placed in the perivitelline space of recipient oocytes through the same slit in the zona pellucida that had been made for enucleation. Then they were fused with the recipient oocytes using LF 101 (TR Tech, Tokyo, Japan) by stimulating direct current pulses of 150 V/mm for 50 μsec in 0.28 M mannitol solution supplemented with 0.1 mM $MgSO_4$ and 0.01% (w/v) PVA (Yin et al., 2003). Throughout the experiments, nuclear transfer was performed by a microinjection method of donor cells into recipient cytoplasts. Only in Experiment 3 did we further perform the electrofusion method.

Activation and culture of SCNT embryos

The activation of embryos was performed as described by Yamanaka et al. (2007). In brief, to activate the SCNT embryos, the reconstructed embryos at 3 h after injection or fusion of donor cells were treated with 15 μM ionomycin (Sigma) in PZM-3 containing fatty acid-free BSA (Yoshioka et al., 2002) for 20 min at 38.5°C in 5% CO_2 in humidified air and then washed with PZM-3. Next, 20 SCNT embryos were cultured in a 100-μL droplet of PZM-3 containing 5 μg/mL cycloheximide (Sigma) with cytochalasin D (Sigma) for 5 h at 38.5°C in 5% CO_2 in humidified air and then washed five times with cycloheximide-free PZM-3 (Sugimura et al., 2008). Last, the 20 embryos were transferred to a 100-μL droplet of PZM-3 and cultured at 38.5°C in an atmosphere of 5% CO_2 in air.

In vitro *fertilization*

IVF was also performed with *in vitro*-matured (IVM) oocytes. After the IVM, oocytes were washed three times with fertilization medium (TU medium) (Miyoshi et al., 1999), and 30 to 40 oocytes were transferred to a 100-μL droplet of IVF medium. Cryopreserved semen from Duroc boars was thawed, and spermatozoa were washed twice by centrifugation (1000×*g* for 4 min) in Dulbecco's PBS supplemented with 1 mg/mL BSA. Spermatozoa were resuspended in IVF medium, and 20 to 30 μL of the suspension was added to the drop containing oocytes to give a final concentration of 3×10^5 cells/mL. At 6 h postinsemination, 20 oocytes were washed three times in 100 μL droplet of PZM-3 and cultured in 100 μL droplet of PZM-3 at 38.5°C in 5% CO_2 in air. In our laboratory IVF system using the boar sperm, the rate of penetration and normal fertilization (both male and female pronuclei and two polar bodies) was 95.1 and 61.5%, respectively (Yamanaka et al., 2009).

Collection of in vivo *produced embryos*

Protocol of "surgical collection of embryos" was according to manual on pig embryo transfer (Kashiwazaki, 1993). Day 160 to 220 prepubertal gilts (Large-White) were treated with 1500 IU eCG, followed 72 h later by 500 IU hCG. Artificial insemination (AI) was performed three times from 24 h after hCG. Embryos on day 4 and 5 (day 0 = the day of first AI) were recovered surgically from the gilts.

Production of parthenogenetically activated embryos

To generate different types of parthenogenetically activated embryos (PA), IVM porcine oocytes without manipulation were activated by using the same methods as those used for generating SCNT embryos. From these oocytes, a small volume of ooplasm was removed, and a minimal amount of PB1 medium was injected (Sham-PA); an enucleated pFF cell was also injected (SCI-PA) in these cells. Enucleation of pFF was performed using the method described by Hayashi et al. (1991) with slight modification. Upon reaching confluence, pFF cells were enucleated by centrifugation (23,000×*g* for 30 min) in DMEM with cytochalasin B (10 μg/mL) and Hoechst 33342. Enucleation was confirmed by visualizing the cells under UV light. Subsequently, plasma membranes of the cells, in which enucleation was confirmed, were disrupted by gently aspirating the cells in and out of the beveled pipette; their cytoplasts were injected into the oocytes.

Measurement of oxygen consumption

Oxygen consumption by individual porcine embryos was noninvasively measured by the SECM system (HV-405; Hokuto Denko Co., Tokyo, Japan) (Abe and Hoshi, 2003; Shiku et al., 2001). Embryos were transferred singularly to a plate filled with 5 mL of embryo respiration assay medium-2 (ERAM-2; Research Institute for the Functional Peptides, Yonezawa, Japan) and dropped individually to the bottom of the microwell. The medium temperature was maintained at 37 C by placing on a warming plate on the microscope stage. The measurement of oxygen consumption was carried out according to the procedure previously described by Shiku et al. (2001). Briefly, Pt-microdisc electrodes, sealed in a tapered soft-glass capillary (PG10165-4: World Precision Instruments, Sarasota, FL), were fabricated according to the procedure described by Matsue et al. (1993). The tip potential was held at −0.6 V versus Ag/AgCl with a potentiostat to monitor the local oxygen concentration in the solution. The tip scanning rate was 31.0 μm/sec. The XYZ-stage and the potentiostat were controlled by a notebook computer. Voltammetry of the Pt-microdisc electrode in ERAM-2 solution showed a steady-state oxygen reduction wave. No response from other electrochemically active species was observed near the embryo surface. The oxygen consumption rate of embryos was calculated by software, in which the oxygen concentration difference between the bulk solution and sample surface (ΔC), and the oxygen consumption rate (F) of a single sample were estimated according to spherical diffusion theories (Shiku et al., 2001). We repeatedly scanned the electrode back and forth, three times, to estimate the mean ± standard deviation ($n \geq 4$) of the ΔC for each sample.

Differential staining

The cell allocation of blastocysts was assessed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cells according to the staining procedure described by Thouas et al. (2001) with slight modification. Briefly, TE cells of the blastocysts were stained with 100 μg/mL propidium iodide fluorochrome containing a permeabilizing solution of 2% (v/v) Triton X-100 ionic detergent for 20 sec. Blastocysts were then incubated overnight at 4°C in a second solution of 25 μg/ml. Hoechst 33342 in ethanol for fixation. Fixed and stained whole blastocysts

were mounted and assessed for cell number using an epifluorescence microscope.

Detection of apoptosis-positive cells by TUNEL assay

Detection of apoptosis-positive cells by TUNEL assay was performed as described by Yamanaka et al. (2009). Blastocysts from SCNT and IVF were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone and fixed in 2% (w/v) paraformaldehyde and 0.2% (v/v) Triton-X PBS solution for 40 h at room temperature (RT). A commercially available kit (ApopTag; Intergen, Purchase, NY, USA) was used for the detection of apoptosis-positive cells. For the positive control, blastocysts were treated with DNase I (10 IU/mL, Sigma). After washing with PBS supplemented with 0.1% (w/v) polyvinyl alcohol three times for 10 min each, the blastocysts were incubated in the equilibration buffer of the kit for 20 sec at RT. Then, they were incubated at 37°C for 2 h in a moist chamber with 70% (v/v) reaction buffer containing 30% (v/v) terminal deoxynucleotidyl transferase, digoxigenin-11-dUTP. The reaction was stopped by addition of 3% (v/v) stop/wash Buffer (Intergen) at 37°C for 10 min. After washing with PBS including 0.2% Triton-X for four times for 2 min each, they were incubated with antidigoxigenin antibody conjugated to horseradish peroxidase at RT for 1 h. After washing with 0.2 % Triton-X and 0.1% PVA PBS four times, blastocysts were stained with 10 μg/mL propidium iodide in PBS for 1 h at RT. All samples were examined under a laser-scanning confocal microscope (MCR-1024; BIO-RAD Hercule). The numbers of apoptosis-positive nuclei and total number of nuclei in blastocysts were determined.

Individual culture of blastocysts

At day 5 after *in vitro* culture, the blastocysts were collected and washed three times in 100-μL droplets of PZM-3. Thereafter, they were individually cultured in 96-well plates containing 100 μL of PZM-3 for 48 h at 38.5°C in 5% CO_2 in humidified air. The individual culture system was used in Experiments 4 and 5 (shown in Figs. 2, 3, and 4). Both IVF and SCNT blastocysts derived from the individual culture system did not differed from those of conversional group culture system, which was used in Experiment 7 (shown in Table 5), on the embryo cell numbers and apoptosis incidence.

Experimental Studies

In Experiment 1, the rates of cleavage and blastocyst formation in IVF and SCNT embryos were assessed on day 2 and on days 5, 6, and 7 (the day of IVF was defined as day 0) of *in vitro* culture, respectively. In each trial, IVF and SCNT embryos were cultured in each drop.

In Experiment 2, oxygen consumption in the preimplantation IVF and SCNT embryos was measured at the two-cell stage on day 1 (2C); four-cell stage on day 2 (4C); morula stage on day 4 (MO); and blastocyst stages on days 5 (D5BL), 6 (D6BL), and 7 (D7BL). In each trial, IVF and SCNT embryos were cultured in each drop, and measured oxygen consumption at each stage. Some of embryos measured oxygen consumption at D5BL, D6BL, and D7BL were counted total cells number with Hoechst 33342 for calculating oxygen consumption per one cell. As physiological control, *in vivo* produced embryos at molura stage on day 4 (MO) and blastocyst stage on day 5 (D5BL) were also measured oxygen consumption.

In Experiment 3, to further explore some of the possible causes of lower oxygen consumption in the SCNT embryos, we examined the effects of the following four parameters on oxygen consumption of SCNT embryos: removal of ooplasm, contamination of cytoplasm of the donor cell, method of nuclear transfer (microinjection or electrofusion), and artificial activation. In each trial, IVF, PA, Sham-PA, SCI-PA, IN-SCNT, and EF-SCNT embryos were cultured in each drop. The embryos at D5BL were measured for oxygen consumption and then counted for total cell numbers with Hoechst 33342.

In Experiment 4, we determined the correlation between oxygen consumption in embryos at D5BL and their subsequent hatching competence at D7BL. In each trial, IVF and SCNT embryos were cultured in each drop and oxygen consumption was measured at D5BL. Furthermore, these blastocysts were individually cultured, and the developmental stage—unexpanded, expanded (i.e., expansion of the blastocoele and decrease in the thickness of the zona pellucida), hatching (i.e., hatching of the embryos from the zona pellucida), and hatched (i.e., those embryos that had hatched from the zona pellucida) (Kameyama, 1998)—was assessed at 48 h after extended culture (D7BL). Expanded and hatching of SCNT could not be strictly assessed. This was because the SCNT embryos already had mechanical slits or holes in their zona pellucid.

In Experiment 5, to gain an in-depth understanding of the reason behind the lower oxygen consumption in the SCNT embryos, we focused on the total, ICM, and TE cell numbers and apoptosis incidence of embryos. First, we examined the total, ICM, and TE cell numbers and apoptosis incidence of embryos at D5BL and D7BL. In each trial, IVF and SCNT embryos were cultured in each drop and oxygen consumption was measured at D5BL. Next, we determined the correlation between oxygen consumption in embryos at D5BL and their total, ICM, and TE cell numbers and at D7BL. In each trial, IVF and SCNT embryos were cultured in each drop and oxygen consumption measured at D5BL. Furthermore, these blastocysts were individually cultured, and total, ICM, and TE cell numbers and apoptosis incidence were assessed at D7BL.

In Experiment 6, to evaluate whether oxygen consumption was altered by the difference in donor cell types such as pFF and pCC, the effect of donor cells on oxygen consumption in SCNT embryos at D5BL was studied. In each trial, IVF and three types of SCNT (pFF, pCC-Auto, and pCC-Allo) embryos were cultured in each drop. The embryos at D5BL were measured oxygen consumption and then counted total cells number with Hoechst 33342.

In Experiment 7, hatchability, embryo cell numbers and apoptosis incidence of SCNT embryos with pFF and pCC at D7BL was evaluated. In each trial, two types of SCNT (pFF and pCC) embryos were cultured in each drop. SCNT embryos with pCC were derived from both pCC-Auto and pCC-Allo. On the days specified in Experiment 1, the rates of cleavage and blastocyst formation were calculated. The embryos at D7BL assessed the rate of hatched blastocyst, ICM, and TE cell numbers and apoptosis incidence.

Statistical analysis

Each experiment was performed in at least triplicate. The correlation coefficients between oxygen consumption and cell allocation or apoptosis were determined by simple regression analysis. Percentage data were arc-sine transformed, and other data were analyzed using analysis of variance (ANOVA), followed by the Bonferroni procedure ($p < 0.05$). The statistical significance of all data was examined using StatView (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Experiment 1. In vitro *development of porcine IVF and SCNT embryos*

The cleavage rate did not significantly differ between the IVF and SCNT embryos (Table 1). On the other hand, at D5BL, D6BL, and D7BL, the blastocyst formation rates in the case of SCNT embryos were significantly lower than those in the case of IVF embryos ($p < 0.05$).

Experiment 2. Oxygen consumption in preimplantation IVF and SCNT embryos

As shown in Figure 1A, oxygen consumption, expressed as mean ± SD, in the preimplantation IVF embryos at the 2C and 4C stages was stable at a low value (0.35 ± 0.12 and $0.38 \pm 0.08 \times 10^{14}$/mol sec^{-1}, respectively). Thereafter, oxygen consumption significantly increased at the MO stage ($0.52 \pm 0.11 \times 10^{14}$/mol sec^{-1}) compared to that at the 2C stage ($p < 0.05$). Subsequently, this value increased 1.5 times at D5BL ($0.82 \pm 0.18 \times 10^{14}$/mol sec^{-1}). In contrast, the SCNT embryos at D5BL did not exhibit any increase in oxygen consumption from the MO stage, even up to D7BL (MO, $0.41 \pm 0.08 \times 10^{14}$/mol sec^{-1}; D5BL, $0.44 \pm 0.12 \times 10^{14}$/mol sec^{-1}; and D7BL, $0.46 \pm 0.11 \times 10^{14}$/mol sec^{-1}) (Fig. 1B). Furthermore, we compared the oxygen consumption calculated by per one cell of the IVF and SCNT embryos at the blastocyst stage (Fig. 1CD). The values in the case of the SCNT embryos were lower than those in the case of the IVF embryos at D5BL, D6BL, and D7BL ($p < 0.05$).

From the MO stage to the D5BL stage, we found the significant difference in the way of changing of oxygen consumption between the IVF and the SCNT embryos (Fig. 1AB). For this confirmation, we further examined the oxygen consumption in *in vivo*-produced embryos at both the MO and D5BL stages. The result revealed that the oxygen consumption in *in vivo* produced embryos increased 1.5 times from the MO stage to the D5BL stage as well as in the IVF embryos (MO: $n = 4$; $0.67 \pm 0.06 \times 10^{14}$/mol sec^{-1} and D5BL: $n = 8$; $0.93 \pm 0.14 \times 10^{14}$/mol sec^{-1}).

Experiment 3. Effect of removal of the ooplasm, contamination of the cytoplasm of the donor cell, method of nuclear transfer, and artificial activation on oxygen consumption at D5BL

As shown in Table 2, there were no significant differences with respect to the oxygen consumption and total cell number of embryos at D5BL between the IVF, PA, Sham-PA, and SCI-PA embryos; further, the values of oxygen consumption were significantly higher in these embryos than in the SCNT embryos ($p < 0.05$). Furthermore, there were no significant differences with respect to the oxygen consumption and total cell number of embryos at D5BL between the method of nuclear transfer; namely, the microinjection (IN-SCNT) and electrofusion (EF-SCNT) methods. Thus, the removal of ooplasm, contamination of the cytoplasm of the donor cell, method of nuclear transfer, and artificial activation did not influence anomalous oxygen consumption in the SCNT embryos.

Experiment 4. Correlation between oxygen consumption at D5BL and subsequent developmental competence

To clarify the correlation between oxygen consumption and subsequent development, the sample embryos that were used for analysis were aligned as previously described (Kameyama, 1998). We selected IVF and SCNT embryos at D5BL with similar morphology—in these embryos, the volume of the blastocoele cavity up to the vacuole was half that of the entire embryo (Fig. 2A). The oxygen consumption (mean ± SD) in the IVF embryos at D5BL definitely reflected their hatching ability at D7BL (Fig. 2B). However, the oxygen consumption was significantly lower at D5BL in the SCNT embryos than in the IVF embryos that underwent expansion at D7BL ($p < 0.05$).

Next, on the basis of the results shown in Figure 2B, oxygen consumption in the IVF and SCNT embryos at D5BL was classified into four classes, as shown in Figure 2C: <0.59, 0.59–0.74, 0.75–0.93, and $>0.93 \times 10^{14}$/mol sec^{-1}, representing embryos with the potential of undergoing one of the following developmental stages, respectively: unexpanded, expanded, hatching, and hatched. These four groups comprised 15.6, 31.3, 28.1, and 25% of the IVF embryos at D5BL, respectively. In contrast, at D5BL, most of the SCNT embryos were categorized as being in the unexpanded or expanded

TABLE 1. *IN VITRO* DEVELOPMENTAL COMPETENCE OF IVF AND 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)*
IVF	114	84 (74.0 ± 11.1)	37 (33.0 ± 11.8)[a]	47 (41.9 ± 11.8)[a]	47 (41.9 ± 11.8)[a]
SCNT	116	76 (65.4 ± 3.9)	19 (15.9 ± 4.9)[b]	28 (24.0 ± 6.2)[b]	30 (26.0 ± 6.2)[b]

[a-b]Different letters within the same stage indicate significant difference ($p < 0.05$).
IVF, *in vitro*-fertilized; SCNT, somatic cell nuclear transfer.

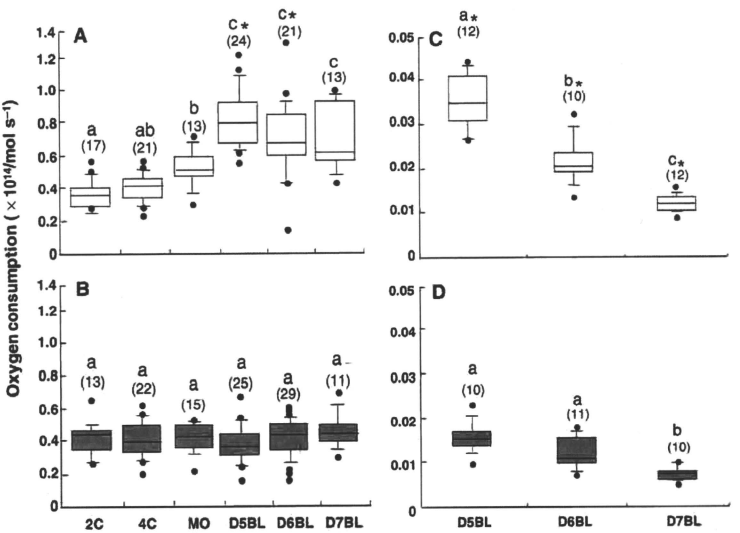

FIG. 1. Oxygen consumption in the preimplantation *in vitro*-fertilized (IVF) and somatic cell nuclear transfer (SCNT) embryos. The box-plot graphs represent oxygen consumption in the preimplantation IVF (A, C) and SCNT (B, C) embryos. The box indicates two quartiles, namely, the 25th and 75th percentiles, 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* ($l \times 10^{14}$ *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

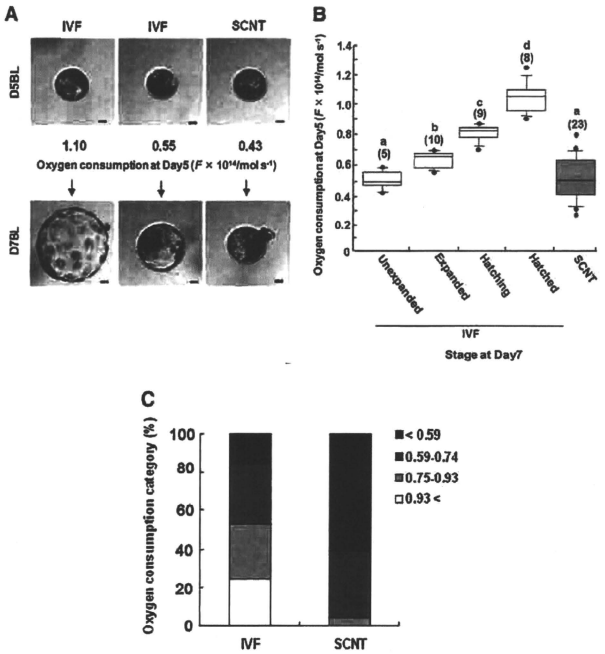

FIG. 2. Oxygen consumption on day 5 after *in vitro* culture and development of the embryo in the blastocyst stage on day 7. (**A**) Morphology of the embryos at D5BL (top) and D7BL (bottom). Bars = 30 μm. (**B**) Oxygen consumption at D5BL in unexpanded, expanded, hatching, and hatched blastocysts at 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).

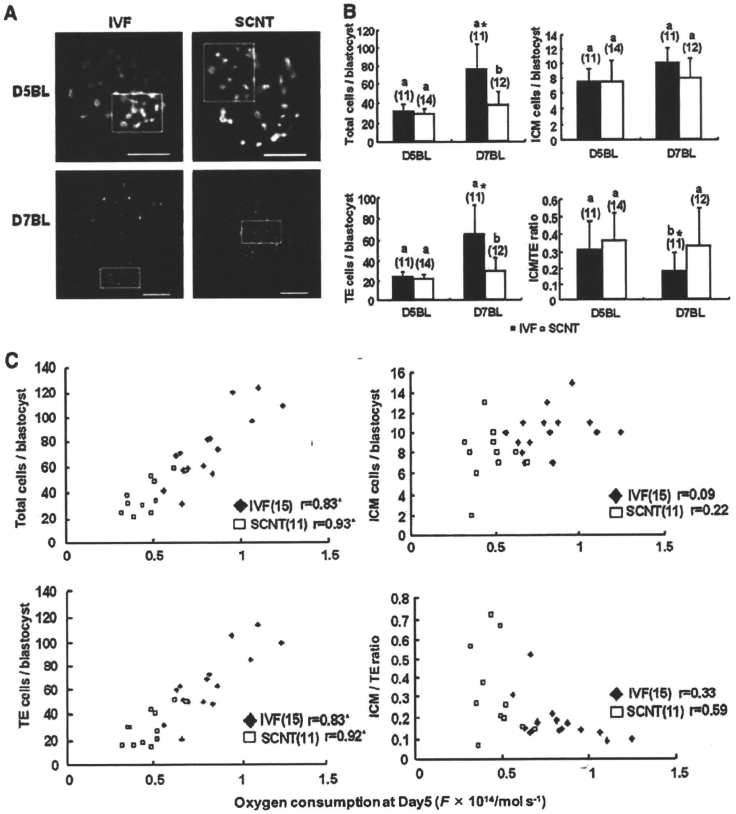

FIG. 3. Total, inner cell mass (ICM), and trophectoderm (TE) cell numbers in the IVF and SCNT embryos at D5BL and D7BL and correlation between oxygen consumption at D5BL and the values of these parameters at D7BL. (**A**) Differentially stained ICM and TE cells in the IVF and SCNT embryos at D5BL and 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

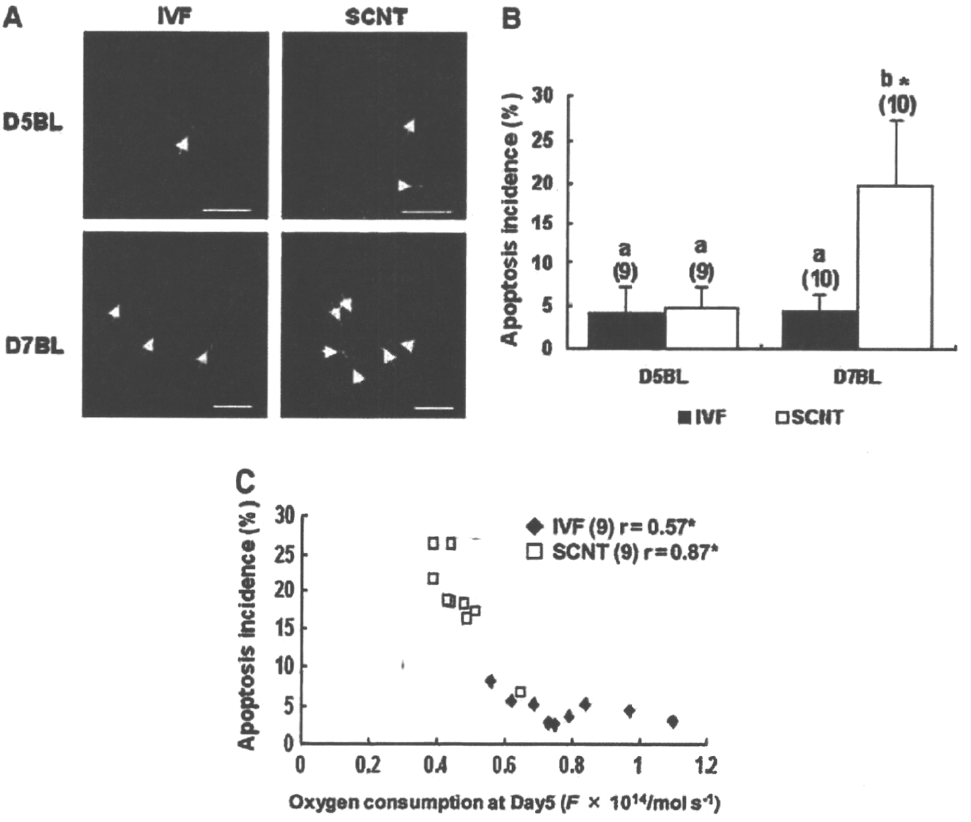

FIG. 4. Number of apoptosis-positive cells in the IVF and SCNT embryos at D5BL and D7BL and correlation between oxygen consumption at D5BL and the number of apoptosis-positive cells at D7BL. (A) Apoptosis-positive cells at D5BL (top) and D7BL (bottom) detected by TUNEL staining. The arrows indicate apoptosis-positive 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.

References

Abe, H., and Hoshi, H. (2003). Evaluation of bovine embryos produced in high performance serum-free media. J. Reprod. Dev. 49, 193–202.

Agung, B., Otoi, T., Abe, H. et al. (2005). Relationship between oxygen consumption and sex of bovine in vitro fertilized embryos. Reprod. Domest. Anim. 40, 51–56.

Beebe, L., McIlfatrick, S., Grupen, C., et al. (2007). A comparison of two in vitro maturation media for use with adult porcine oocytes for adult somatic cell nuclear transfer. Cloning Stem Cells 9, 564–570.

Boiani, M., Eckardt, S., Leu, N.A., et al. (2003). Pluripotency deficit in clones overcome by clone–clone aggregation: epigenetic complementation? EMBO J. 22, 5304–5312.

Boiani, M., Gambles, V.V., and Scholer, H.R. (2004). ATP level in clone mouse embryos. Cytogenet. Genome Res. 105, 270–278.

Chae, J.I., Cho, S.K., Seo, J.W., et al. (2006). Proteomic analysis of the extraembryonic tissue from cloned porcine embryos. Mol. Cell Proteomics 5, 1559–1566.

Dumollard, R., Duchen, M., and Carroll, J. (2007). The role of mitochondrial function in the oocyte and embryo. Curr. Top. Dev. Biol. 77, 21–49.

Funahashi, H., and Day, B.N. (1993). Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes in vitro. J. Reprod. Fertil. 98, 179–185.

Gao, S., Chung, Y.G., Williams, J.W., et al. (2003). Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol. Reprod. 69, 48–56.

Han, Z., Vassena, R., Chi, M.M., et al. (2008). Role of glucose in cloned mouse embryo development. Am. J. Physiol. Endocrinol. Metab. 295, 798–809.

Hao, Y., Lai, L., Mao, J., et al. (2003). Apoptosis and in vitro development of preimplantation porcine embryos derived in vitro or by nuclear transfer. Biol. Reprod. 69, 501–507.

Harvey, A.J., Kind, K.L., and Thompson, J.G. (2002). REDOX regulation of early embryo development. Reproduction 123, 479–486.

Hayashi, J., Ohta, S., Kikuchi, A., et al. (1991). Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. Proc. Natl. Acad. Sci. USA 88, 10614–10618.

Houghton, F.D., Thompson, J.G., Kennedy, C.J., et al. (1996). Oxygen consumption and energy metabolism of the early mouse embryo. Mol. Reprod. Dev. 44, 476–485.

Im, G.S., Lai, L., Liu, Z., et al. (2004). In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61, 1125–1135.

Jincho, Y., Sotomaru, Y., Kawahara, M., et al. (2007). Identification of genes aberrantly expressed in mouse embryonic stem cell-cloned blastocysts. Biol. Reprod. 78, 568–576.

Kameyama, K. (1998). Examination of embryo. In: *Japanese Society for Development of Swine New Technology. Manual on Pig Embryo Transfer Prosedures.* Nishigo: National Livestock Breeding Center, pp. 70–75.

Kashiwazaki, N. (1998). Surgical collection of embryos. In: *Japanese Society for Development of Swine New Technology. Manual on Pig Embryo Transfer Procedures.* Nishigo: National Livestock Breeding Center, pp. 53–61.

Kato, Y., Tani, T., Tsunoda, Y. (2000). Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J. Reprod. Fertil. 120, 231–237.

Kawahara, M., Wakai, T., Yamanaka, K., et al. (2005). Caffeine promotes premature chromosome condensation formation and in vitro development in porcine reconstructed embryos via a high level of maturation promoting factor activity during nuclear transfer. Reproduction 130, 351–357.

Kikyo, N., Wade, P.A., Guschin, D., et al. (2000). Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. Science 289, 2360–2362.

Koo, D.B., Kang, Y.K., Choi, Y.H., et al. (2002). Aberrant allocations of inner cell mass and trophectoderm cells in bovine nuclear transfer blastocysts. Biol. Reprod. 67, 487–492.

Lee, S.Y., Park, J.Y., Choi, Y.J., et al. (2007). Comparative proteomic analysis associated with term placental insufficiency in cloned pig. Proteomics 7, 1303–1315.

Leese, H.J. (2003). What does an embryo need? Hum. Fertil. (Camb.), 6, 180–185.

Lopes, A.S., Larsen, L.H., Ramsing, N, et al. (2005). Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. Reproduction 130, 669–679.

Manser, R.C., Leese, H.J., and Houghton, F.D. (2006) Effect of inhibiting nitric oxide production on mouse preimplantation embryo development and metabolism. Biol. Reprod. 71, 528–533.

Matsue, T., Koike, S., and Uchida, I. (1993). Microamperometric estimation of photosynthesis inhibition in a single algal protoplast. Biochem. Biophys. Res. Commun. 197, 1283–1287.

Miyoshi, K., Umezu, M., and Sato E. (1999). Effect of hyaluronic acid on the development of porcine 1-cell embryos produced by a conventional or new in vitro maturation/fertilization system. Theriogenology 51, 777–784.

Miyoshi, K., Saeki, K., and Sato, E. (2000). Improvement in development of porcine embryos reconstituted with cells from blastocyst-derived cell lines and enucleated oocytes by optimization of reconstruction methods. Cloning 2, 175–184.

Mohana Kumar, B., Jin, H.F., Kim, J.G., et al. (2006). DNA methylation levels in porcine fetal fibroblasts induced by an inhibitor of methylation, 5-azacytidine. Cell Tissue Res. 325, 445–454.

Nilsson, B.O., Magnusson, C., Widéhn, S., et al. (1982). Correlation between blastocyst oxygen consumption and trophoblast cytochrome oxidase reaction at initiation of implantation of delayed mouse blastocysts. J. Embryol. Exp. Morphol. 71:75–82.

Quinn, P., Barros, C., and Whittingham, D.G. (1982). Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J. Reprod. Fertil. 66, 161–168.

Santos, F., Zakhartchenko, V., Stojkovic, M., et al. (2003). Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr. Biol. 13, 1116–1121.

Shiku, H., Shiraishi, T., Ohya, H., et al. (2001). Oxygen consumption of single bovine embryos probed by scanning electrochemical microscopy. Anal. Chem. 73, 3751–3758.

Sugimura, S., Kawahara, M., Wakai, T., et al. (2008). Effect of cytochalasins B and D on the developmental competence of somatic cell nuclear transfer embryos in miniature pigs. Zygote 16, 153–159.

Thompson, J.G. (2000). In vitro culture and embryo metabolism of cattle and sheep embryos—a decade of achievement. Anim. Reprod. Sci. 60–61, 263–275.

Thompson, J.G., Partridge, R.J., Houghton, F.D., et al. (1996). Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J. Reprod. Fertil. 106, 299–306.

Thouas, G.A., Korfiatis, N.A., French, A.J., et al. (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod. Biomed. Online 3, 25–29.

Thouas, G.A., Trounson, A.O., and Jones, G.M. (2006). Developmental effects of sublethal mitochondrial injury in mouse oocytes. Biol. Reprod. 74, 969–977.

Trimarchi, J.R., Liu, L., Porterfield, D.M., et al. (2000). Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. Biol. Reprod. 62, 1866–1874.

Vassena, R., Han, Z., Gao, S., et al. (2007). Tough beginnings: alterations in the transcriptome of cloned embryos during the first two cell cycles. Dev. Biol. 304, 75–89.

Wakai, T., Sugimura, S., Yamanaka, K., et al. (2008). Production of viable cloned miniature pig embryos using oocytes derived from domestic pig ovaries. Cloning Stem Cells 10, 249–262.

Wakayama, T., Perry, A.C., Zuccotti, M., et al. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394, 369–374.

Yamanaka, K, Sugimura, S., Wakai, T., et al. (2007). Effect of activation treatments on actin filament distribution and in vitro development of miniature pig somatic cell nuclear transfer embryos. J. Reprod. Dev. 53, 791–800.

Yamanaka, K., Sugimura, S., Wakai, T., et al. (2009). Difference in sensitivity to culture condition between in vitro fertilized and somatic cell nuclear transfer embryos in pigs. J. Reprod. Dev. 55, 299–304.

Yang, F., Hao, R., Kessler, B., et al. (2007a). Rabbit somatic cell cloning: effects of donor cell type, histone acetylation status and chimeric embryo complementation. Reproduction 133, 219–230.

Yang, X., Smith, S.L., Tian, X.C., et al. (2007b). Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat. Genet. 39, 295–302.

Yin, X.J., Cho, S.K., Park, M.R., et al. (2003). Nuclear remodelling and the developmental potential of nuclear transferred porcine oocytes under delayed-activated conditions. Zygote 11, 167–174.

Yoshioka, K., Suzuki, C., Tanaka, A., et al. (2002). Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol. Reprod. 66, 112–119.

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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.

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.

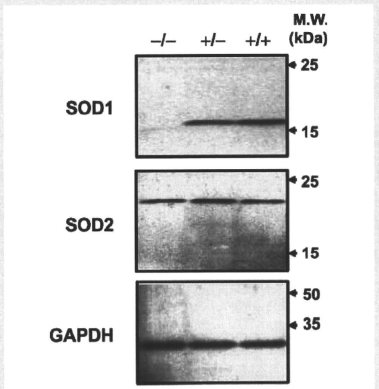

Figure 1 Western blot analysis of SOD1 and SOD2 in oocytes. Sixty superovulated MII oocytes from each genotypic female mouse were subjected to SDS–PAGE followed by immunoblot analysis. Antibodies against SOD1 (upper panel), SOD2 (middle panel) and GAPDH (lower panel, protein loading control) were used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

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 ($P < 0.05$).

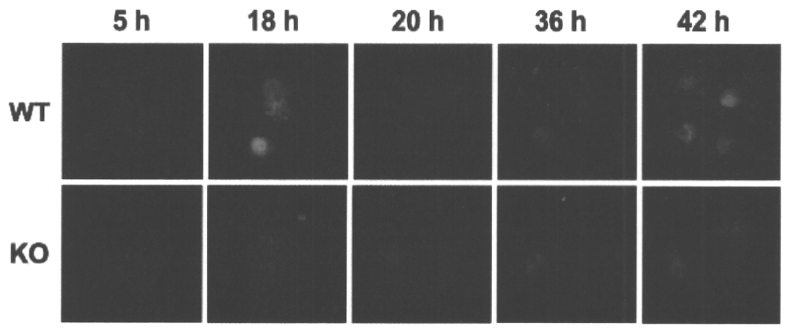

Figure 2 Chromosome labeling of zygotes/embryos. Superovulated COCs from wild-type (WT) or SOD1-deficient (KO) mice were subjected to IVF and incubated under atmospheric oxygen conditions. The zygotes/embryos were stained with Hoechst33342 at 5, 18, 20, 36 and 42 h after IVF and observed under a fluorescent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

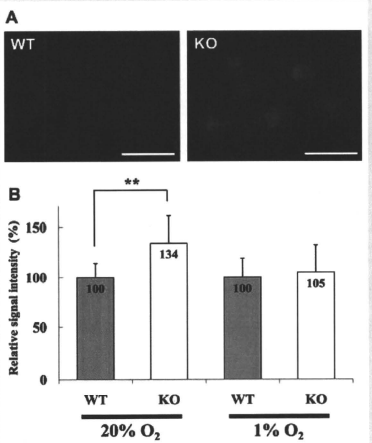

Figure 3 Fluorescent images and signal intensity of 2-cell embryos stained with dihydroethidium. (**A**) Two-cell embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes under 20% O_2 after dihydroethidium staining. Scale bars, 100 μm. (**B**) Embryos from wild-type (WT) and SOD1-deficient (KO)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

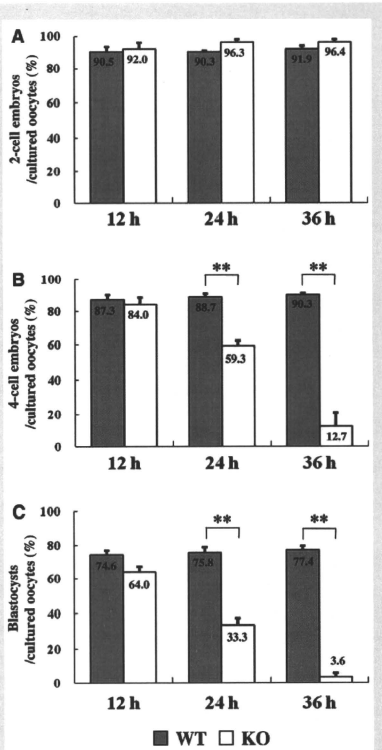

Figure 4 Time required for 2-cell arrest in oocytes from SOD1-deficient mice. Superovulated COCs from wild-type or SOD1-deficient mice were subjected to *in vitro* fertilization (IVF) and incubated under atmospheric oxygen conditions, and then transferred to 1% O_2 at 12, 24 and 36 h after IVF. (**A**)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

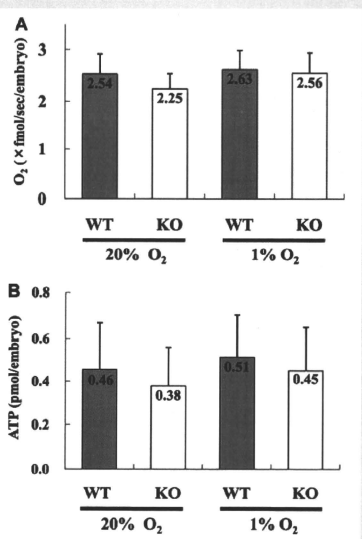

Figure 5 Respiratory activity and ATP levels of 2-cell embryos. (**A**) Embryos from wild-type (WT) or SOD1-deficient (KO) mouse oocytes were cultured under 1% or 20% O_2 for 36 h after IVF, and oxygen consumption in the individual embryos was measured by an SECM measuring system ($n = 10–13$). (**B**)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

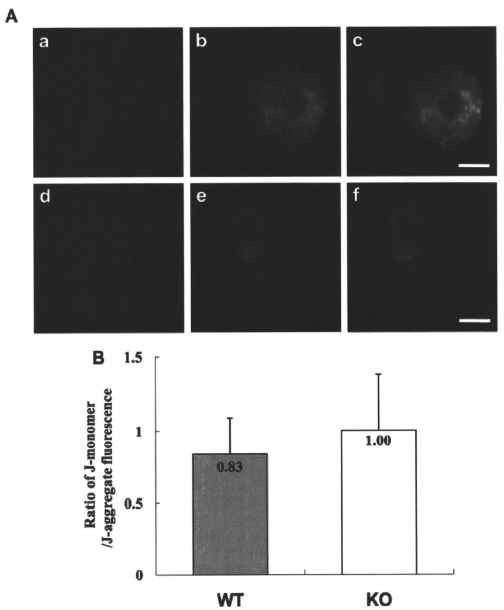

Figure 6 Fluorescent images and mitochondrial membrane potential of 2-cell embryos stained with JC-1. (**A**) Fluorescent images of JC-1-stained 2-cell embryos from wild-type (a–c) or SOD1-deficient (d–f) mouse oocytes under 20% O_2. (a and d) J-aggregate; (b and e) J-monomer; (c and f) Merge. Scale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

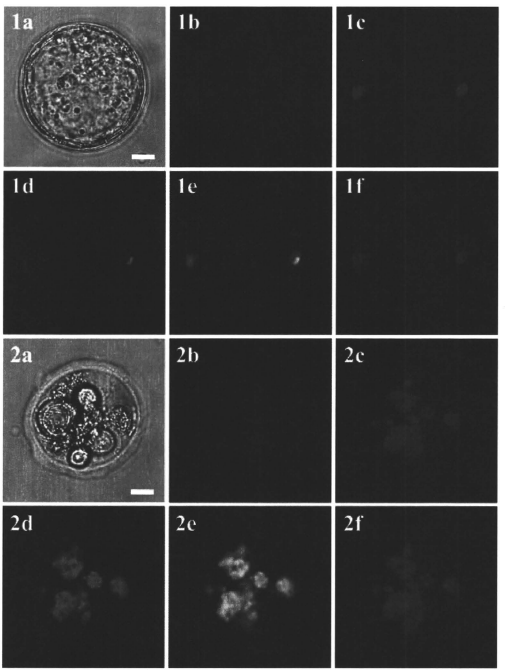

Figure 7 Cytological examination of embryos with abnormality. Superovulated COCs from wild-type mice (1a–1f) or SOD1-deficient mice (2a–2f) were subjected to IVF and incubated for 42 h after IVF under 1% O_2. Four-cell embryos were then transferred to atmospheric oxygen conditions and incubated further. 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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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.