学センシングなど種々の系で用いられている[3-6]。例えば，酸素の還元電位を検出できるマイクロ電極を用いることで細胞の酸素消費量（呼吸）を高感度・非侵襲的にリアルタイムで測定することができる（図1）。

従来，SECMは微量な酸素消費を検出できることから，金属錆の検出装置として用いられてきた。筆者らは，従来型SECMを生物試料，特に受精卵の呼吸計測に応用するために，呼吸測定に関連した要素技術の開発を行ってきた。その結果，SECMをベースに受精卵や微小組織などの球状試料の酸素消費量を非侵襲的に測定できる「受精卵呼吸測定装置」の開発に成功した[7]。この呼吸測定システムは，倒立型顕微鏡，マイクロ電極の電位を一定に保持するポテンショスタット，マイクロ電極の移動を制御するコントローラー，酸素消費量算出のための専用解析ソフトを内蔵したノート型コンピューターにより構成されている（図2）。倒立型顕微鏡のステージ上には，マイクロ電極の3次元走査を可能とするXYZステージが設置されており，生物試料の呼吸計測のために気相条件制御が可能な測定用チャンバーや保温プレートが設置できる。また，計測

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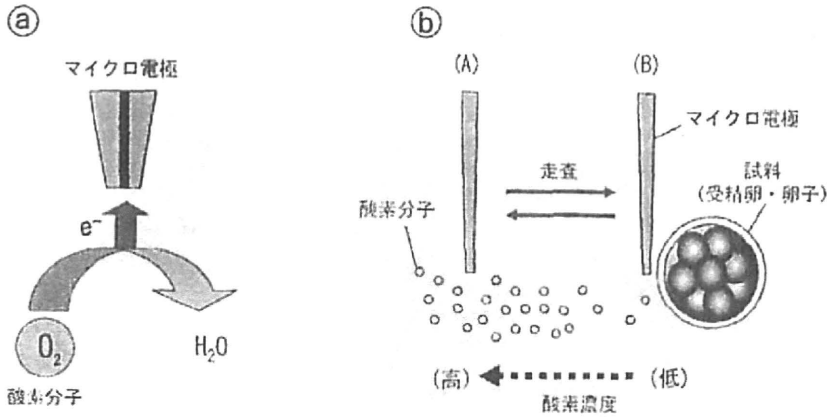


図1　マイクロ電極を用いた受精卵呼吸測定法
ⓐマイクロ電極は酸素の還元電位を検出する。
ⓑ走査型電気化学顕微鏡による呼吸測定。呼吸により胚近傍の溶存酸素が減少するため，沖合との間に溶存酸素の濃度勾配が生じる。その酸素濃度差（電流値の差：ΔC）から球面拡散理論式[8]を用いて試料の酸素消費量を算出する。

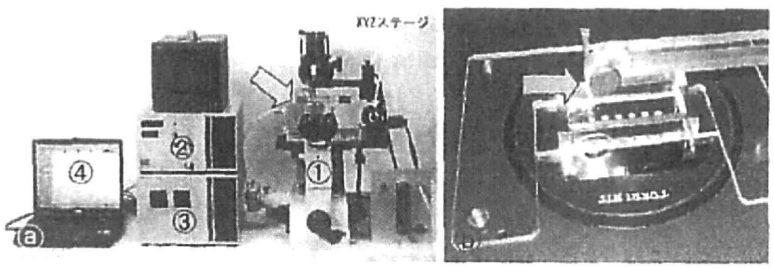


図2　走査型電気化学顕微鏡を改良した「受精卵呼吸測定装置」（北斗電工㈱製）
①倒立型顕微鏡，②ポテンショスタット，③コントローラー，④ノートパソコン（呼吸解析ソフトを内蔵）。矢印は，専用の測定チャンバーを示す。
ⓑ測定ステージ部。矢印は，ホルダーに設置したマイクロ電極を示す。

精度と操作性の向上を目的に，マイクロ電極の改良と専用の多検体測定プレートおよび測定液の開発を行っている。従来型SECMは，金属などの腐食部位を検出する装置として先端径10～20 μmのマイクロ電極を探針として用いているが，酸素消費量が非常に小さい細胞や受精卵の呼吸測定には電極の感度が不十分であるため，より高感度のマイクロ電極が必要である。マイクロ電極の計測感度は先端径が小さいほど高いため，受精卵や細胞の呼吸測定では先端径2～4 μmにエッチング加工した白金電極をガラスキャピラリー先端部に封止したディスク型マイクロ電極（図3ⓐ，ⓑ）を使用している。

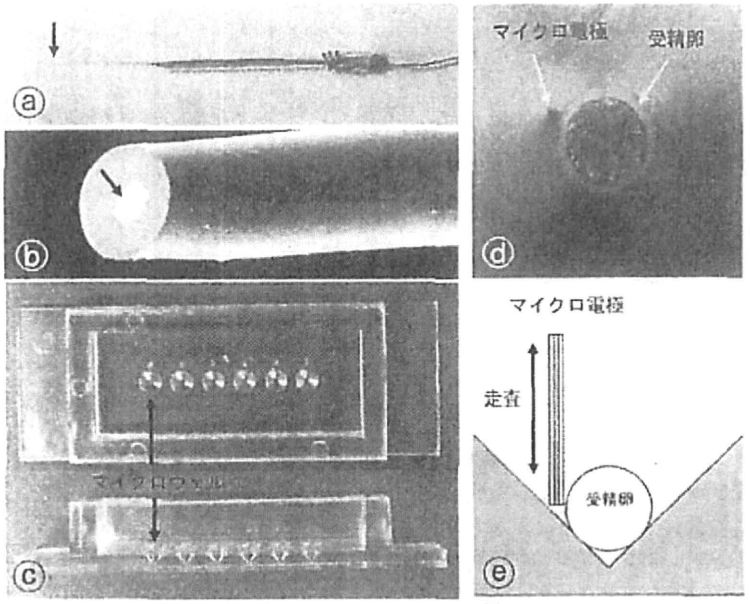


図３　受精卵呼吸測定用のマイクロ電極ⓐと多検体測定プレートⓒ
ⓐディスク型白金マイクロ電極。
ⓑマイクロ電極の走査電子顕微鏡像。先端部（矢印）が直径２～４μmにエッチング加工された白金電極がガラスキャピラリーに封止されている。
ⓒ多検体プレート底面には円錐形のマイクロウェルが６穴施されている。
ⓓマイクロウェル底部に静置したウシ胚。
ⓔマイクロ電極を胚に対して鉛直方向に走査し酸素消費量を測定する。

5.3　単一受精卵の呼吸量測定

「受精卵呼吸測定装置」を用いた呼吸測定には，専用の多検体測定プレートと測定液を用いる。多検体測定プレートは測定操作の簡易化を目的に開発され，プレートの底面には逆円錐形のマイクロウェルが施されている（図３ⓒ）。マイクロ電極が検出する微弱な電流は溶液の成分によって影響を受けるため，呼吸量測定にはマイクロ電極の測定感度に影響を与えず，胚に対して無侵襲な専用の測定液を用いる。測定液を満たしたマイクロウェル内に試料（胚）を導入した後，マイクロウェルの底部中心に静置させる（図３ⓓ）。胚のサイズ（半径値）を解析ソフトに入力した後，マイクロ電極を胚の透明帯直近に手動で移動する。マイクロ電極は酸素が還元可能な−0.6 V *vs.* Ag/AgClに電位を保持した後，移動速度20～30 μm/sec，走査距離150～300 μmの条件に設定し，コンピューター制御により透明帯近傍を鉛直方向に自動的に走査する（図３ⓔ）。通常，１回の呼吸測定ではマイクロ電極を２～３回走査した後，胚の酸素消費量は球面拡散理論式[8,9]を基本に開発した解析ソフトを用いて算出する。

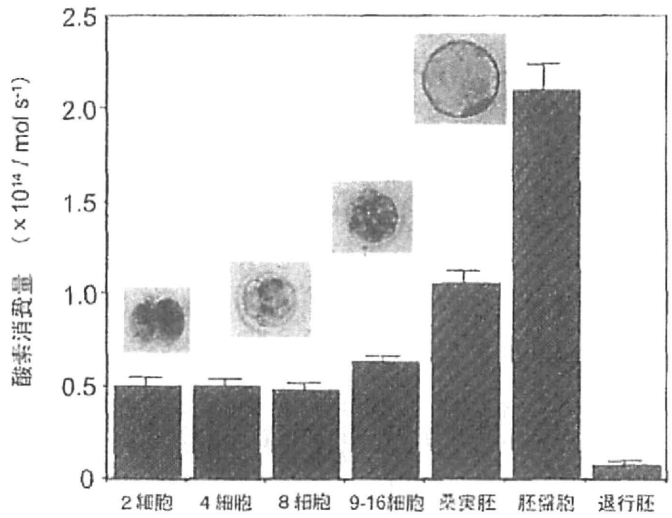


図4　ウシ体外受精胚の発生過程における呼吸量変化
桑実胚から胚盤胞期にかけて呼吸量が増加する。退行胚ではほとんど呼吸量は検出されない。

これまでに「受精卵呼吸測定装置」を用いて，ウシ，ブタ，マウスの単一胚の呼吸量測定を行っている。ほとんどの動物胚において，８細胞期までの発生初期では酸素消費量は少なく，桑実胚から胚盤胞にかけて顕著に呼吸量が増加する（図４）。呼吸測定の有用性を評価するために呼吸能とミトコンドリアの関係を調べた結果，呼吸活性の低い８細胞期まではほとんどのミトコンドリアは未成熟であるが，桑実胚から胚盤胞にかけてミトコンドリアの顕著な発達（クリステの拡張）が認められた[10]。このように，ミトコンドリアの発達と呼吸量の増加が一致して起こることから，「受精卵呼吸測定装置」はミトコンドリア呼吸を高精度でモニタできることがわかる。

5.4　呼吸測定による受精卵の品質評価

体外受精・胚移植技術は，不妊治療の最も有効な治療法となっている。不妊治療では，質的に良好な胚の選択は妊娠率向上，多胎妊娠回避，流産率低下のために不可欠である。現在，胚の品質は割球の数や形態を基準に評価されているが，このような形態的特徴は定量性に欠けるため判定基準が観察者の主観に左右される可能性が指摘されている。筆者らは，形態良好胚ではミトコンドリアは正常に発達しているが，形態不良胚や発生停止胚ではミトコンドリアの多くは未成熟あるいは退行していることを発見した[11-13]。そこで，ミトコンドリア呼吸活性を指標に受精卵の品質を評価するという独創的発想に至り，「受精卵呼吸測定装置」を用いた精度の高いシングルセルレベルでの細胞呼吸計測システム開発を進めてきた。これまでに，ウシを用いた動物実験によって，呼吸活性の高い胚は呼吸測定後に追加培養を行うと高い確率でクオリティ・良好な胚

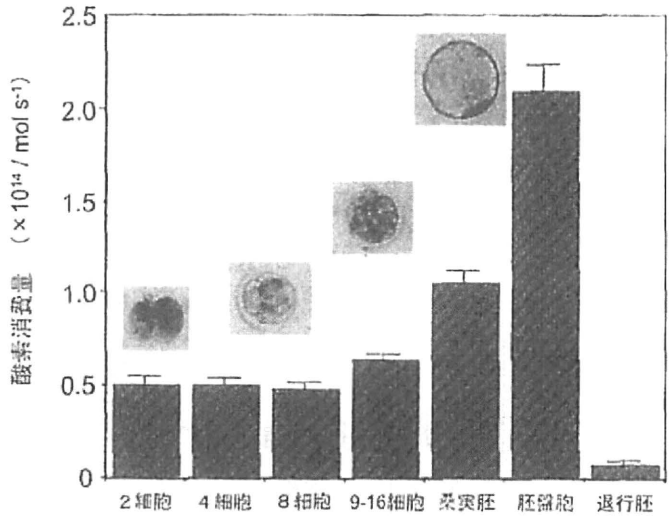
表1　ウシ胚の呼吸量と妊娠率の関係[9]

移植時の発生ステージ	酸素消費量（$F\times10^{14}$/mol s^{-1}）	受胎胚数/移植胚数（妊娠率%）
胚盤胞	$F\geq1.0$	21/36 (58.3)
	$F<1.0$	0/6 (0)
初期胚盤胞	$F\geq0.8$	16/25 (64.0)
	$F<0.8$	0/6 (0)
桑実胚	$F\geq0.5$	17/28 (60.7)
	$F<0.5$	1/12 (8.3)

盤胞へと発生すること[14]，凍結時に呼吸量の大きい胚盤胞は融解した後の生存率も良好であることを明らかにしている[15]。さらに，呼吸測定後の胚を借腹牛に移植し胚の呼吸活性と受胎率の関係を調べた結果，移植前の呼吸量が基準値以上（胚盤胞で1.0×10^{14}/mol s^{-1}，初期胚盤胞で0.8×10^{14}/mol s^{-1}，桑実胚で0.5×10^{14}/mol s^{-1}）の胚を移植した場合，60%以上の高い確率で妊娠するが，基準値に満たない胚のほとんどは受胎しないことが確認されている（表1）。このように，「受精卵呼吸測定装置」を用いた呼吸測定は，妊娠が期待できる品質良好胚の有効な選別法になると考えられる。

5.5　呼吸測定システムの医療応用

「受精卵呼吸測定装置」は，短時間で非侵襲的に細胞の呼吸量を測定できることから，不妊治療や移植医療における受精卵や微小組織のクオリティー評価への応用が可能である。ウシやマウスを用いた動物実験により，呼吸測定した胚を移植し誕生した個体の染色体や行動などを調べているが，染色体異常や奇形，行動異常などの事例は確認されていない。電気化学呼吸計測技術は，医療応用へ向けて安全面での問題もクリアできると考えられる。現在，探索的臨床研究として「受精卵呼吸測定装置」を用いたヒト胚の呼吸量測定と品質評価システムの確立を試みている。これまでに，ヒト胚（余剰胚）ではミトコンドリアの発達に伴い呼吸量が増加すること（表2，図5），呼吸活性の高い胚は胚盤胞への発生率が高い傾向にあることが示されている[16]。これらの研究成果を踏まえ所定の倫理承認を得た後，不妊治療への臨床応用を目的に呼吸測定胚の移植試験を予定している。



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これまでに「受精卵呼吸測定装置」を用いて，ウシ，ブタ，マウスの単一胚の呼吸量測定を行っている。ほとんどの動物胚において，8細胞期までの発生初期では酸素消費量は少なく，桑実胚から胚盤胞にかけて顕著に呼吸量が増加する（図4）。呼吸測定の有用性を評価するために呼吸能とミトコンドリアの関係を調べた結果，呼吸活性の低い8細胞期まではほとんどのミトコンドリアは未成熟であるが，桑実胚から胚盤胞にかけてミトコンドリアの顕著な発達（クリステの拡張）が認められた[10]。このように，ミトコンドリアの発達と呼吸量の増加が一致して起こることから，「受精卵呼吸測定装置」はミトコンドリア呼吸を高精度でモニタできることがわかる。

5.4　呼吸測定による受精卵の品質評価

体外受精・胚移植技術は，不妊治療の最も有効な治療法となっている。不妊治療では，質的に良好な胚の選択は妊娠率向上，多胎妊娠回避，流産率低下のために不可欠である。現在，胚の品質は割球の数や形態を基準に評価されているが，このような形態的特徴は定量性に欠けるため判定基準が観察者の主観に左右される可能性が指摘されている。筆者らは，形態良好胚ではミトコンドリアは正常に発達しているが，形態不良胚や発生停止胚ではミトコンドリアの多くは未成熟あるいは退行していることを発見した[11-13]。そこで，ミトコンドリア呼吸活性を指標に受精卵の品質を評価するという独創的発想に至り，「受精卵呼吸測定装置」を用いた精度の高いシングルセルレベルでの細胞呼吸計測システム開発を進めてきた。これまでに，ウシを用いた動物実験によって，呼吸活性の高い胚は呼吸測定後に追加培養を行うと高い確率でクオリティ・良好な胚

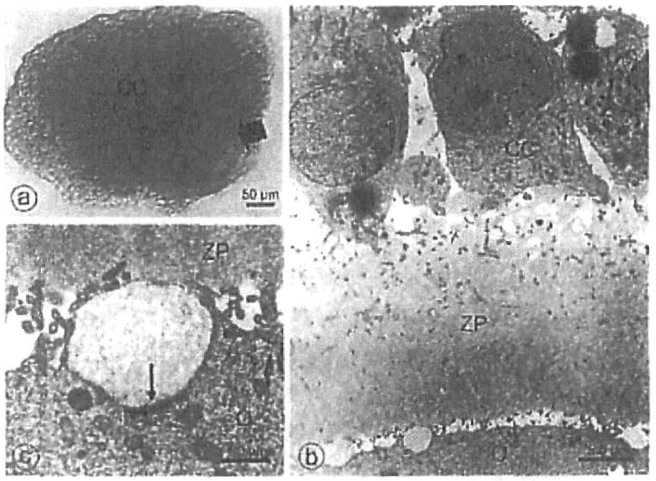

図6　ヒト卵丘細胞―卵子複合体（COC）の形態
ⓐ光学顕微鏡像。矢印は，卵子を示す。
ⓑ，ⓒ電子顕微鏡像。矢印は，卵丘細胞と卵子間のギャップ結合を示す。
CC：卵丘細胞，O：卵子，ZP：透明帯。

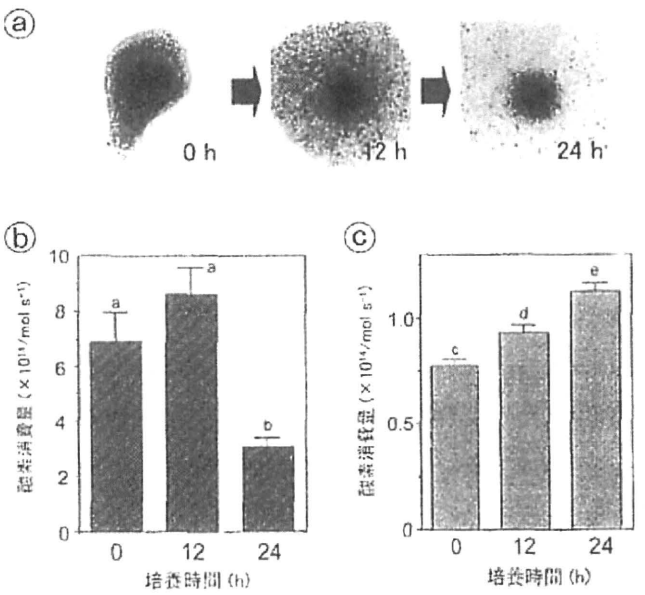

図7　ウシ卵子成熟過程における呼吸活性変化
ⓐ卵子成熟過程における卵丘細胞層の形態。
ⓑ卵丘細胞―卵子複合体（COC）の酸素消費量。
ⓒ卵子の酸素消費量。

卵子は単一の細胞であるため，受精卵のように割球の数や形態を基準に品質を評価することは困難である。このため，卵細胞質の形態（透明度や顆粒の分布状態など）や卵丘細胞の付着状態を基準にクオリティーが評価されている[17~19]。一般に，卵丘細胞が密にほぼ均一に付着し卵丘細胞間とのギャップ結合が正常に発達している卵子は，成熟率が高くクオリティー良好胚へと発生する割合も大きい[20,21]。このことは，卵子と卵丘細胞の細胞間ネットワークが正常に機能していることが卵子の機能維持に極めて重要であることを示している。「受精卵呼吸測定装置」による呼吸測定は，卵子―卵丘細胞間のネットワークをリアルタイムで解析することができ，これまで困難であった卵子品質評価の有効な方法になるものと期待される。

5.7 おわりに

本節で解説した電気化学計測技術は，細胞呼吸を無侵襲的に測定できることから，細胞の代謝機能解析，受精卵や卵子の品質診断システムへの応用が期待できる。さらに，この技術は超高感度計測であることからシングルセルレベルでの呼吸能解析やミトコンドリア機能解析が可能であり，卵子においては卵子―卵丘細胞間ネットワーク解析に有用な方法であることが示された。細胞呼吸は動物・植物問わずほとんどの細胞に共通する生物現象であることから，電気化学計測技術および装置の応用範囲は極めて広い。今後，筆者らが開発した「受精卵呼吸測定装置」がシングルセル機能解析や不妊治療などの医療において活用されることを期待している。

文　献

1) R. C. Engstrom, C. M. Pharr, *Anal. Chem.*, **61**, 1099A (1989)
2) A. J. Bard, M. V. Mirkin, "Scanning Electrochemical Microscopy", Marcel Dekker , New York (2001)
3) H. Shiku, T. Matsue, I. Uchida, *Anal. Chem.*, **68**, 1276 (1996)
4) S. Kasai, A. Yokota, H. Zhou, M. Nishizawa, K. Niwa, T. Onouchi, T. Matsue, *Anal. Chem.*, **72**, 5761 (2000)
5) K. Yamashita, M. Takagi, K. Uchida, H. Kondo, S. Takenaka, *Analyst*, **26**, 1210 (2001)
6) D. Oyamatsu, N. Kanaya, H. Shiku, M. Nishizawa, T. Matsue, *Sens. Actuat. B*, **91**, 199 (2003)
7) H. Abe, H. Shiku, S. Aoyagi, H. Hoshi, *J. Mamm. Ova Res.*, **21**, 22 (2004)
8) H. Shiku, T. Shiraishi, H. Ohya *et al.*, *Anal. Chem.*, **73**, 3751 (2001)
9) H. Shiku, T. Shiraishi, S. Aoyagi *et al.*, *Anal. Biochim. Acta*, **522**, 51 (2004)

10) H. Abe, *J. Mamm. Ova Res.*, 24, 70 (2007)
11) H. Abe, T. Otoi, S. Tachikawa *et al.*, *Anat. Embryol.*, **199**, 519 (1999)
12) H. Abe, S. Yamashita, T. Satoh, H. Hoshi, *Mol. Reprod. Dev.*, **61**, 57 (2002)
13) H. Abe, S. Matsuzaki, H. Hoshi, *Theriogenology*, **57**, 1273 (2002)
14) H. Abe, H. Shiku, M. Yokoo *et al.*, *J. Reprod. Dev.*, **52** (Suppl.), S55 (2006)
15) H. Shiku, Y. Torisawa, A. Takagi *et al.*, *Sens. Actuat. B*, **108**, 597 (2005)
16) T. Utsunomiya, K. Goto, M. Nasu *et al.*, *J. Mamm. Ova Res.*, **25**, 2 (2008)
17) R. Boni, A. Cuomo, E. Tosti, *Biol. Reprod.*, **66**, 836 (2002)
18) F. De Loos, P. Kastrop, P. van Maurik *et al.*, *Mol. Reprod. Dev.*, **28**, 255 (1991)
19) H. Aktas, M. B. Wheeler, N. L. First *et al.*, *J. Reprod. Fertil.*, **105**, 237 (1995)
20) P. Blandin, M. A. Sirard, *Mol. Reprod. Dev.*, **39**, 54 (1995)
21) N. L. Hazeleger, D. J. Hill, R. B. Stubbings *et al.*, *Theriogenology*, **43**, 509 (1995)

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呼吸活性を指標とした胚の品質評価
—マウス胚移植試験の成績と産子の正常性について

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今日の話題

呼吸活性を指標とした胚の品質評価 —マウス胚移植試験の成績と産子の正常性について

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われわれは電気化学的計測技術を応用した「受精卵呼吸測定装置」を開発し，呼吸活性を指標とした新しい胚の品質評価法を確立した。本研究では，マウスをモデル動物として，この胚品質評価法の有効性と安全性を検証した。胚移植試験の結果，形態評価に加え呼吸活性の高い胚を移植することで，移植成績が有意に上昇した。また，得られた産子の発育，血液生化学値，染色体，主要臓器の組織，行動，繁殖能力の各検査において異常所見は認められなかった。以上の結果から，この技術はヒト胚の新しい品質評価法としても臨床応用が十分に期待できる。

はじめに

近年，生殖補助医療の普及とともに多胎妊娠が増加しており，それを防止する最も有効な手段として「単一胚移植」に注目が集まっている。そのため，臨床現場においては移植に供する個々の胚の品質を正確に評価，選別することの重要性がこれまで以上に高まっている。現在，ヒト胚の品質評価法としては，割球の数や形態を基準とした方法（Veeck 分類，Gardner 分類など）が広く普及しているが，これらの方法は主観性が強く，観察者によって検査結果に差が生じる可能性が高い。そのため，客観的で高精度な胚の評価方法が望まれている。

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われわれはこれまでに，マイクロ電極をプローブとして用いて，電気化学的に細胞の酸素消費量を計測する技術を開発している[1]。さらに，この技術を基盤とする画期的な「受精卵呼吸測定装置」を開発した[2]（図 1）。現在までに，哺乳動物胚（マウス，ブタ，ウシ，ヒト）の呼吸活性の測定に成功しており，胚の呼吸活性と品質とが相関することを明らかにしている[3,4]。この装置の特徴としては，① 胚の酸素消費量を数値化できる（客観的），② 単一胚で測定ができる（高感度），③ 胚に電極を触れることなく測定できる（非侵襲），④ 測定が 1 分以内（迅速）といった点が挙げられる。これらの特徴から，将来的に生殖補助医療におけるヒト胚の品質評価方法として臨床応用が十分に期待される。そこで，本研究ではこの測定技術が胚移植時における胚の品質評価法として有効かつ安全であることを検証する目的で，マウスを実験モデルとして胚移植試験および産子の表現型解析を実施した。