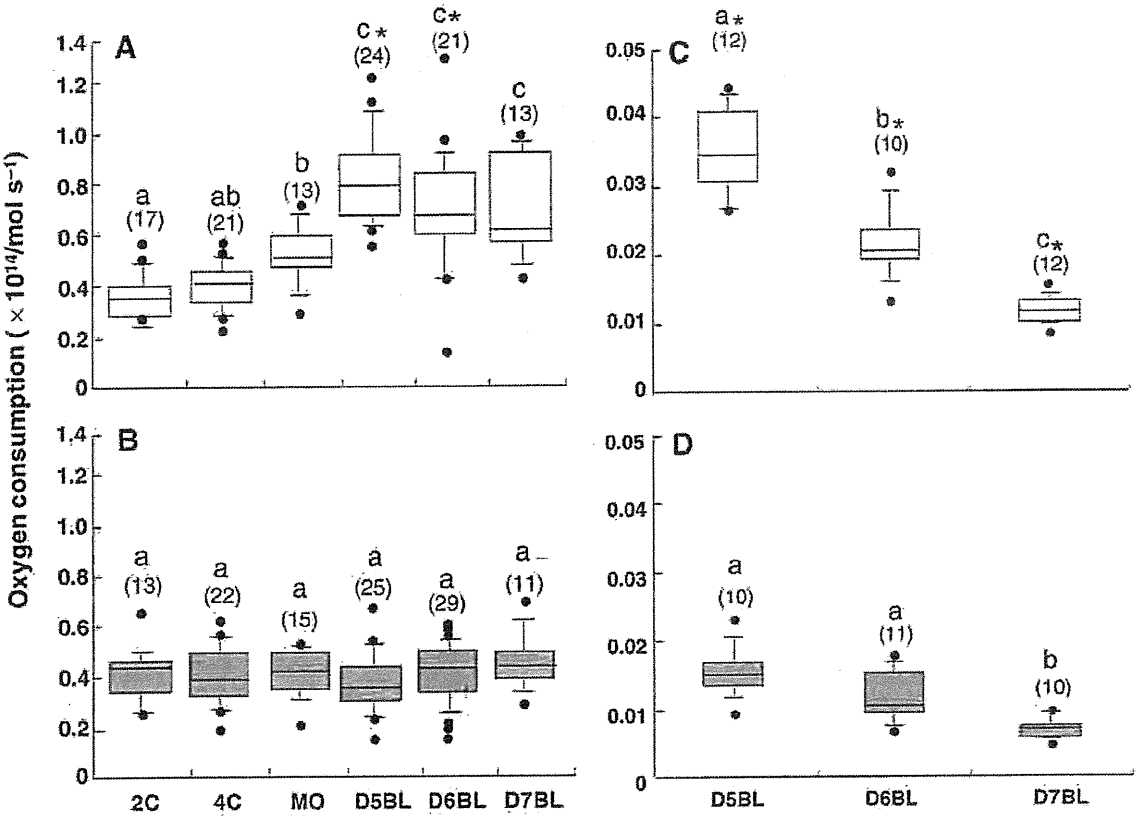

FIG. 1. Oxygen consumption in the preimplantation *in vitro*-fertilized (IVF) and somatic cell nuclear transfer (SCNT) embryos. The box-plot graphs represent oxygen consumption in the preimplantation IVF (A, C) and SCNT (B, C) embryos. The box indicates two quartiles, namely, the 25th and 75th percentiles, and the line indicates the median. The whiskers indicate the maximum and minimum values within the acceptable range that is defined by the two quartiles. The circles denote the outliers. 2C: two-cell stage; 4C: four-cell stage; MO: morula stage; D5BL, D6BL, and D7BL: days 5, 6, and 7, respectively, in the blastocyst stage after *in vitro* culture. The data of oxygen consumption per one cell at D5BL, D6BL, and D7BL are presented in C and D. The number of evaluated embryos in respective stages is indicated within parentheses. [a–c]Different letters indicate significant differences within each panel ($p < 0.05$). *Significant difference with respect to oxygen consumption between the IVF and SCNT embryos ($p < 0.05$).

TABLE 2. EFFECT OF REMOVAL OF OOPLASM, CONTAMINATION OF THE CYTOPLASM OF THE DONOR CELL, METHOD OF NUCLEAR TRANSFER, AND ARTIFICIAL ACTIVATION ON OXYGEN CONSUMPTION AT D5BL

Embryos	*No. of embryos evaluated*	*Oxygen consumption* ($E \times 10^{14}$/mol sec^{-1})	*No. of total cells/ blastocysts*
IVF	19	0.80 ± 0.24^{a}	28.4 ± 6.8
PA	8	0.82 ± 0.16^{a}	30.8 ± 3.0
Sham-PA	9	0.76 ± 0.10^{a}	31.7 ± 5.2
SCI-PA	9	0.73 ± 0.10^{a}	31.0 ± 4.5
IN-SCNT	17	0.41 ± 0.12^{b}	31.0 ± 9.8
EF-SCNT	9	0.49 ± 0.06^{b}	28.1 ± 4.1

[a–b]Different letters within the same column indicate significant difference ($p < 0.05$).

PA, parthenogenetically activated embryos; Sham-PA, parthenogenetically activated embryos from which a small volume of ooplasm was removed and a minimal amount of PB1 medium was injected; SCI-PA, parthenogenetically activated embryos in which only the cytoplasm of the donor cell was injected; IN-SCNT, SCNT embryos generated by microinjection; EF-SCNT, SCNT embryos generated by electrofusion. Data are presented as mean ± SD.

stages (60.9 and 34.8%, respectively), but none of them were classified as hatched embryos.

Experiment 5. Correlation between oxygen consumption and embryo cell numbers and apoptosis incidence

As shown in Figure 3A and B and Figure 4A and B, no significant differences with respect to the total, ICM, and TE cell numbers and incidence of apoptosis were observed between the IVF and SCNT embryos at D5BL. However, at D7BL, significant differences were observed between the IVF and SCNT embryos ($p < 0.05$) with regard to the following parameters: the total (IVF, 75.9 ± 27.5 vs. SCNT, 38.3 ± 13.0) and TE cell numbers (IVF, 65.8 ± 27.5 vs. SCNT, 29.9 ± 13.5); ICM/TE ratio (IVF, 0.18 ± 0.11 vs. 0.33 ± 0.22); and incidence of apoptosis (IVF, 4.8 ± 2.4 vs. SCNT, 19.7 ± 7.6).

Next, the correlation between oxygen consumption in embryos at D5BL and the total, ICM, and TE cell numbers and incidence of apoptosis in the embryos at D7BL was determined. As shown in Figure 3C and 4C, a significant correlation ($p < 0.01$) was observed between the oxygen

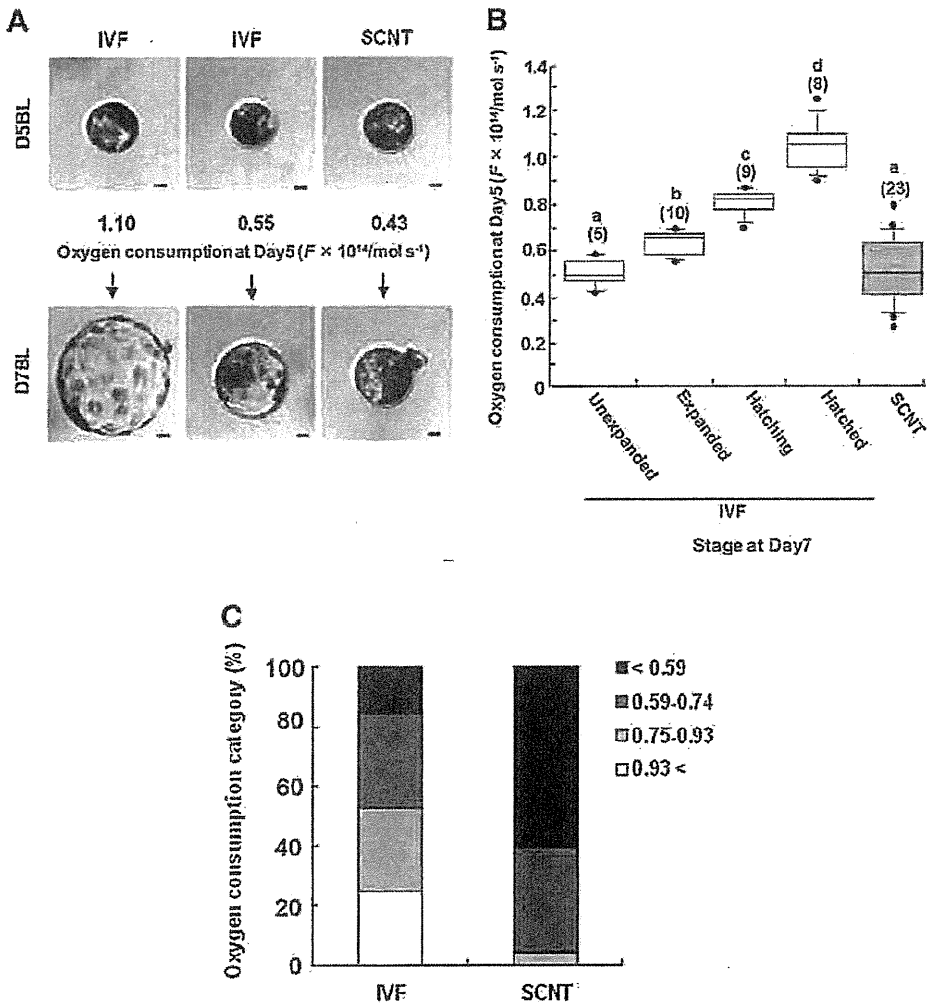

FIG. 2. Oxygen consumption on day 5 after *in vitro* culture and development of the embryo in the blastocyst stage on day 7. (A) Morphology of the embryos at D5BL (top) and D7BL (bottom). Bars = 30 μm. (B) Oxygen consumption at D5BL in unexpanded, expanded, hatching, and hatched blastocysts at D7BL. The box plots are similar to those shown in Figure 3. The number of embryos evaluated is indicated within parentheses. $^{a-d}$Different letters indicate significant differences. (C) On the basis of the data presented in B, the IVF and SCNT embryos at D5BL were placed in four categories according to the estimated oxygen consumption. The number of IVF and SCNT embryos examined was 32 and 23, respectively.

consumption in embryos at D5BL and the total (IVF: $r = 0.83$, SCNT: $r = 0.93$) and TE cell numbers (IVF: $r = 0.83$, SCNT: $r = 0.92$) and incidence of apoptosis (IVF: $r = 0.57$, SCNT: $r = 0.84$) in embryos at D7BL in the IVF and SCNT embryos; however, the ICM cell number did not significantly differ between the IVF and SCNT embryos. This finding indicates that the oxygen consumption in the embryos at D5BL correlated with the total and TE cell numbers and incidence of apoptosis at D7BL.

Experiment 6. Effect of donor cell type on oxygen consumption in the SCNT embryos at D5BL

As shown in Table 3, oxygen consumption was significantly higher in the SCNT embryos generated using cumulus cells (pCC-Auto and pCC-Allo) as donor cells than in the pFF embryos; however, oxygen consumption was significantly lower in these embryos than in the IVF embryos ($p < 0.05$). Furthermore, oxygen consumption and the total cell number did not significantly differ between the pCC-Auto and pCC-Allo embryos.

Experiment 7. Hatchability, embryo cell numbers, and apoptosis incidence in SCNT embryos reconstructed with cumulus cells at D7BL

At D7BL, the hatched blastocyts were observed in pCC embryos (3/30), but not in the pFF embryos (0/27) (Table 4). Both the total and TE cell numbers of pCC embryos significantly increased compared with those of pFF embryos; in contrast, the apoptosis incidence in pCC embryos significantly decreased ($p < 0.05$) (Table 5).

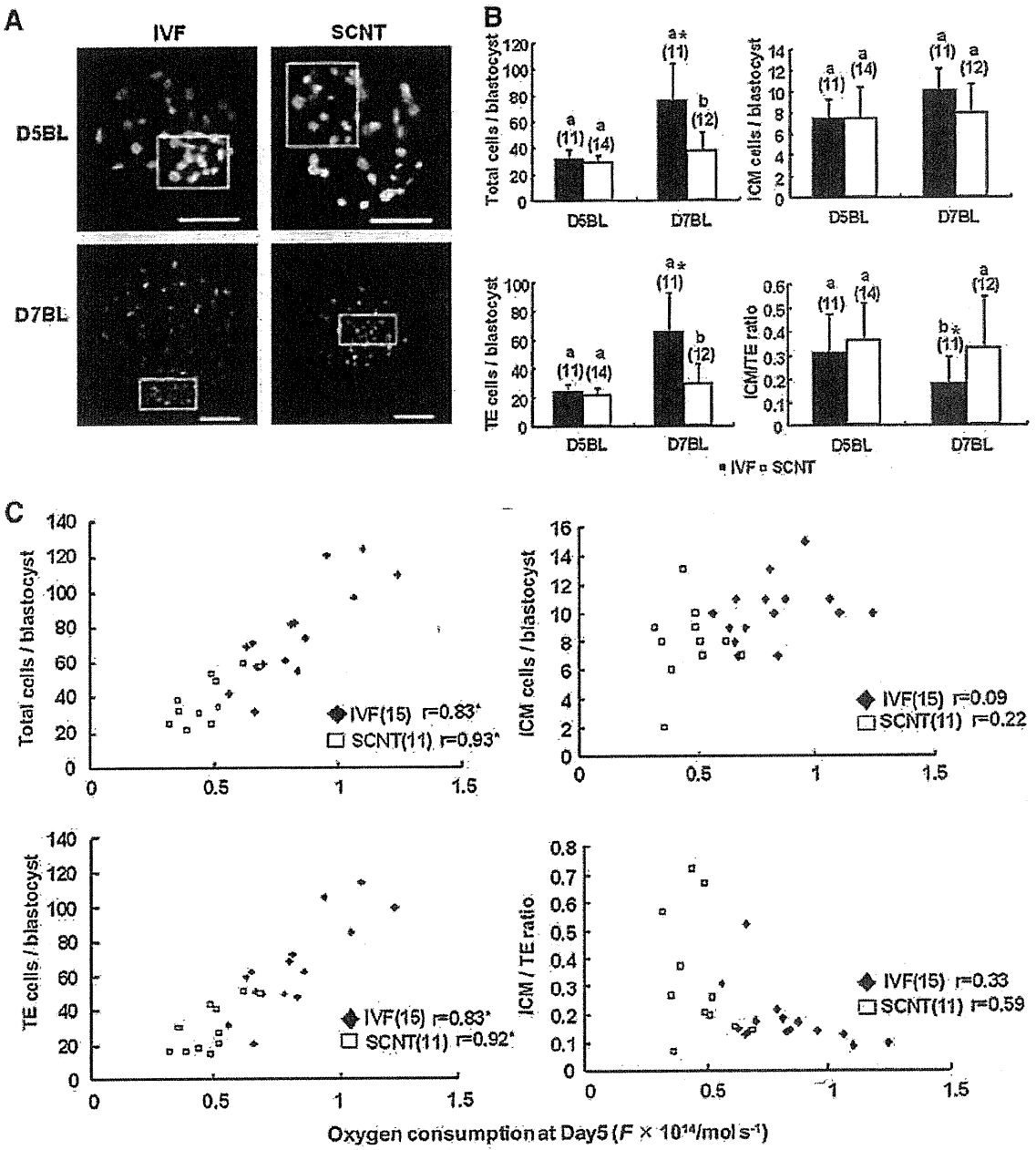

FIG. 3. Total, inner cell mass (ICM), and trophectoderm (TE) cell numbers in the IVF and SCNT embryos at D5BL and D7BL and correlation between oxygen consumption at D5BL and the values of these parameters at D7BL. (A) Differentially stained ICM and TE cells in the IVF and SCNT embryos at D5BL and D7BL. The regions enclosed within the squares indicate ICM. Bars = 60 μm. (B) The total, ICM, and TE cell numbers and ratio of ICM to TE at D5BL and D7BL. The number of embryos evaluated is indicated within parentheses. Data are presented as mean ± SD. $^{a-b}$Different letters indicate significant differences within the same stage ($p < 0.05$). *Significant difference in the values at D7BL compared to those at D5BL ($p < 0.05$). (C) Correlation between oxygen consumption at D5BL and the total, ICM, and TE cell numbers at D7BL. The number of embryos evaluated is indicated within parentheses. *The coefficient of determination "r" was statistically significant ($p < 0.01$).

Discussion

To evaluate the overall metabolic activity in individual SCNT embryos during the preimplantation stages, we measured their oxygen consumption levels by SECM. Our results clearly indicate lower oxygen consumption in the SCNT embryos than in the IVF embryos at blastocyst stage (Fig. 1). Oxygen consumption in the IVF and *in vivo*-produced embryos rapidly increased from the MO stage to the D5BL stage. In contrast, no such increase in oxygen consumption was observed in the SCNT embryos.

In the present study, we hypothesized that low embryo numbers (Boiani et al., 2003; Koo et al., 2004) and high

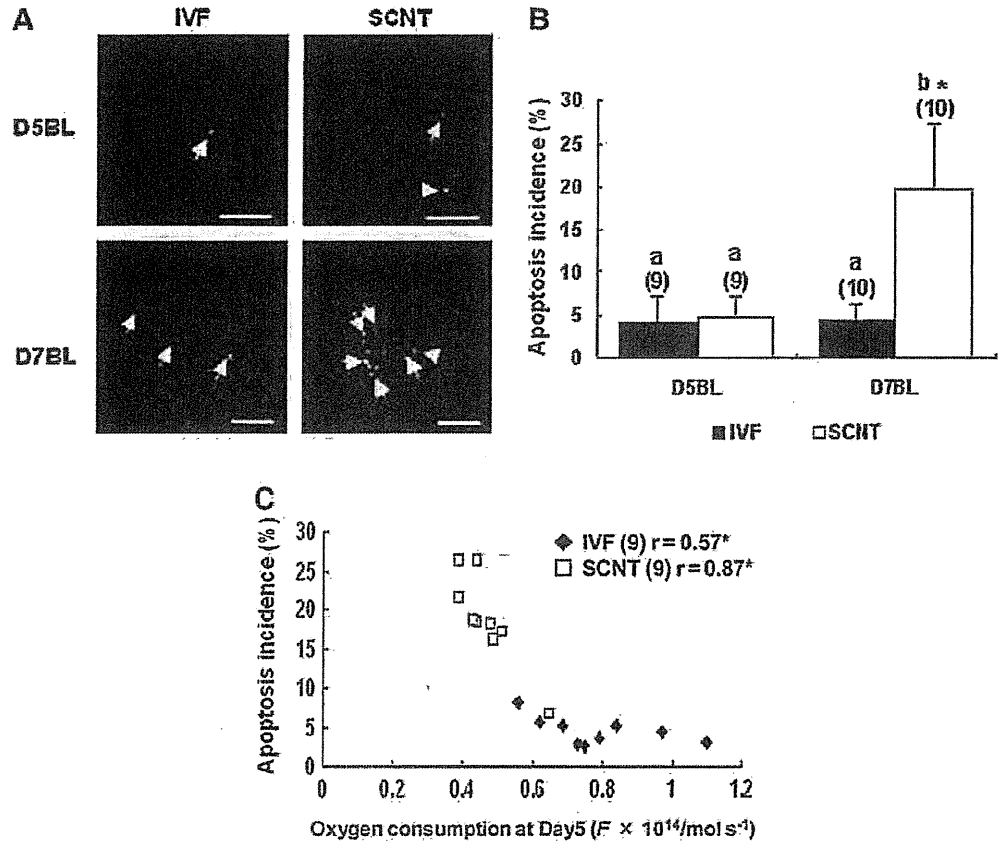

FIG. 4. Number of apoptosis-positive cells in the IVF and SCNT embryos at D5BL and D7BL and correlation between oxygen consumption at D5BL and the number of apoptosis-positive cells at D7BL. (A) Apoptosis-positive cells at D5BL (top) and D7BL (bottom) detected by TUNEL staining. The arrows indicate apoptosis-positive cells. Bars = 60 μm. (B) The number of apoptosis-positive cells at D5BL and D7BL. The number of embryos evaluated is indicated within parentheses. Data are presented as mean (SD). [a–b]Different letters indicate significant differences within the same stage ($p < 0.05$). *Indicates significant difference between apoptosis incidence at D5BL and D7BL ($p < 0.05$). (C) Correlation between oxygen consumption at D5BL and apoptosis incidence at D7BL. The number of embryos evaluated is indicated within parentheses. *The coefficient of determination "r" was statistically significant ($p < 0.05$).

incidence of apoptosis (Hao et al., 2003) in the SCNT embryos might have resulted in the low oxygen consumption at D5BL. We, therefore, analyzed both the ICM and TE cell numbers and apoptosis incidence in the SCNT embryos. Although at D7BL significantly lower embryo cell numbers and higher apoptosis incidence were observed in the SCNT embryos than in the IVF embryos, these differences were not significant at D5BL (Figs. 3 and 4). Thus, the low embryo cell numbers and high apoptosis incidence in the SCNT could not have accounted for the low oxygen consumption in the SCNT embryos at D5BL. Furthermore, we examined the effects of the nuclear transfer methods on the oxygen consumption in the SCNT embryos—removal of ooplasm; methods of introduction of the donor nuclei into the enucleated oocyte, such as injection or electrofusion; contamination of the cytoplasm of the somatic cell, artificial activation, and treatment with cytochalasins. We found that the abovementioned methods did not appear to be the major causes of lower oxygen consumption in the SCNT embryos at D5BL (Table 2). The sex difference of donor cells also did not affect the lower oxygen consumption at D5BL (male pFF, $0.45 \pm 0.11 \times 10^{14}$/mol sec^{-1} vs. female pFF, $0.48 \pm 0.16 \times 10^{14}$/mol sec^{-1}) (Supplementary Table 1). This is in accordance with the previous reports, which show no significant difference between sexes on oxygen consumption of bovine *in vitro* produced blastocysts (Agung et al., 2005; Lopes et al., 2005). On the other hand, the oxygen

TABLE 3. EFFECT OF DONOR CELL TYPE ON OXYGEN CONSUMPTION IN SCNT EMBRYOS AT D5BL

Embryos	*No. of embryos evaluated*	*Oxygen consumption* ($F \times 10^{14}$/mol sec^{-1})	*No. of total cells/ blastocyst*
IVF	10	0.77 ± 0.13^{a}	34.1 ± 12.1
pFF	13	0.48 ± 0.04^{c}	30.8 ± 10.2
pCC-Allo	13	0.57 ± 0.05^{b}	33.2 ± 7.4
pCC-Auto	9	0.62 ± 0.08^{b}	32.8 ± 3.1

[a–c]Different letters within the same column indicate significant difference ($p < 0.05$).

pFF, SCNT embryos with fetal fibroblast; pCC-Allo and pCC-Auto represent allogeneic and autologous SCNT embryos with cumulus cells, respectively. Data are presented as mean ± SD.

TABLE 4. EFFECT OF DONOR CELL TYPE ON *IN VITRO* DEVELOPMENT IN SCNT EMBRYOS

Embryos	No. of embryos cultured	No. (percent ± SD) of embryos cleaved on day 2	No. (percent ± SD) of embryos developed to the blastocyst stage (BL) on Day 5 (D5BL)	Day 6 (D6BL)	Day 7 (D7BL)	No. (percent ± SD) of embryos developed to the hatched blastocyst stage on day 7
pFFf	120	87 (72.5 ± 8.8)	13 (10.8 ± 5.8)	16 (13.3 ± 2.6)[b]	27 (22.5 ± 2.7)	0 (0)
pCC	125	92 (73.7 ± 12.0)	19 (15.2 ± 4.5)	23 (18.5 ± 3.9)[a]	30 (24.0 ± 2.0)	3 (2.3 ± 2.6)

[a–b]Different letters within the same stage indicate significant difference ($p < 0.05$).
pFF and pCC represent SCNT embryos with fetal fibroblasts and cumulus cells, respectively.

TABLE 5. EFFECT OF DONOR CELL TYPE ON EMBRYO CELL NUMBERS AND APOPTOSIS INCIDENCE IN SCNT EMBRYOS AT D7BL

Embryos	No. of blastocysts at day 7 evaluated	No. of cells/blastocysts Total	ICM	TE	ICM/TE ratio	No. of blastocysts at day 7 evaluated	Apoptosis incidence (%)
Pff	15	30.2 ± 3.7[b]	7.7 ± 1.8	22.5 ± 4.2[b]	0.36 ± 0.15	12	22.3 ± 7.4[b]
pCC	16	47.8 ± 15.3[a]	9.6 ± 3.3	38.2 ± 13.6[a]	0.28 ± 0.14	14	11.2 ± 4.2[a]

[a–b]Different letters within the same column indicate significant difference ($p < 0.05$).
pFF and pCC represent SCNT embryos with fetal fibroblast and cumulus cell, respectively. Data are presented as mean ± SD.

consumption levels in the SCNT embryos at D5BL that were reconstructed with cumulus cells was neutralized, regardless of the difference between the recipient cytoplasm and donor nucleus in an individual. This result indicates that the type of donor nucleus might be responsible for the low oxygen consumption in the SCNT embryos at D5BL. In a previous study, incomplete reprogramming of donor nuclei that were transferred to recipient cytoplasts was suggested to cause aberrant expression of genes associated with the mitochondria; this aberrant expression was associated with developmental arrest and various other disorders in the cloned embryos (Jincho et al., 2008). Although further investigation is necessary for clarifying the mechanism underlying the lower oxygen consumption in the SCNT embryos than in the IVF embryos, incomplete reprogramming of the donor nucleus might have led to mitochondrial dysfunction, resulting in anomalous oxygen consumption in the SCNT embryos at D5BL.

The activation of oxygen consumption at the blastocyst stage was associated with the energy demand for protein synthesis and activity of the plasma membrane Na^+-K^+-dependent ATPase during blastocoele formation and embryo implantation (Harvey et al., 2002; Nilsson et al., 1982). Hence, the low oxygen consumption in the SCNT embryos at D5BL appears to be involved in various disorders, including low TE cell number (Koo et al., 2002), abnormal placentation (Yang et al., 2007b), and high apoptosis incidence in cells of extra-embryonic tissues such as TE (Chae et al., 2006; Lee et al., 2007). In the present study, we observed that when the IVF embryos actively consumed oxygen at D5BL ($>0.81 \times 10^{14}$/mol sec^{-1}), they exhibited higher hatching and hatched rates at D7BL (Fig. 2). In fact, >50% of the IVF embryos at D5BL exhibited active oxygen consumption along with hatching competence, whereas only 4% of the SCNT embryos at D5BL exhibited active oxygen consumption with a similar level of competence (Fig. 2). Furthermore, it has been suggested that the low level of mitochondrial metabolism causes a decrease in the TE cell number (Thouas et al., 2006), and that low oxygen consumption is associated with the apoptosis incidence (Manser and Houghton, 2006; Trimarchi et al., 2000). Our results also suggest the involvement of low oxygen consumption at D5BL in the low TE cell number and high apoptosis incidence at D7BL (Figs. 3 and 4).

In conclusion, we showed that most SCNT embryos exhibited anomalous oxygen consumption at D5BL and low TE cell number and high apoptosis incidence at D7BL. However, we found that anomalous oxygen consumption at D5BL and limited hatching competence, low TE cell number, and high apoptosis incidence at D7BL in the SCNT embryos could be improved by using cumulus cells as donor nuclei (Tables 3, 4, and 5). These results are consistent with the results of previous studies that reported that in the case of many species, cumulus cells might be the appropriate donor nuclei for SCNT embryos (Kato et al., 2000; Wakayama et al., 1998; Yang et al., 2007a). Thus, although embryo transfer after measurement of oxygen consumption needs to be investigated in the future, our findings suggest that measurement of oxygen consumption by using SECM could be useful as a noninvasive and physiological evaluation method for the developmental competence of porcine SCNT embryos.

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Author Disclosure Statement

The authors do not have any competing financial interests.

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MHR ORIGINAL RESEARCH

Intrinsic oxidative stress causes either 2-cell arrest or cell death depending on developmental stage of the embryos from SOD1-deficient mice

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ABSTRACT: Oxidative stress characterized by elevated reactive oxygen species is a well-known cause of developmental arrest and cellular fragmentation in the development of *in vitro*-produced embryos. To investigate the effects of intrinsic oxidative stress on the early development of embryos, oocytes from superoxide dismutase 1 (SOD1)-deficient mice resulting from *in vitro* fertilization, followed by culture for 4 days, were examined. Development of all embryos from SOD1-deficient oocytes was arrested at the 2-cell stage under conventional culture conditions with atmospheric oxygen (20% O_2). Significantly higher levels of superoxide were detected in SOD1-deficinet embryos cultured under 20% O_2 using dihydroethidium. Among treatments with antioxidants, only hypoxic culture with 1% O_2 negated the 2-cell arrest and advanced the development of the embryos with efficacy similar to that in wild-type embryos. Mitochondrial function was investigated because its malfunction was a suspected cause of 2-cell arrest. However, respiratory activity, ATP content and mitochondrial membrane potential in the 2-cell embryos were not markedly affected by culture with 20% O_2. When embryos from SOD1-deficient oocytes were first developed to the 4-cell stage under 1% O_2 culture and were then transferred to 20% O_2, most of them developed to the morula stage but underwent total degeneration thereafter. Thus, oxidative stress was found to damage embryos differentially, depending on the developmental stage. These results suggest that embryos derived from SOD1-deficient mouse oocytes are an ideal model to investigate intrinsic oxidative stress-induced developmental abnormality.

Key words: SOD1 deficiency / oxidative stress / 2-cell arrest / mitochondria

Introduction

It is universally accepted that mammalian preimplantation embryos are sensitive to their environment and that culture conditions, including collection and manipulation, have a momentous impact on the developmental potential of the embryos (Loutradis *et al.*, 2000; Summers and Biggers, 2003). *In vitro* fertilized (IVF) embryos in most mouse strains often show developmental arrest, e.g. 2-cell arrest or cellular fragmentation before the blastocyst stage, due to various deterioration factors during culture (Chatot *et al.*, 1990; Jurisicova and Acton, 2004). Among factors that have a deteriorating effect on fertilizability and subsequent developmental competence, oxidative stress, a condition with an overabundance of oxidants relative to antioxidants, is a well-known cause of developmental arrest, cell death by necrosis or by apoptosis, suppression of sperm motility and sperm–oocyte fusion (Noda *et al.*, 1991; Feugang *et al.*, 2004; Aitken and Baker, 2006). During culture under atmospheric conditions (approximately 20% O_2), embryos are exposed to a higher oxygen concentration than is physiologically normal in the oviduct and uterus (2% to 8% O_2; Fischer and Bavister, 1993). Elevated oxygen concentration advances the generation of cytotoxic reactive oxygen species (ROS), which can induce lipid peroxidation and functionally alter proteins and DNA (Nasr-Esfahani *et al.*, 1990; Takahashi *et al.*, 2000; Aitken and Baker, 2006). Hence, oxidative stress must be kept at a low level. Moreover, oxidative stress is augmented by various stimuli and maternal aging and exerts deteriorating effects on oocytes, consequently impairing reproductive ability (Fujii *et al.*, 2005; Agarwal *et al.*, 2008; Ruder *et al.*, 2008).

Transient metal ions, such as iron and copper, in the presence of superoxide and hydrogen peroxide, result in the generation of hydroxyl radicals, the most harmful reactive ROS, via the Fenton reaction (Halliwell and Gutteridge, 1999). The mechanism by which ethylenediamine tetraacetic acid (EDTA) supports embryo development *in vitro* (Suzuki *et al.*, 1988; Jinno *et al.*, 1989) is, at least in part, based on elimination of the free transition metal ions. ROS are also produced during the consumption of respired oxygen and other biological reactions involving reduction-oxidation reactions. In fact, the mitochondrial electron transfer system is a major source of ROS because it consumes more than 90% of the oxygen molecules respired by ordinary cells (Halliwell and Gutteridge, 1999).

The body has multiple antioxidative systems to suppress oxidative stress. Among the known antioxidative proteins, superoxide dismutase (SOD) is thought to play a central role because of its ability to scavenge superoxide anions, the primary ROS generated from molecular oxygen in cells, at the initial stage of the radical chain reaction (Fridovich, 1995). There are three kinds of mammalian SOD genes (Valentine *et al.*, 2005): the SOD1 product is localized in the cytosol and the intermembrane space of mitochondria; the SOD2 product is exclusively located in the mitochondrial matrix and the SOD3 product circulates in plasma. Regardless of the pivotal role of SOD1, SOD1-deficient mice show relatively mild phenotypes and grow normally (Ho *et al.*, 1998; Matzuk *et al.*, 1998), compared with mice lacking SOD2 protein, which die due to dilated cardiomyopathy during the neonatal stage (Li *et al.*, 1995). The prominent phenotype of SOD1-deficient mice is female infertility (Ho *et al.*, 1998; Matzuk *et al.*, 1998). Matzuk *et al.* (1998) have found a decrease in serum follicle stimulating hormone and luteinizing hormone levels and have proposed that ovary dysfunction may be secondary to decreased gonadotrophins and/or decreased responsiveness of the ovaries to physiological concentrations of gonadotrophins. On the other hand, Ho *et al.* (1998) showed that the number of 4 embryos in uteri of SOD1-deficient mice were not significantly different from those in wild-type mice and concluded that embryonic lethality was a cause of infertility. Thus, there are conflicting data, and it is yet unclear what actually causes female infertility in SOD1-deficient mice.

The authors are attempting to elucidate the underlying mechanism of female infertility in SOD1-deficient mice. This communication reports that intrinsic oxidative stress-injured embryos from SOD1-deficient mice respond differentially, in a development stage-specific manner.

Materials and Methods

Experimental animals

Three pairs of C57BL/6 *SOD1*$^{+/-}$ mice, originally established by Matzuk *et al.* (1998), were purchased through Jackson Laboratories (Bar Harbor, ME, USA) and bred at our institute, giving rise to *SOD1*$^{+/+}$ and *SOD1*$^{-/-}$ littermates. They were genotyped by PCR amplification as described previously (Iuchi *et al.*, 2007). Mice backcrossed to C57BL/6 mice more than eight times were used in this study. Four- to 6-week-old female mice were used to maximize the yield of oocytes in response to hormone stimulation, and 8- to 16-week-old male mice were used to collect epididymal spermatozoa. The animal room climate was kept under specific pathogen-free conditions at a constant temperature of 20–22°C with a 12-h alternating light–dark cycle, with food and water available *ad libitum*. Animal experiments were performed in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee of Yamagata University.

Oocyte collection, IVF and embryo culture

SOD1-deficient (*SOD1*$^{-/-}$) female mice and C57BL/6 background genetic controls (*SOD1*$^{+/+}$) were superovulated with 5 IU of equine chorionic gonadotrophin (Sankyo-elu, Tokyo, Japan), followed 48 h later with 5 IU of human chorionic gonadotrophin (Asuka-seiyaku, Tokyo, Japan) administered intra-peritoneally. Human tubal fluid (HTF) medium was used for IVF (Quinn *et al.*, 1985), and potassium simplex optimization medium (KSOM) was used for mouse oocytes/embryos cultures (Erbach *et al.*, 1994). For IVF, spermatozoa from *SOD1*$^{+/+}$ male mice were collected by squeezing the epididymal cauda and were pooled into a drop of HTF, supplemented with 0.5% bovine serum albumin (BSA fraction V, Sigma-Aldrich, St Louis, MO, USA). They were adjusted to a final concentration of $1–2 \times 10^6$ sperm/ml with the same medium and were then pre-incubated for 1 h. Ovulated cumulus–oocyte complexes (COCs) were collected by tearing the oviductal ampulla at 14–15 h after hCG injection and were pooled into the droplet with sperm. COCs were co-incubated with spermatozoa for 5 h. After IVF, presumptive zygotes were stripped of cumulus and sperm cells in KSOM supplemented with 0.5% BSA for embryo culture. Cleavage rates were assessed at 24 h after the start of embryo culture. Normally cleaved embryos remained in culture for 4 days (up to the blastocyst stage).

Each culture was kept in 200 μl droplets (groups of 10–20 oocytes/embryos) of medium overlaid with mineral oil in plastic dishes. Incubation was conducted at 37°C under a humidified atmosphere of either 20% O_2 (5% CO_2 in air) or 1% O_2 (1% O_2/5% CO_2/94% N_2). The effects of antioxidants, culturing under supplementation with 100 μM β-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan) or 500 μg/ml human SOD1 protein, on fertilization and embryo development were also investigated in normoxic conditions.

Western blot analysis

To detect SOD1 and SOD2 protein expression, ovulated COCs derived from each genotype (*SOD1*$^{-/-}$, *SOD1*$^{+/-}$, *SOD1*$^{+/+}$) of mice were denuded by hyaluronidase treatment (300 μg/ml in KSOM, type IVs, Sigma-Aldrich) and presumptive metaphase II (M II) oocytes with the first polar body were collected. Each genotype sample of 60 oocytes was washed in PBS containing 3 mg/ml polyvinylalcohol (PVA, Sigma-Aldrich), sonicated for 1 s, and then lysed in 2% SDS and 60 mM Tris–HCl-based sampling buffer (pH 6.8). The lysates were separated by electrophoresis in 15% SDS–PAGE and electrically transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% (w/v) skim milk in 0.1% (v/v) Tween-20-tris-buffered saline (T-TBS) for 1 h, the membrane was incubated overnight at 4°C with a goat antihuman SOD1 polyclonal antibody (Ishii *et al.*, 2005) diluted 1:1000 in Can Get Signal® solution I (Toyobo, Oosaka, Japan). Following three washes of 5 min each in T-TBS, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase (HRP)-conjugated anti-goat second antibody (Zymed Laboratories, South San Francisco, CA, USA) diluted 1:20 000 in Can Get Signal® solution II. After three washes in T-TBS, the blots were visualized using an ECL detection kit (Amersham Pharmacia Biosciences, Piscataway, NJ, USA). The membrane was then incubated for 30 m at 50°C in stripping buffer (2% SDS, 100 μM β-mercaptoethanol and 60 mM Tris–HCl) to remove the antibodies. In the same manner described above, SOD2 (Suzuki *et al.*, 1993) and glyceraldehydes-3-phosphate dehydrogenase (G3PDH, Santa Cruz Biotechnology, Santa Cruz, CA, USA) proteins were detected by rabbit antihuman SOD2 polyclonal antibody diluted 1:1200 or by rabbit antihuman

G3PDH diluted 1:1000, as the first antibody, and HRP-conjugated anti-rabbit diluted 1:40 000, as the second antibody.

Chromosome labeling of zygotes/embryos

To successively compare the transition from zygote to 2-cell stage embryo in SOD1-deficient mice and wild-type mice, at 5, 18, 20, 36 and 42 h after IVF under 20% O_2, the zygotes/embryos were fixed with 2% formaldehyde in PBS-PVA for 30 min and stained with 10 μg/ml Hoechst33342 (Sigma-Aldrich) in PBS-PVA for 15 min at room temperature. After three washes in PBS-PVA, the embryos were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Detection of superoxide in 2-cell stage embryos

The level of intracellular superoxide in 2-cell stage embryo was determined using dihydroethidium (Molecular Probes, Eugene, OR, USA), a specific indicator of superoxide. At 36 h after IVF, the embryos were incubated with 2.5 μM dihydroethidium in KSOM for 10 min at 37°C in the dark, then washed and continuously cultured in KSOM for 30 min. Superoxide-specific fluorescence was detected using a fluorescent microscope (Leica DMI3000B, Leica Microsystems, Wetzlar, Germany) at fluorescence maximum 515/590 nm (excitation/emission). An image was obtained for the optical section of each embryo when two divided cells lined a plane. Individual images were analyzed using Image J software ver.1.38 (developed by NIH), which allows for quantification of signal intensity of dihydroethidium staining. Signal intensity was expressed by the integration of average pixels in each embryo. Relative values of signal intensity were calculated by the mean value of a wild-type embryo to the individual value of wild-type or SOD1-deficient embryos under the same oxygen conditions.

Respiration assay of 2-cell stage embryos

At 36 h after IVF, the oxygen consumption of individual 2-cell embryos was quantified non-invasively by a modified scanning electrochemical microscope (SECM) measuring system (Shiku *et al.*, 2001; Abe, 2007). The oxygen consumption of the embryos was indicated by the oxygen concentration difference between the bulk solution and the sample surface, using voltammetry of the Pt-microdisc electrode (Shiku *et al.*, 2004). The tip potential was held at −0.6 V versus Ag/AgCl with a potentiostat to monitor the local oxygen concentration in the solution. Modified HTF medium was employed for the measurement of oxygen consumption. Its composition includes only salt electrolyte, glucose, sodium pyruvate, sodium lactate, HEPES and gentamicin sulfate. Voltammetry of the Pt-microdisc electrode in the modified HTF medium showed a steady-state oxygen reduction wave. No response from other electrochemically active species was observed near the embryo surface. The single embryo was transferred into a cone-shaped microwell filled with modified HTF medium at 37°C on a warming plate (MATS502NLR; Tokai Hit, Shizuoka, Japan) where it fell to the bottom of the well and remained at the lowest point. The microelectrode was scanned according to the z-direction from the side point of the sample. The motor-driven XYZ stage was located on the microscope stage for electrode tip scanning. The XYZ stage and potentiostat were controlled by a computer. The oxygen consumption rate of each embryo was calculated by software based on the spherical diffusion theory (Shiku *et al.*, 2004). Each embryo was scanned three times.

Measurement of ATP content of 2-cell stage embryos

At 36 h after IVF, the ATP content of individual embryos was measured using a commercial assay based on the luciferin–luciferase reaction, using a BacTiter-GloTM Microbial Cell Viability Assay Kit (Promega, Madison, WI, USA). Briefly, samples were rinsed three times in PBS-PVA, suspended in 50 μl PBS, and stored at −80°C until use. After the addition of a 50-μl reaction mixture followed by incubation at 25°C for 5 min, chemiluminescence of the sample was measured by a luminometer (Berthold Lumat LB9507, Bad Wildbad, Germany). A 5-point standard curve (0–100 pmol/tube) was routinely included in each assay. The ATP content was determined from the formula for a standard curve (linear regression). The linear relationship was observed between luciferin luminescence and ATP content from a 0.01–10 pmol/assay, which fully covered ATP contents in each embryo.

Measurement of mitochondrial membrane potential in 2-cell stage embryos

Mitochondrial membrane potential ($\Delta\Psi m$) was determined by staining 2-cell stage embryos with the mitochondrial stain JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide, Mitochondrial Membrane Potential Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA). At 36 h after IVF under 20% O_2, the embryos were incubated in JC-1 at half the manufacturer's recommended concentration (1:200 in KSOM) for 15 min at 37°C in the dark, then washed and immediately examined using a fluorescent microscope (Leica DMI3000B, Leica Microsystems). One optical section was imaged for each embryo, in the plane where both cleaved cells could be visualized. Individual images of fluorescently labeled embryos were analyzed using Image J software ver.1.38 (developed by NIH), which allows for quantification of signal intensity of JC-1 staining. $\Delta\Psi m$ was estimated by the representing integration of average red pixels (J-aggregate, high membrane potential) as a ratio of average green pixels (J-monomer, low membrane potential) in the same area for each embryo.

Analysis of degenerated embryos

To characterize cellular damage, at Day 4 after IVF, embryos were reacted with 10 μg/ml Hoechst33 342, 5 μg/ml propidium iodide (PI) or FITC-labeled annexin V using a MEBCYTO® Apoptosis Kit (Medical and Biological Laboratories, Woburn, MA, USA) and 5 μg/ml Hoechst33342 for 15 min at room temperature. After washing three times with PBS-PVA, samples were examined with a fluorescent microscope (Leica DMI3000B, Leica Microsystems).

Statistical analysis

Data showing embryo development in normoxic and hypoxic culture consist of at least three independent replicates. A Fisher's exact test was performed to evaluate the developmental ability among individual groups. Statistical analyses of signal intensity by dihydroethidium staining or JC-1 staining, respiration assay and ATP content were carried out using the Bonferroni test. A P-value of <0.05 was considered statistically significant.

Results

Two-cell arrest of embryos from SOD1-deficient mouse oocytes and rescue under hypoxic culture

Western blot analysis indicated that SOD1 protein was present in oocytes from wild- and hetero-type mice, but was totally absent in oocytes from SOD1-deficient mice, whereas a similar amount of SOD2 protein was present in both (Fig. 1). The developmental ability of fertilized oocytes from SOD1-deficient mice after IVF was

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.

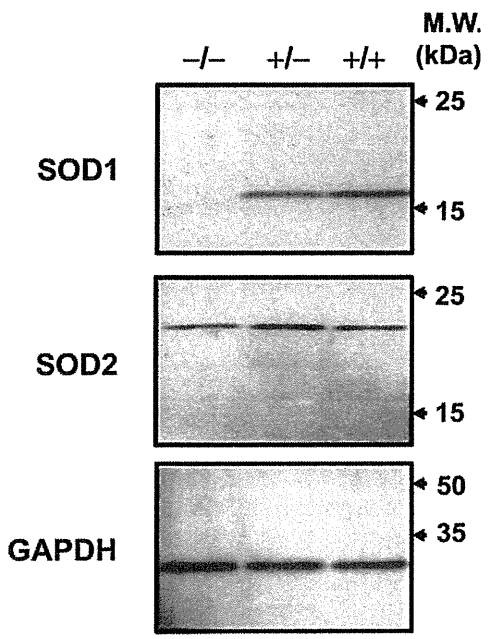

Figure 1 Western blot analysis of SOD1 and SOD2 in oocytes. Sixty superovulated MII oocytes from each genotypic female mouse were subjected to SDS–PAGE followed by immunoblot analysis. Antibodies against SOD1 (upper panel), SOD2 (middle panel) and GAPDH (lower panel, protein loading control) were used 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 I 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 ($P < 0.05$).

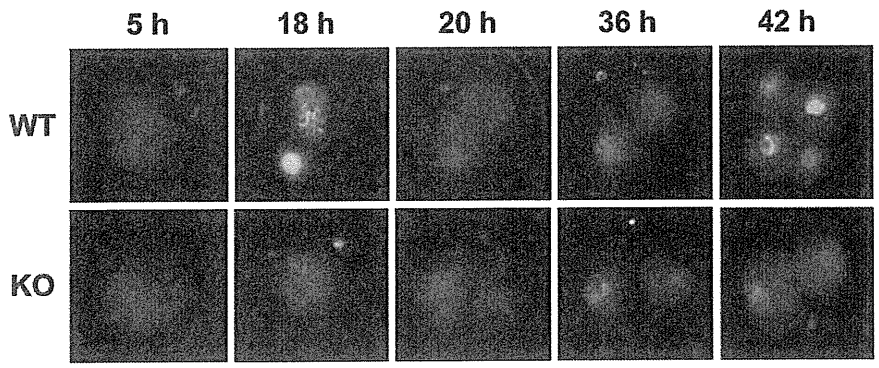

Figure 2 Chromosome labeling of zygotes/embryos. Superovulated COCs from wild-type (WT) or SOD1-deficient (KO) mice were subjected to IVF and incubated under atmospheric oxygen conditions. The zygotes/embryos were stained with Hoechst33342 at 5, 18, 20, 36 and 42 h after IVF and observed under a fluorescent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

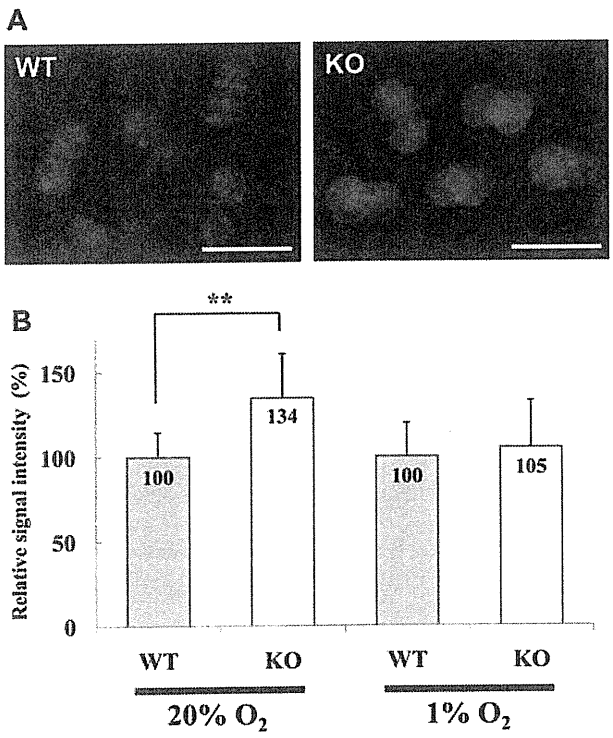

Figure 3 Fluorescent images and signal intensity of 2-cell embryos stained with dihydroethidium. (**A**) Two-cell embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes under 20% O_2 after dihydroethidium staining. Scale bars, 100 μm. (**B**) Embryos from wild-type (WT) and SOD1-deficient (KO) 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.

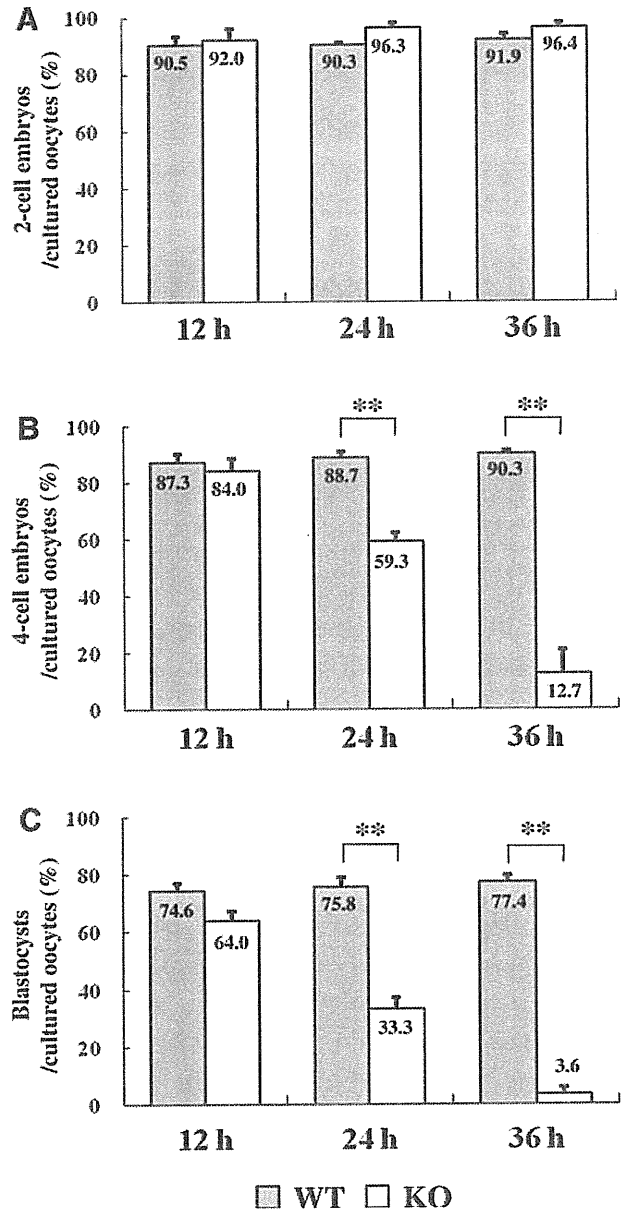

Figure 4 Time required for 2-cell arrest in oocytes from SOD1-deficient mice. Superovulated COCs from wild-type or SOD1-deficient mice were subjected to *in vitro* fertilization (IVF) and incubated under atmospheric oxygen conditions, and then transferred to 1% O_2 at 12, 24 and 36 h after IVF. (**A**) 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

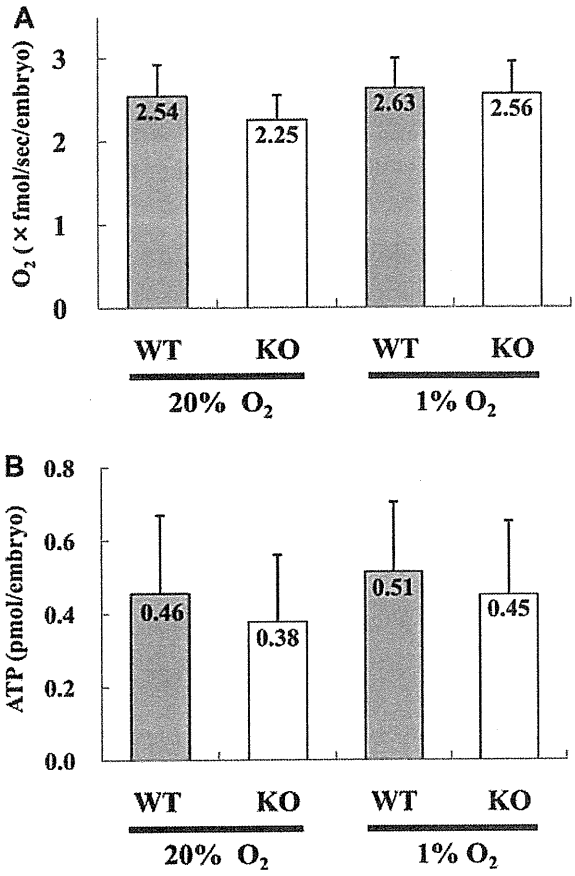

Figure 5 Respiratory activity and ATP levels of 2-cell embryos. (**A**) Embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes were cultured under 1% or 20% O_2 for 36 h after IVF, and oxygen consumption in the individual embryos was measured by an SECM measuring system ($n = 10–13$). (**B**) 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

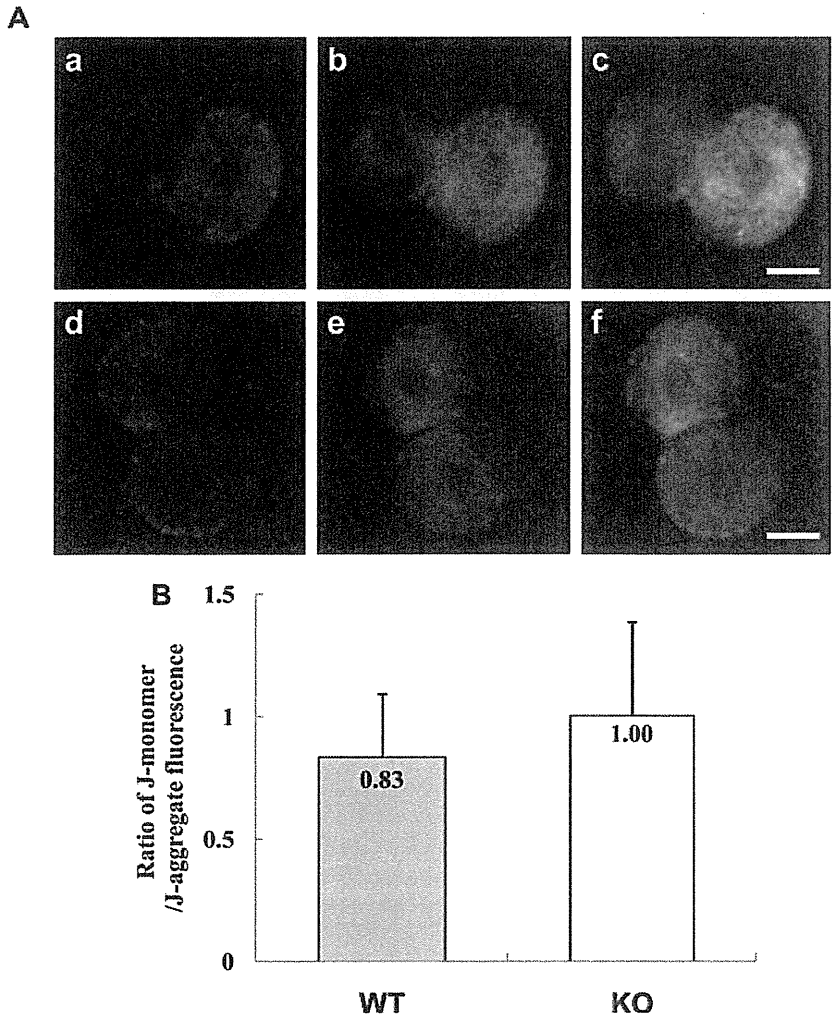

Figure 6 Fluorescent images and mitochondrial membrane potential of 2-cell embryos stained with JC-1. (**A**) Fluorescent images of JC-1-stained 2-cell embryos from wild-type (a–c) or SOD1-deficient (d–f) mouse oocytes under 20% O_2. (a and d) J-aggregate; (b and e) J-monomer; (c and f) Merge. Scale 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

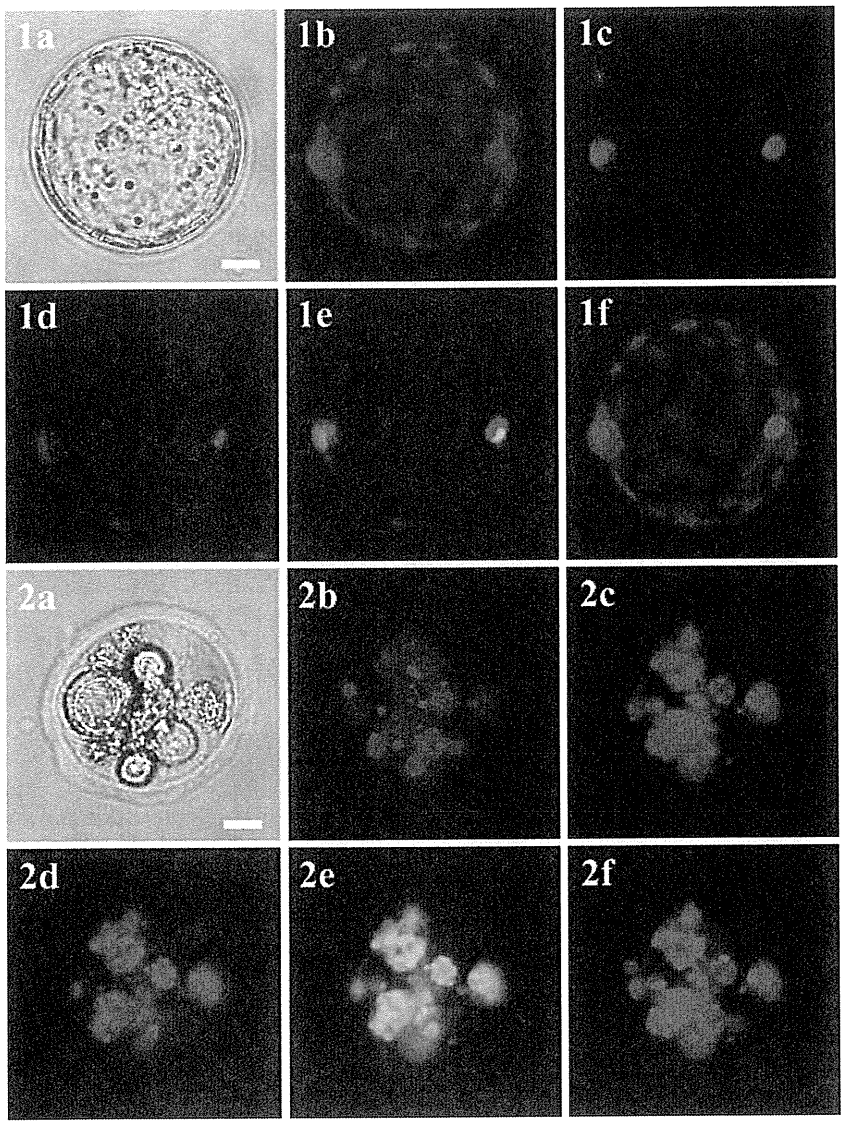

Figure 7 Cytological examination of embryos with abnormality. Superovulated COCs from wild-type mice (1a–1f) or SOD1-deficient mice (2a–2f) were subjected to IVF and incubated for 42 h after IVF under 1% O_2. Four-cell embryos were then transferred to atmospheric oxygen conditions and incubated further. 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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電気化学的呼吸計測技術におけるヒト胚クオリティー評価と安全性

Evaluating the Safety and Quality of Human Embryos with Measurement of Oxygen Consumption by Scanning Electrochemical Microscopy

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要旨：プローブ電極を用いた走査型電気化学顕微鏡(SECM)は，局所領域における生物反応を電気化学的にモニタリングできる。本研究では，電気化学呼吸計測技術を応用したヒト胚クオリティー評価法の安全性と胚評価の可能性を検討した。体外受精－胚移植または凍結胚移植を施行した後の未移植胚に対し，個々の胚の透明帯近傍をマイクロ電極で走査し胚の酸素消費量を測定した。測定時の最適状態を保つため，顕微鏡に設置されている保温プレートの温度について検討した。呼吸量測定群と非測定群にて胚の胚盤胞発生率も比較した。測定群は51.8%であり非測定群は57.0%と差は認めなかった。測定時のDay 3における分割は4細胞期～10細胞期であったが，平均呼吸量は各分割期で偏りはみられず，また，Veeckの分類とは相関のない呼吸活性を示した。測定後の胚を培養した結果，呼吸量が0.26-0.56 × 10^{14}/mol·s^{-1}であった胚の胚盤胞発生率は65.8%であり，0.26未満または0.56より大きい場合の胚盤胞発生率は39%と有意差が認められた。SECMを用いて胚の呼吸活性を計測することにより，従来のVeeck分類では知りえなかった胚の質を安全により厳密に評価することができる可能性が示唆された。

キーワード：呼吸量，走査型電気顕微鏡，安全性，胚評価，ヒト胚

ABSTRACT: Respiration is a useful parameter for evaluating embryo quality as it provides important information about metabolic activity. A scanning electrochemical microscopy (SECM) measuement system provides a non-invasive, simple, accurate, and consistent measurement of the respiration activity of single human embryos. In this study, we describe an accurate method for assessing the quality of individual human embryos. We measured oxygen consumption rates of human embryos at various cleavage stages. We monitored the temperature of the hot plate which was installed in the microscope to keep the most suitable state at the time of the measurement. We compared the blastocyst development of embryos in the measurement group with those of the no-measurement group. The measured embryos (51.8%) showed the same developmental rate to the blastocyst as the no-measurement group embryos (57.0%). There were no significant differences in the mean rates of oxygen consumption at each cleavage stage. Embryos with moderate respiration rates (0.26–0.56 × 10^{14}/mol·s^{-1}) showed a high developmental rate (65.8%) to the blastocyst. The developmental rate of embryos with lower and higher respiration rates (<0.26 × 10^{14}/mol·s^{-1} and >0.56 × 10^{14}/mol·s^{-1}) was 39.0%. These results support the suggestion that measuring embryonic respiration provides safety and valuable information about embryo quality.

Key words: Respiration, scanning electrochemical microscopy, safety, evaluation of embryos, human embryo

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緒　言

現在，体外受精における移植胚の選択は，移植時の胚の形態を基に行っている。妊娠率の向上，多胎率，流産率を減少させるには，より着床しうる能力を持った胚を

選択することが不可欠である。現在，胚のクオリティー評価は簡単で非侵襲的な方法である形態評価法が最も普及している。しかし，形態評価法は観察者の主観により判定結果に差が生じる可能性があるため，より客観的で，精度が高く，安全な胚クオリティー評価法の開発が望まれている。

筆者らは形態観察に比べてより客観的な方法として，細胞呼吸能測定による胚クオリティー評価方法を提案している。これは，高感度で生体反応を検出できる電気化学的計測技術の基盤である走査型電気化学顕微鏡（SECM）を用いて非侵襲的に胚の呼吸量を測定し，呼吸能を指標に胚のクオリティーを評価する新しい方法である。これまでにウシ胚を用いた研究により，呼吸活性を基準とする胚クオリティー評価法の開発に成功している[1,2]。さらに，SECMを用いて単一のヒト胚の呼吸量測定に初めて成功した[3]。そこで本研究は，SECMを用いた胚の評価が安全でかつ着床能を有する胚の選択が可能となるかを検討するために，まず胚の発育を観察した。Day 3胚（体外受精：Day 0）の呼吸量を測定後，追加培養を行い，呼吸量測定胚と非測定胚の発育能を比較することで，胚への影響を調査した。胚呼吸量の違いと胚発育の関係についても検討した。また，物理的影響を避けるため，最適顕微鏡ステージ温度についても検討を行った。

方　法

患者背景および測定方法

体外受精胚移植または凍結胚移植を施行し，患者の同意が得られた未移植胚を研究に用いた。対象期間は2006年7月から2007年7月で73周期188個の胚を測定した。平均年齢は34.5 ± 4.5歳で平均体外受精施行回数は2.7 ± 2.3回だった。受精後1日から3日までは，Sydney IVF Cleavage Medium（Cook社）を用いて5% O_2, 6% CO_2, 90% N_2の気相下に37℃で培養を行った。受精日をDay 0とし，Day 3にて4～10細胞期胚に発生した胚をVeeckの分類法[4]を基に評価した。胚を形態観察により評価した後，SECMをベースに開発した「受精卵呼吸測定装置」を用いて個々の胚の呼吸量を測定した。呼吸測定後，胚発生能を調べるために個々の胚をSydney IVF Blastocyst medium（Cook社）に培養液の交換を行い，Day 5–7まで培養した。装置は，倒立型顕微鏡，ポテンショスタット，呼吸解析ソフトを内蔵したノートパソコンにより構成されている。呼吸量の測定には，マイクロ電極と参照電極，測定プレートを用いた。測定プレートに10% Synthetic serum substitude（SSS：IS Japan）を添加したmodified-Human Follicle Fluid（m-HFF；扶桑薬品工業株式会社）を5 ml入れ，マイクロウェルの中にミネラルオイルが混入しないように胚を洗浄した後，マイクロウェルの底部中心に胚を静置した。参照電極を測定プレートの端に設置した後，マイクロ電極を透明帯の間近に移動させた。ポテンショスタットの電位を－0.6 V vs. Ag/AgCl（参照電極）に保持した後，移動速度30 μm/sec，走査距離160 μmの条件で透明帯に対して鉛直方向（Z軸方向）に掃引した（図1）。1個の胚に対する測定時間は約30秒である。測定中のマイクロウェル内温度や浸透圧変化を最小に留めるため，最適顕微鏡ステージ温度の検討を行った。浸透圧測定にはOSMOSTAT（アークレイ株式会社）を用い測定を行った。

結　果

顕微鏡ステージ温度について検討を行った。経時的に温度と浸透圧変化を調べた（図2）。40度に顕微鏡ステージ温度を設定した場合20分以後より温度低下を示し，45度では5分後より温度の上昇を認めた。浸透圧変化では，45度に設定した場合15分後には290 mOsm/kgを上回り上昇が早くなる結果を示した。42.5度では培養液の液温も35度を保ち浸透圧も30分までは300 mOsm/kgを上回ることはなく，比較的最適な条件を長く保つことが可能であると考えられる。

呼吸量測定による，胚発育への影響を評価するために呼吸量測定群と呼吸量非測定群で胚盤胞発生率も比較した。呼吸量測定群で胚盤胞発生率は57.0%，非測定群で51.8%と差は認めなかった（図3）。

同一分割期において，それぞれの胚の呼吸能に顕著な違いが認められた。例えば，Veeckの分類によって4分割グレード1と形態的に同じクオリティーと評価された胚の呼吸量を比較した結果，呼吸測定値はそれぞれ①0.25，②0.44，6分割グレード2では③0.57，④0.23，8分割グレード2では⑤0.71，⑥0.35であり胚によって顕著な違いが認められた（図4）。このように本研究では，Veeckの分類法による形態的評価と胚の呼吸能には明確な相関は認められなかった。Day 3における4～10細胞期胚（n=187）の呼吸量を測定した。各分割期の酸素消費（呼吸）量を表1に示す。呼吸量（$F \times 10^{14}$/mol s^{-1}）の平均値は，0.34–0.50であり各分割期間に顕著な差はなかった。Day 3胚の呼吸量測定後，胚盤胞期まで培養し，呼吸能と胚盤胞発生率の関係を調べた。その結果，Day 3において呼吸量が0.26-0.56 $\times 10^{14}$/mol s^{-1}であった胚は65.8%が胚盤胞に発生した。一方，呼吸量が0.26 $\times 10^{14}$/mol s^{-1}未満及び0.56 $\times 10^{14}$/mol s^{-1}より大きい場合の胚盤胞発生率は，39.0%であった（図5）。