mKRB–egg yolk medium containing 11.12 mM glucose, 1 mM pyruvate, and 43.16 mM lactate (control); glucose-free control (containing pyruvate and lactate); pyruvate-free control (containing glucose and lactate); lactate-free control (containing glucose and pyruvate); and substrate-free raffinose–mKRB–egg yolk medium. The final concentrations of the various substrates in medium containing sperm were 5.56 mM glucose, 0.5 mM pyruvate, and 21.58 mM lactate. The osmotic pressure of these solutions was adjusted to 400 mOsm with sucrose and the pH to 7.3 with HCl. The oxygen consumption rates of the sperm were measured by using Clark-type oxygen electrodes (Rank Brothers, Cambridge, UK) maintained at 37 °C for 10 min and calibrated with air-saturated water at 37 °C, which was assumed to contain 406 nmol oxygen/mL.[26] A sperm sample in a volume of 1 mL was suspended in the reaction chamber by stirring carefully to prevent the addition of any external air. The final concentration of sperm in the incubation chamber was approximately 1×10^7 sperm/mL. Data were acquired by using a commercial software program (LabChart version 5.2, AD Instruments, Castle Hill, Australia). The oxygen consumed by the sperm was calculated as:[26]

> Oxygen concentration (nmol oxygen/mL) = oxygen (U) × oxygen concentration of air-saturated water (that is, 406 nmol oxygen/mL) ÷ oxygen full-chart span (U)

The rate of oxygen consumption by sperm was expressed as nmol/min/1×10^7 sperm.

Experiment 2b. The effect of various concentrations of lactate in the raffinose–mKRB–egg yolk extender on the oxygen uptake of sperm was analyzed. Sperm were collected from the caudae epididymides of 5 rats and suspended in lactate-free raffinose–mKRB–egg yolk medium and then incubated for 5 min at 37 °C. Equal volumes of raffinose–mKRB–egg yolk medium containing 0, 21.58, 43.16, 64.74, and 86.32 mM lactate were diluted with lactate-free raffinose–mKRB–egg yolk medium, resulting in solutions with final lactate concentrations of 0, 10.79, 21.58, 32.37, and 43.16 mM, respectively. The osmotic pressure and the pH of all these solutions were adjusted to 400 mOsm and 7.3, respectively, except the solution containing 43.16 mM lactate (430 mOsm and pH 7.3). The oxygen consumption of each sperm suspension was determined in relation to air-saturated medium as described for experiment 2b.

Experiment 2c. The effect of adding ATP to raffinose–mKRB–egg yolk medium containing 32.37 mM lactate on the rate of oxygen consumption of sperm was examined. Sperm from 5 rats were flushed the sperm out by using the medium, and then the suspensions in ATP-free raffinose–mKRB–egg yolk medium were incubated for 5 min at 37 °C. Each treated sample was placed in raffinose–mKRB–egg yolk medium containing 0, 1.84, 3.70, 7.4, or 11.1 mM ATP. Subsequently, equal volumes of extended sperm were added to these solutions, resulting in solutions with final ATP concentrations of 0, 0.92, 1.85, 3.70, and 5.55 mM (400 mOsm and pH 7.3). The oxygen consumption of the sperm was determined as described for experiment 2a.

Statistical analysis. The data were subjected to ANOVA and the Fisher protected least-significant difference post hoc test (StatView, Abacus Concepts, Berkeley, CA). All data are expressed as mean ± SEM. A P value of less than 0.05 indicated statistical significance.

Results

Effect of various substrates in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm (experiment 1a). The first experiment in this series was aimed at assessing the effect of various energy-yielding substrates in the raffinose–mKRB–egg yolk extender on the motility characteristics of fresh and frozen–thawed sperm. The motility of sperm added to the medium without the substrates glucose, pyruvate, and lactate was significantly ($P < 0.05$) lower than that of sperm added to the medium containing all 3 of these substrates (control; Table 1); this result was obtained from both fresh and frozen–thawed sperm. In contrast, the sperm motility and motion parameters did not differ significantly between fresh and frozen–thawed sperm when glucose-free and pyruvate-free solutions were used. The medium that contained glucose, pyruvate, and lactate resulted in the highest motility of frozen–thawed sperm. The percentage of intact acrosomes did not differ significantly among sperm treated with the various extenders for both fresh and frozen–thawed sperm (Table 1).

Effect of lactate in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm (experiment 1b). According to the results of experiment 1a, lactate was the most effective agent for increasing the motility of both fresh and frozen–thawed sperm. We therefore investigated the effect of adding lactate at 0, 10.79, 21.58, 32.37, and 43.16 mM to the raffinose–mKRB–egg yolk medium on the motility of sperm. Sperm diluted in lactate-free extender showed significantly ($P < 0.05$) lower motility than did sperm diluted in extender containing 21.58 or 32.37 mM lactate (Table 2). The data revealed that sperm frozen in the raffinose–mKRB–egg yolk extender containing 32.37 mM lactate showed significantly ($P < 0.05$) higher motility after thawing than did sperm frozen in substrate-free extender. The proportion of sperm with intact acrosomes either before or after thawing did not differ significantly among extenders containing 0, 10.79, 21.58, 32.37, or 43.16 mM lactate.

Effect of ATP in raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on fresh and frozen–thawed sperm (experiment 1c). The effect of adding 0, 0.92, 1.85, 3.70, or 5.55 mM ATP to raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on the cryosurvival of the sperm are summarized in Table 3. Sperm frozen in extender containing 32.37 mM lactate and 1.85 mM ATP exhibited significantly ($P < 0.05$) higher motility than that of sperm frozen in ATP-free extender. The sperm frozen and thawed in extender supplemented with 1.85 mM ATP maintained significantly ($P < 0.05$) higher motility throughout the 3-h incubation at 37 °C than did sperm frozen and thawed in the ATP-free extender (Figure 1). The addition of ATP to the extender increased the proportion of intact acrosomes in after collected sperm, and among all concentrations of ATP tested, the percentage of intact acrosomes was highest at 1.85 mM ATP. Similar results were obtained for the acrosome status of frozen–thawed sperm.

Effect of glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk medium on the oxygen consumption of sperm (experiment 2a). In the next series of experiments, we examined the effect of the substrates glucose, pyruvate, and lactate in the raffinose–mKRB–egg yolk medium on the rate of oxygen consumption of sperm. Incubation of the sperm suspension with lactate-free medium resulted in a significant ($P < 0.05$) decline in the rate of oxygen consumption during incubation as compared with the incubation of the sperm suspension with medium containing glucose, pyruvate, and lactate (Figure 2). The oxygen consumption of sperm in medium lacking any added substrates tended to be decreased compared with that of sperm in the complete

Table 1. Effect of the substrates glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm

	Sperm characteristics	Control	–Glucose	–Pyruvate	–Lactate	Substrate-free
Fresh sperm	Motility (%)	78.2 ± 8.0	70.8 ± 2.6	67.3 ± 8.0	54.9 ± 6.1	44.8 ± 3.9[a]
	VSL (μm/s)	17.4 ± 1.2	16.5 ± 3.3	10.9 ± 1.4	15.4 ± 1.0	11.1 ± 1.9
	VCL (μm/s)	124.2 ± 3.1	115.1 ± 8.6	109.8 ± 1.9	108.3 ± 2.9	102.4 ± 4.1
	ALD (μm)	6.9 ± 0.1	6.5 ± 0.5	6.5 ± 0.2	6.5 ± 0.1	5.8 ± 0.3
	BCF (Hz)	22.2 ± 0.8	23.3 ± 1.8	25.4 ± 1.6	23.4 ± 1.0	29.0 ± 0.6
	Acrosomal integrity (%)	84.5 ± 2.9	76.2 ± 1.9	79.6 ± 4.9	77.1 ± 2.4	81.4 ± 4.4
Frozen–thawed sperm	Motility (%)	21.5 ± 1.4	23.2 ± 0.9	19.8 ± 1.3	13.8 ± 2.4[a]	13.7 ± 1.5[a]
	VSL (μm/s)	3.9 ± 0.4	3.2 ± 0.5	3.9 ± 0.4	3.9 ± 0.3	4.4 ± 0.8
	VCL (μm/s)	85.4 ± 8.7	71.8 ± 4.7	89.2 ± 13.2	77.8 ± 5.5	69.3 ± 7.4
	ALD (μm)	4.2 ± 0.5	3.3 ± 0.2	4.1 ± 0.5	3.8 ± 0.2	3.2 ± 0.5
	BCF (Hz)	32.2 ± 3.7	31.3 ± 1.6	36.2 ± 1.8	34.1 ± 0.9	31.7 ± 2.5
	Acrosomal integrity (%)	68.1 ± 4.0	71.3 ± 5.8	70.0 ± 2.0	64.1 ± 5.3	70.6 ± 2.3

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity.
Data are presented as mean ± SEM ($n = 3$).
[a]Value significantly ($P < 0.05$) different from control value.

Table 2. Effect of lactate in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm

		Lactate concentration (mM)				
	Sperm characteristics	0	10.79	21.58	32.37	43.16
Fresh sperm	Motility (%)	47.4 ± 4.9	46.3 ± 13.2	61.3 ± 2.1	67.5 ± 3.8[a]	55.1 ± 3.7
	VSL (μm/s)	12.5 ± 1.7	8.8 ± 1.0	16.5 ± 2.6	12.3 ± 1.2	9.8 ± 3.0
	VCL (μm/s)	98.3 ± 6.3	86.0 ± 2.7	94.6 ± 10.0	92.9 ± 3.1	95.9 ± 6.5
	ALD (μm)	5.3 ± 0.4	5.3 ± 0.3	4.8 ± 0.6	4.7 ± 0.3	5.1 ± 0.3
	BCF (Hz)	26.4 ± 1.4	24.7 ± 1.3	22.5 ± 0.8	24.5 ± 0.8	25.6 ± 1.3
	Acrosomal integrity (%)	75.9 ± 0.5	71.7 ± 4.0	73.6 ± 5.9	75.1 ± 0.5	71.8 ± 1.8
Frozen–thawed sperm	Motility (%)	11.3 ± 2.2	17.5 ± 3.5	19.7 ± 2.8	22.3 ± 4.0[a]	12.6 ± 4.3
	VSL (μm/s)	3.5 ± 0.6	2.8 ± 0.3	3.6 ± 0.2	3.8 ± 0.1	4.9 ± 0.5
	VCL (μm/s)	78.6 ± 8.2	75.0 ± 3.5	78.5 ± 4.2	95.0 ± 14.1	126.9 ± 5.1[a]
	ALD (μm)	3.4 ± 0.6	3.3 ± 0.1	3.5 ± 0.2	4.5 ± 0.6	5.5 ± 0.4[a]
	BCF (Hz)	44.8 ± 1.5	36.7 ± 1.8	39.8 ± 0.8	36.5 ± 2.3	42.9 ± 2.1
	Acrosomal integrity (%)	69.3 ± 2.9	71.6 ± 0.4	69.0 ± 0.2	69.2 ± 0.2	61.5 ± 1.8

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity.
Data are presented as mean ± SEM ($n = 3$).
[a]Value significantly ($P < 0.05$) different from control value.

medium. In contrast, oxygen consumption did not differ significantly between sperm in glucose- or pyruvate-free media and those in the medium containing glucose, pyruvate, and lactate.

Effect of lactate in raffinose–mKRB–egg yolk medium on the oxygen consumption of sperm (experiment 2b). The respiration capacity of sperm was tested after their incubation in a lactate-free raffinose–mKRB–egg yolk medium or in a medium supplemented with 10.79, 21.58, 32.37, or 43.16 mM lactate (Figure 3). Oxygen uptake was significantly ($P < 0.05$) higher in sperm incubated in medium containing 32.37 mM lactate than in sperm incubated in lactate-free medium.

Effect of ATP in the raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on the oxygen consumption of sperm (experiment 2c). This experiment evaluated the influence of supplementation of the raffinose–mKRB–egg yolk medium containing 32.37 mM lactate with various concentrations of ATP (0, 0.92, 1.85, 3.70, and 5.55 mM) on the oxygen consumption of sperm during incubation at 37 °C for 10 min (Figure 4). When the medium was supplemented with 1.85 mM ATP, the rate of oxygen consumption tended to be increased compared with that in ATP-free medium, but difference is not significant.

Discussion

The present study demonstrated that an extender of raffinose–mKRB–egg yolk containing 32.37 mM lactate enhanced the metabolic capacity and survival of rat sperm after cryopreservation. The cryosurvival of rat sperm was further improved by the addition of 1.85 mM exogenous ATP to the freezing extender.

When the oxidizable substrate lactate was not added to the raffinose–mKRB–egg yolk extender, the motility, viability, and rate of oxygen consumption decreased considerably in both fresh and frozen–thawed sperm. In contrast, sperm frozen and thawed in extender supplemented with 32.37 mM lactate exhibited higher motility than those frozen and thawed in lactate-free extender. This finding indicates that exogenous lactate in the freezing extender is a potent inducer that enhances the oxygen consumption of rat sperm and their motility after collection and freezing–thawing.

Table 3. Effect of ATP in raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on fresh and frozen–thawed sperm

		ATP concentration (mM)				
		0	0.92	1.85	3.70	5.55
Fresh sperm	Motility (%)	74.7 ± 1.8	72.8 ± 8.1	79.2 ± 3.3	73.8 ± 6.6	55.6 ± 4.2[a]
	VSL (μm/s)	9.1 ± 1.7	10.1 ± 0.5	14.7 ± 3.3	7.9 ± 0.6	8.4 ± 1.4
	VCL (μm/s)	110.2 ± 9.9	116.9 ± 8.5	123.6 ± 5.2	101.5 ± 8.8	104.9 ± 7.9
	ALD (μm)	6.0 ± 0.1	7.6 ± 0.6	6.3 ± 0.6	6.8 ± 1.0	5.7 ± 0.4
	BCF (Hz)	29.9 ± 2.8	34.0 ± 1.7	29.7 ± 2.7	32.9 ± 2.6	31.2 ± 1.5
	Acrosomal integrity (%)	75.5 ± 6.3	78.9 ± 6.5	83.2 ± 1.6	82.5 ± 7.2	77.1 ± 7.1
Frozen–thawed sperm	Motility (%)	20.6 ± 0.3	24.6 ± 0.9	35.3 ± 1.3[a]	26.9 ± 1.4	25.7 ± 7.2
	VSL (μm/s)	6.0 ± 1.2	5.0 ± 0.2	4.1 ± 0.6	4.4 ± 0.6	3.0 ± 0.4
	VCL (μm/s)	99.0 ± 8.3	89.3 ± 4.5	84.5 ± 4.1	85.8 ± 2.7	80.9 ± 3.9
	ALD (μm)	5.2 ± 0.8	4.4 ± 0.4	4.3 ± 0.1	4.3 ± 0.1	3.4 ± 0.1
	BCF (Hz)	37.1 ± 2.0	34.4 ± 0.6	35.0 ± 1.6	34.6 ± 5.0	37.2 ± 2.2
	Acrosomal integrity (%)	67.7 ± 2.3	68.4 ± 8.8	70.6 ± 4.6	61.7 ± 3.5	66.5 ± 3.8

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity.
Data are presented as mean ± SEM ($n = 3$).
[a]Value significantly ($P < 0.05$) different from control value.

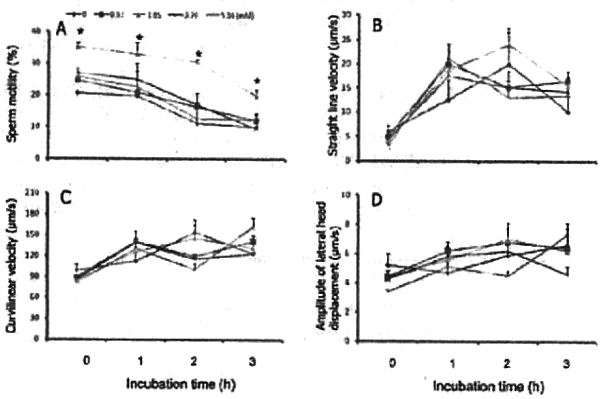

Figure 1. Effect of ATP in raffinose–mKRB–egg yolk medium containing 32.37 mM lactate on the (A) motility, (B) straight line velocity, (C) curvilinear velocity, and (D) amplitude of lateral head displacement of frozen–thawed sperm during incubation at 37 °C for 3 h. Data are presented as mean ± SEM ($n = 3$). *, Value significantly ($P < 0.05$) different from control value.

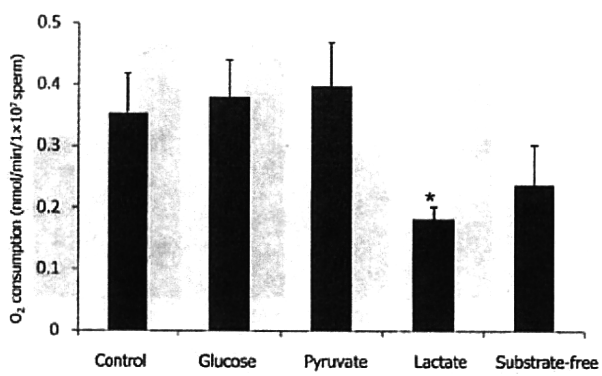

Figure 2. Effect of glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk medium on the oxygen consumption of fresh sperm during incubation at 37 °C for 10 min. Data are presented as mean ± SEM ($n = 5$). *, Value significantly ($P < 0.05$) different from control value.

The sperm-specific enzyme lactate dehydrogenase isozyme C_4 is located in the cytosol and the matrix of the mitochondria in the midpiece of rat sperm. Further, a study[9] has revealed that both a shuttle involving the redox couple lactate–pyruvate and lactate dehydrogenase isozyme C_4 are active in rat sperm mitochondria. In another study,[12] the lactate concentration in oviductal fluids was 10-fold higher than the glucose concentration, and the lactate concentration in the uterine fluids was 15-fold higher than the glucose concentration during the murine estrous cycle. Therefore, it is very likely that lactate is used by rat sperm as an essential substrate to maintain highly regulated ATP production and dissipation: lactate in the cytosol and mitochondrial matrix is oxidized to pyruvate by mitochondrial lactate dehydrogenase isozyme C_4, and pyruvate is oxidized through the Krebs cycle and electron transport chain.[4,5,23,24] To our knowledge, our findings are the first evidence showing that rat sperm can use exogenous lactate in the cryodiluent as an essential substrate to maintain highly regulated metabolic capacity and that this lactate acts as an energy substrate for mitochondria to the mobilization of fresh and frozen–thawed sperm.

Mitochondria, the site of ATP generation due to oxidative phosphorylation, are localized solely in the midpiece of sperm.[22] The oxidative production of ATP through the Krebs cycle is an essential function of the midpiece mitochondria for motility.[31] The mitochondrial biochemical pathways of oxidative phosphorylation are 15 times more efficient than is anaerobic glycolysis for ATP production.[7,28] These findings also support our arguments that the energy production and dissipation in rat sperm are highly dependent on the mitochondria.

The present study showed that supplementation of raffinose–mKRB–egg yolk extender with 32.37 mM lactate and 1.85 mM exogenous ATP considerably increases sperm motility before freezing, thus improving the survivability of sperm after cryopreservation. Exogenous ATP in the freezing medium may be responsible for the generation of multiple metabolic signals that appear to be related to the sperm motility through a rise in calcium levels;[10,17,18,20,25,27] this reaction increases de novo ATP synthesis before freezing and may contribute to the remobilization of sperm after freezing–thawing. The motility of ram sperm was restored by exogenous ATP that crossed plasma membrane when the membrane was damaged by cryopreservation.[13] In light of that finding,[13] we cannot discount that our result is caused by the facultative transport of ATP across plasma membrane because of damage during freezing, thereby allow-

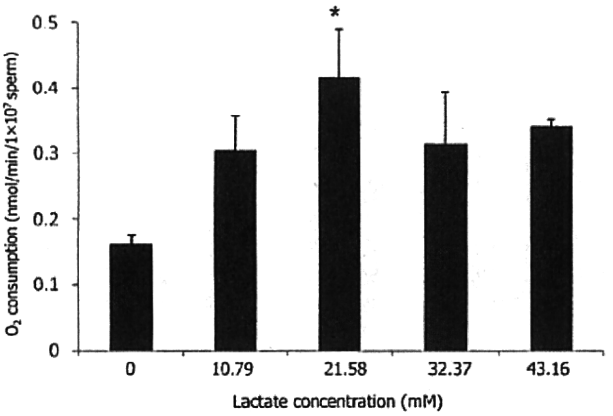

Figure 3. Effect of lactate in raffinose–mKRB–egg yolk medium on oxygen consumption of fresh sperm during incubation at 37 °C for 10 min. Data are presented as mean ± SEM (n = 5). *, Value significantly ($P < 0.05$) different from control value.

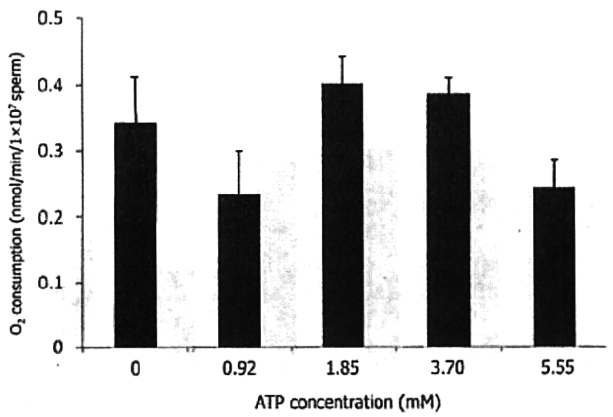

Figure 4. Effect of ATP in raffinose–mKRB–egg yolk medium containing 32.37 mM lactate on oxygen consumption of fresh sperm during incubation at 37 °C for 10 min. Data are presented as mean ± SEM (n = 5). *, Value significantly ($P < 0.05$) different from control value.

ing substrates to directly access ATP and allowing adenosine triphosphatase to use ATP directly to generate energy for the mobilization of rat sperm.

In conclusion, the current study demonstrated that the addition of lactate and ATP to the raffinose–mKRB–egg yolk extender before freezing increases the number of motile sperm and mediates the energy-dependent synthetic processes of rat epididymal sperm. In turn, these effects may increase the cryosurvival of rat sperm. Further investigation of species-specific differences in the energy-dependent synthetic processes in sperm may prove valuable in defining the ideal components of a cryodiluent, which interact to regulate the cryosurvival of rat sperm, and in clarifying the adaptations needed for cryopreservation of sperm from other species.

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References

1. **Aboagla EM, Terada T.** 2003. Trehalose enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol Rèprod **69**:1245–1250.
2. **Barbas JP, Mascarenhas RD.** 2009. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank **10**:49–62.
3. **Brokaw CJ.** 1972. Flagellar movement: a sliding filament model. Science **178**:455–462.
4. **Brooks GA, Dubouchaud H, Brown M, Sicurello JP, Butz CE.** 1999. Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. Proc Natl Acad Sci USA **96**:1129–1134.
5. **Brooks GA.** 2002. Lactate shuttles in nature. Biochem Soc Trans **30**:258–264.
6. **Cancel AM, Lobdell D, Mendola PP, Perreault SD.** 2000. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. Hum Reprod **15**:1322–1328.
7. **Cardullo RA, Baltz JM.** 1991. Metabolic regulation in mammalian sperm: mitochondrial volume determines sperm length and flagellar beat frequency. Cell Motil Cytoskeleton **19**:180–188.
8. **Ford WCL.** 2006. Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? Hum Reprod Update **12**:269–274.
9. **Gallina FG, Deburgos NMG, Burgos C, Coronel CE, Blanco A.** 1994. The lactate–pyruvate shuttle in spermatozoa: operation in vitro. Arch Biochem Biophys **308**:515–519.
10. **Gibbons IR.** 1963. Studies on the protein components of cilia from *Tetrahymena pyriformis*. Proc Natl Acad Sci USA **50**:1002–1010.
11. **Halang KW, Bohnensack R, Kunz W.** 1985. Interdependence of mitochondrial ATP production and extramitochondrial ATP utilization in intact spermatozoa. Biochim Biophys Acta **808**:316–322.
12. **Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM.** 2005. Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology **64**:992–1006.
13. **Holt WV, Head MF, North RD.** 1992. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. Biol Reprod **46**:1086–1094.
14. **Jones AR.** 1997. Metabolism of lactate by mature boar spermatozoa. Reprod Fertil Dev **9**:227–232.
15. **Kamp G, Busselmann G, Lauterwein J.** 1996. Spermatozoa: models for studying regulatory aspects of energy metabolism. Experientia **52**:487–494.
16. **Klingenberg M.** 1979. The ADP–ATP shuttle of the mitochondrion. Trends Biochem Sci **4**:249–252.
17. **Kinukawa M, Oda S, Shirakura Y, Okabe M, Ohmuro J, Baba SA, Nagata M, Aoki F.** 2006. Roles of cAMP in regulating microtubule sliding and flagellar bending in demembranated hamster spermatozoa. FEBS Lett **580**:1515–1520.
18. **Litvin TN, Kamenetsky M, Zarifyan A, Buck J, Levin LR.** 2003. Kinetic properties of 'soluble' adenylyl cyclase: synergism between calcium and bicarbonate. J Biol Chem **278**:15922–15926.
19. **Long JA.** 2006. Avian semen cryopreservation: what are the biological challenges? Poult Sci **85**:232–236.
20. **Luria A, Rubinstein S, Lax Y, Breitbart H.** 2002. Extracellular adenosine triphosphate stimulates acrosomal exocytosis in bovine spermatozoa via P2 purinoceptor. Biol Reprod **66**:429–437.
21. **Medrano A, Fernández-Novell JM, Ramió L, Alvarez J, Goldberg E, Rivera MM, Guinovart JJ, Rigau T, Rodríguez-Gil JE.** 2006. Utilization of citrate and lactate through a lactate-dehydrogenase- and ATP-regulated pathway in boar spermatozoa. Mol Reprod Dev **73**:369–378.
22. **Millette CF, Spear PG, Gall WE, Edelman GM.** 1973. Chemical dissection of mammalian spermatozoa. J Cell Biol **58**:662–675.
23. **Montamat EE, Vermouth NT, Blanco A.** 1988. Subcellular localization of branched-chain amino acid aminotransferase and lactate dehydrogenase C4 in rat and mouse spermatozoa. Biochem J **255**:1053–1056.
24. **Poole RC, Halestrap AP.** 1993. Transport of lactate and other monocarboxylates across mammalian plasma membranes. Am J Physiol **264**(4Pt 1):C761–C782.
25. **Ren D, Navarro B, Perez G, Jackson AC, Hsu SQ, Shi Q, Tilly JL, Clapham DE.** 2001. A sperm ion channel required for sperm motility and male fertility. Nature **413**:603–609.

26. **Reynafarje B, Costa LE, Lehninger AL.** 1985. O_2 solubility in aqueous media determined by a kinetic method. Anal Biochem 145:406–418.
27. **Rodriguez-Miranda E, Buffone MG, Edwards SE, Ord TS, Lin K, Sammel MD, Gerton GL, Moss SB, William CJ.** 2008. Extracellular adenosine 5′-triphosphate alters motility and improves the fertilizing capability of mouse sperm. Biol Reprod 79:164–171.
28. **Ruiz-Pesini E, Díez-Sánchez C, López-Pérez MJ, Enríquez JA.** 2007. The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? Curr Top Dev Biol 77:3–19.
29. **Storey BT, Kayne FJ.** 1978. Energy metabolism of spermatozoa. VII. Interactions between lactate, pyruvate and malate as oxidative substrates for rabbit sperm mitochondria. Biol Reprod 18:527–536.
30. **Storey BT.** 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int J Dev Biol 52:427–437.
31. **Suarez SS, Marquez B, Harris TP, Schimenti JC.** 2007. Different regulatory systems operate in the midpiece and principal piece of the mammalian sperm flagellum. Soc Reprod Fertil Suppl 65:331–334.
32. **Turner RM.** 2003. Tales from the tail: what do we really know about sperm motility? J Androl 24:790–803.
33. **Yamashiro H, Han YJ, Sugawara A, Tomioka I, Hoshino Y, Sato E.** 2007. Freezability of rat epididymal sperm induced by raffinose in modified Krebs–Ringer bicarbonate (mKRB) based extender solution. Cryobiology 55:285–294.

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臨床経験

選択的単一胚移植（eSET）における移植胚選別困難例に対する呼吸量測定の有用性

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eSETは最も発育能のある胚を見分け移植することが望まれる。移植胚選別において，Day 3の形態評価に呼吸量評価を加えることで発育能のある胚が選別できるか検討した。Day 3の分割別に呼吸量の違いによる胚盤胞到達率を調べたところ，形態評価のみで高率に胚盤胞へ到達する8分割を除いた9分割以上，7，6分割で呼吸量を測ることで胚盤胞到達胚の選別が可能となった。呼吸量が最も大きい胚を移植した場合，形態評価のみの移植と比較して，高い妊娠率と低い流産率が得られた。また呼吸量測定した胚移植で健常な出生を確認した。

はじめに

近年の妊娠率向上，多胎率の上昇に伴い体外受精の移植胚は1個とすることが求められている[1]。着床能のある1個の胚を見分け移植することが望まれるが，移植時に形態が等しく，選別困難である場合も少なくはない。妊娠率を低下させることなく多胎率，流産率を減少させるには，より着床しうる能力を持った1個の胚を選択することが不可欠である。現在，胚の評価は簡単で非侵襲的な方法である顕微鏡下での形態評価のみで行われている。しかし，形態評価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，安全な胚評価法の開発が望まれている。電気化学計測技術を基盤とする走査型電気化学顕微鏡は受精卵の呼吸活性を非侵襲的に測定することができる。

この細胞呼吸能測定による胚評価法を形態評価に加えることで，最も着床能のある1個の胚を見分けることが可能となるか検討した。呼吸量測定後，出産した症例についての出生結果も報告する。

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1. 対象および方法

呼吸量測定装置と，測定の概略を示す（図1）。方法は那須らの方法に順じた[2]。

1. 検討1：Day 3（媒精後3日目）の分割数と呼吸量の関係

2006年7月～2009年9月に，Day 3-eSET（elective Single Embryo Transfer）予定となり，移植時Veeckの分類にてグレード1もしくは2である良好胚が2個以上存在し移植胚の選別が困難と思われる307症例574個の胚を対象とした。Day 3に観察した分割数別に，胚呼吸量を調べ，胚盤胞到達率を調べた。測定後移植した胚は今回の検討から除いている。

2. 検討2：eSETにおける検討

2007年3月～2009年9月にVeeckによる形態評価がまったく等しい良好胚が2個以上存在

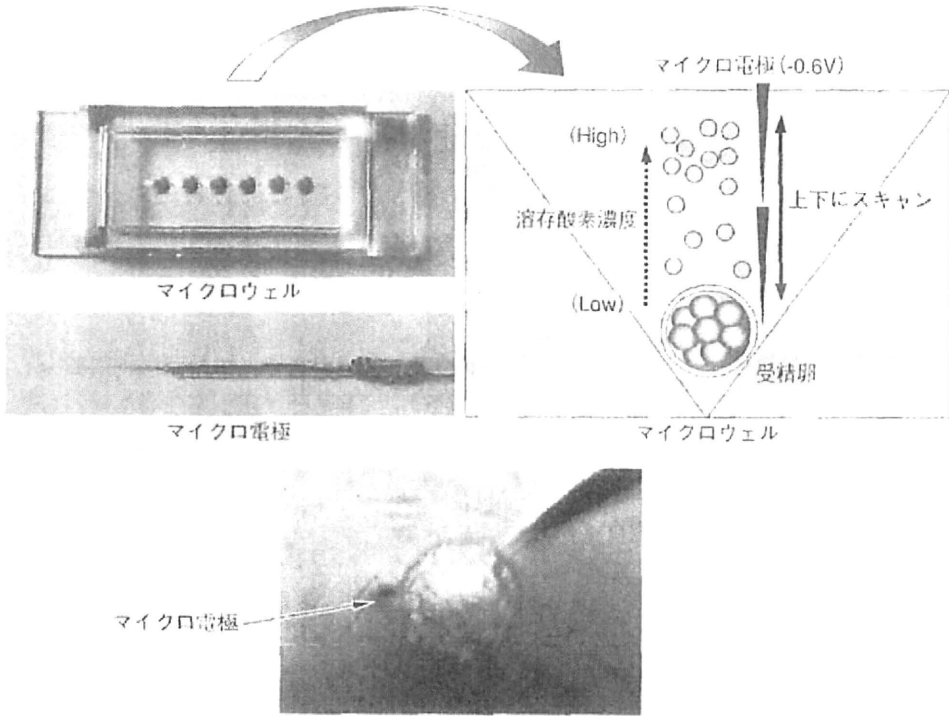

図1　呼吸量測定の概略

し，Day 3-eSET を行った症例 68 周期を対象とした。前方視的検討として Day 3 の形態がまったく同じ良好胚が 2 個以上存在した場合，移植胚の選択は無作為に呼吸量測定群と形態評価のみの群に振り分け，妊娠率，流産率，継続妊娠率を検討した。

3．検討 3：出生について

呼吸量評価が高く移植胚となり，出産に至った出生結果を形態評価のみで出産した場合と比較した。

II．結　果

1．検 討 1

Day 3 で 9 割球以上に分割していた胚の胚盤胞到達率は呼吸量 $0.3\times10^{14}/mols^{-1}$ 未満で 46.2%，0.3～0.4 未満で 81.8%，0.4～0.5 未満は 66.7%，0.5 以上で 65.5% と 0.3 未満と比較し 0.3～0.4 未満で胚盤胞到達率は高くなる結果を示した（$p<0.05$）。8 分割胚では呼吸量の差による発育の違いは認めなかった（77.1%，70.2%，76.7%，83.3%）（図 2）。

7 分割胚ではどの群にも有意差は認めなかったが，呼吸量が大きくなるに従い胚盤胞到達率が高くなる傾向を示した（50.0%，61.3%，56.0%，69.4%）。6 分割胚ではその傾向も顕著となり，呼吸量 0.3 未満と比べ，0.5 以上で胚盤胞到達率は有意に高くなる結果を認めた（$p<0.05$）（29.4%，38.2%，50.0%，57.1%）（図 3）。

2．検 討 2

形態評価がまったく等しい良好胚が 2 個以上ある移植胚選別困難例において，呼吸量測定群の妊娠率は 50.0%，形態評価のみの群は 26.5% で呼吸量の大きい胚を移植することにより，高い妊娠率が得られた（$p<0.05$）（図 4）。流産率は呼吸量評価を加えた群で 5.9%，形態評価のみでは 22.2% と呼吸量評価を加えた群で低くなる傾向を示した。継続妊娠率は，従来の形態評価のみでは 20.6% であったのに対し，呼吸量評価を行うことで 47.1% にまで向上した（$p<0.05$）。

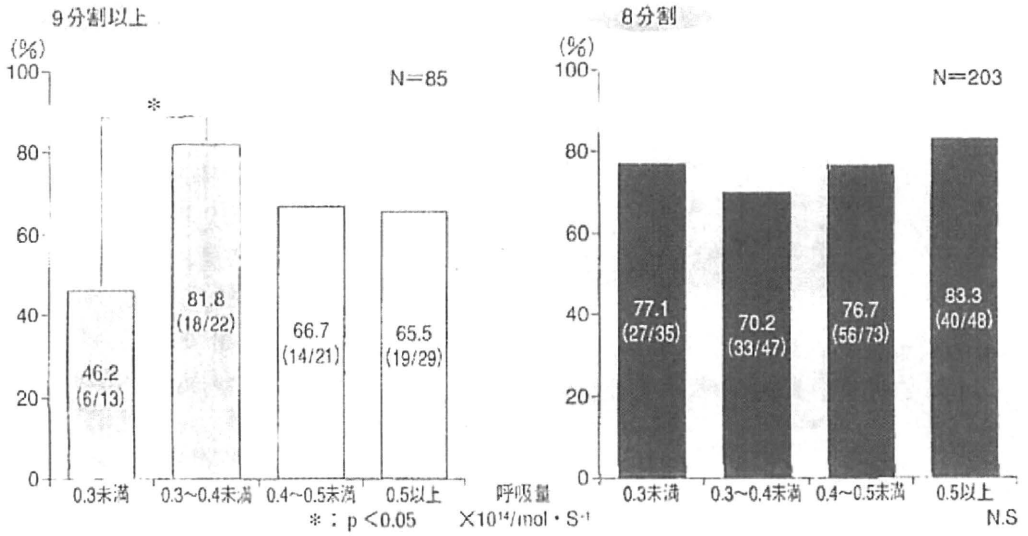

図2　分割数別にみた胚呼吸量と胚盤胞到達率

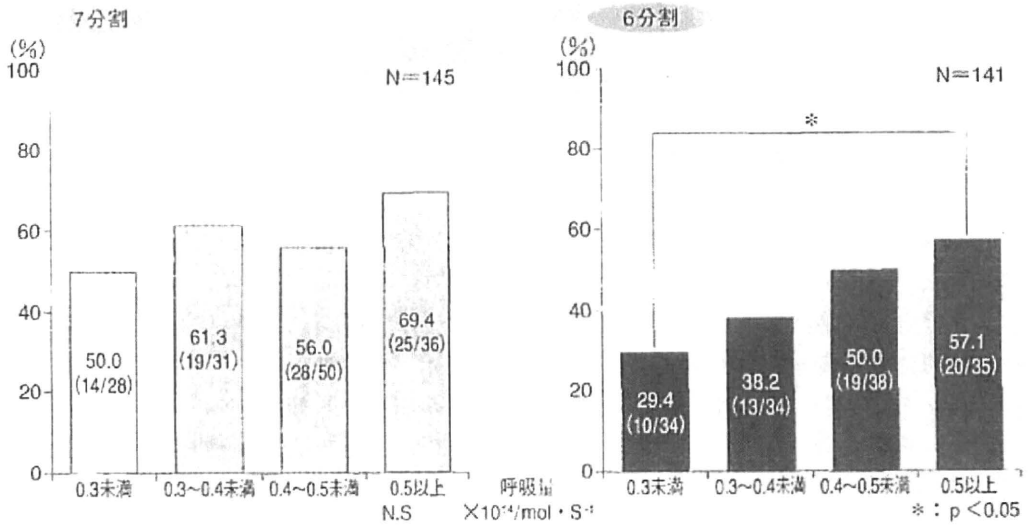

図3　分割数別にみた胚呼吸量と胚盤胞到達率

3. 検討3

現在まで判明しているそれぞれの出生結果を示す。呼吸量評価が高く移植胚となり，妊娠が成立し，出産に至ったのは11名で，男児6名，女児5名，平均体重は2,906 gだった。形態評価のみで出産に至った7名は，男児4名，女児3名，平均体重は2,720 gだった。両群に有意差を認めず，呼吸量測定を行っても健常な出生を認めた。

III. 考　察

体外受精が登場して以来，妊娠率向上のため多くの施設で2個以上の胚移植が行われてきた。近年の体外受精技術の発展により，妊娠率は上昇し，それに伴い多胎率の増加も報告されている[1]。体外受精技術を提供しているわれわれにとって，妊娠率を低下させることなく，危険な合併症や周産期異常が危惧される多胎妊娠

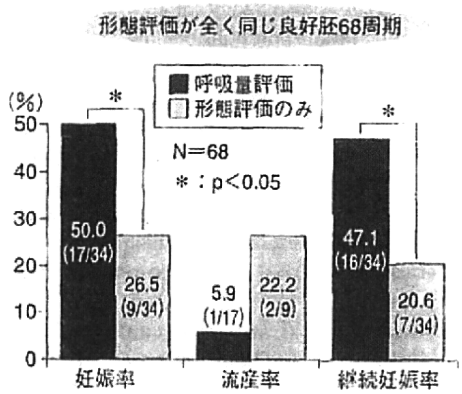

図4　選択的単一胚移植（eSET）の妊娠率

を防ぐことは，重要な課題である。2008年2月，日本産科婦人科学会から条件付きで移植胚は原則1個とするという改定案が出された。このため最も着床能力のある1個の胚を見分けることがさらに要求される。

現在まで妊娠率向上のため多くの研究者より様々な胚の形態評価法が報告された。前核期ではZollnerら[3]，Scottら[4]，Tesarikら[5]が前核の接着や核小体数の評価に基づく判定を提案している。2細胞期胚ではそれぞれの細胞の核の局在判定と早期分割による胚発生能の評価法が提案されている[6]。1998年頃からは，胚の長期培養による選別法が考えられ，胚盤胞期移植が注目されるようになった。分割期と胚盤胞期移植の比較検討では，Gardnerら[7]が，胚盤胞期移植で着床率は有意に上昇したと報告した。しかし，Coskunら[8]やUtsunomiyaら[9]は，胚盤胞まで発育が進まず移植キャンセルとなった症例を含めると，妊娠率は分割期移植と有意差はないという報告もしている。これらの評価法はすべて形態評価であり，観察者の主観によって差が生じる可能性がある。このことからもいまだ決定的な胚の選別方法は確立されていないと考えられる。

阿部らは，細胞活動に必要なエネルギー（ATP）を産生するミトコンドリアの酸化的リン酸化反応（呼吸）に着目した[10][11]。胚の呼吸量を測定することにより，品質評価が可能となるか検討するため，非侵襲的な呼吸測定装置を開発している[12]~[14]。これまでに，ウシ，ブタ，マウスの呼吸量解析に成功しており，胚評価への有効性を示した[15]。そこで，われわれはヒト胚への応用を目的に，余剰胚の呼吸量測定を行った。呼吸量測定の安全性を確認するため，呼吸量測定胚および非測定胚において，胚盤胞到達率に差がないか検討したが，両群で差は認めなかった[16]。また，胚の品質と呼吸量の関連性を検討した結果，Veeckの分類法による形態評価は呼吸量と必ずしも一致しないことを示し，呼吸量測定は，形態観察では評価できない胚の状態を数値化して示すことが可能となることを報告した[17]。また，ミトコンドリアの発達と呼吸量の増加が一致することも明らかにされている[18]。

以前のわれわれの検討では，移植胚の選択は呼吸量評価のみで行うより，形態評価のみで行うほうが妊娠率は高くなることを示した（形態評価のみ39.3%，呼吸量評価のみ26.7%，2008年日本生殖医学会シンポジウムにて）。そこで今回は形態評価に呼吸量評価を加えることで，形態評価のみで選ぶよりも着床能を持った1個の胚を選択することができるか検討した。当院で移植時良好胚が2個以上存在する移植胚選別困難例での，Day 3時形態評価のみの胚盤胞到達率は9分割以上で67.1%，8分割は76.8%，7分割は59.3%，6分割は44.0%で8分割を示す胚で最も胚盤胞到達率が高かった。今回検討した9分割以上，7分割および6分割胚では呼吸量が大きくなるほど胚盤胞到達率は高くなる結果を示し，形態評価のみでも高率に胚盤胞へ発育する8分割では呼吸量の違いによる差は認めなかった。吉田[19]はDay 3に観察したとき，8分割を示す胚は他の分割を示す胚よりも，最も着床率が高くなることを報告しており，このことからもDay 3に良好な8分割を示していた胚はそれだけで十分高い発育能があることを示唆している。しかし，それ以外の胚では胚の呼吸量を測定することで，胚の品質を数値化して客観的に確認することができ，呼吸量の大きい

胚を選ぶことで，その後の発育可能な胚を見分けることが可能となる。

検討2のDay 3-eSETの検討では，前方視的検討として無作為に呼吸量を加えた群と形態評価のみで移植を行った群に振り分けたが，呼吸量を加えた群で妊娠率は高く，流産率も低くなり，それに伴い継続妊娠率が高くなる結果が得られた。形態評価がまったく等しく見える胚でもその後の発育能は異なり，このことが，呼吸量を測定することにより知りえたための結果であると考えられる。

呼吸量測定胚の出生結果でも呼吸量測定胚，非測定胚で差はみられないことが確認できた。

おわりに

胚の呼吸量を測定することは，形態評価のみでは知りえなかった胚の状態を数値化し，客観的に確認することができる。呼吸量測定を形態評価に加えることで，最も着床能のある1個の胚を選別することが可能となることが示された。

文　献

1) Mullin CM, Fino ME, Talebian S, et al : Comparison of pregnancy outcomes in elective single blastocyst transfer versus double blastocyst transfer stratified by age. Fertil Steril, 93 : 1837-1843, 2010. Epub Feb 27. 2009.
2) 那須　恵，後藤香里，熊迫陽子，他：電気化学的呼吸計測によるヒト胚のクオリティー評価．産婦の実際，57 : 289-294, 2008.
3) Zollner U, Zollner KP, Hartl G, et al : The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts : the German experience. Hum Reprod, 17 : 1327-1333, 2002.
4) Scott L, Alvero R, Leondires M, et al : The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum Reprod. 15 : 2394-2403, 2000.
5) Tesarik J, Greco E : The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum Reprod, 14 : 1318-1323, 1999.
6) Ciray HN, Karaqenc L, Uluq U, et al : Early cleavage morphology affects the quality and implantation potential of day 3 embryos. Fertil Steril, 85 : 358-365, 2006
7) Gardner DK, Schoolcraft WB, Wargley L, et al : A prospective randomized trial of blastocyst culture and transfer in *in-vitro* fertilization. Fertil Steril, 13 : 3434-3440, 1998.
8) Coskun S, Hollanders J, Al-Hassan S, et al : Day 5 versus day 3 embryo transfer : a controlled randomized trial. Hum Reprod, 15 : 1947-1952, 2000.
9) Utsunomiya T, Ito H, Nagaki M, et al : A Prospective, Randomized Study : Day-3 versus Hatching Blastocyst Stage. Hum Reprod. 19 : 1598-1603, 2004.
10) Abe H, Matsuzaki S, Hoshi H : Ultrastructural differences in bovine morulae classified as high and low qualiies by morphological evaluation. Theriogenology, 57 : 1273-1283, 2002.
11) 阿部宏之：電気化学的イメージング技術を応用した胚のクオリティー評価．産婦の実際，55 : 207-216, 2006.
12) Abe H, Shiku H, Aoyagi S, et al : *In vitro* culture and evaluation of embryos for production of high quality bovine embryos. J Mamm Ova Res, 21 : 22-30, 2004.
13) Shiku H, Shiraishi T, Ohya H, et al : Oxygen consumption of single bovine embryos probed with scanning electrochemical microscopy. Anal Chem. 73 : 3751-3758, 2001.
14) Shiku H, Shiraishi T, Aoyagi S, et al : Respiration activity of single bovine embryos entrapped in a cone-shaped microwell monitored by scanning electrochemical microscopy. Anal Chim Acta, 522 : 51-58, 2004.
15) Abe H, Shiku H, Yokoo M, et al : Evaluating the quality of individual embryos with a non-invasive and highly sensitive measurement of oxygen consumption by scanning electrochemical microscopy. J Reprod Dev, 52 (Suppl): S55-S64, 2006.
16) 後藤香里，小池　恵，熊迫陽子，他：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．日受精着床会誌，27 : 53-58, 2010.
17) Utsunomiya T, Goto K, Nasu M, et al : Evaluating the quality of human embryos with a measurement of oxygen consumption by scanning electrochemical microscopy. J Mamm Ova Res. 25 : 2-7, 2008.
18) 阿部宏之，横尾正樹，荒木康久，他：電気化学的イメージング法を応用した単一ヒト胚の呼吸能解析．産婦の実際，56 : 2053-2057, 2007.
19) 吉田　淳：胚の選別と胚移植，ART実践マニュアル，p 63，永井書店，2005.

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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) measurement 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周辺に移動させた。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）。

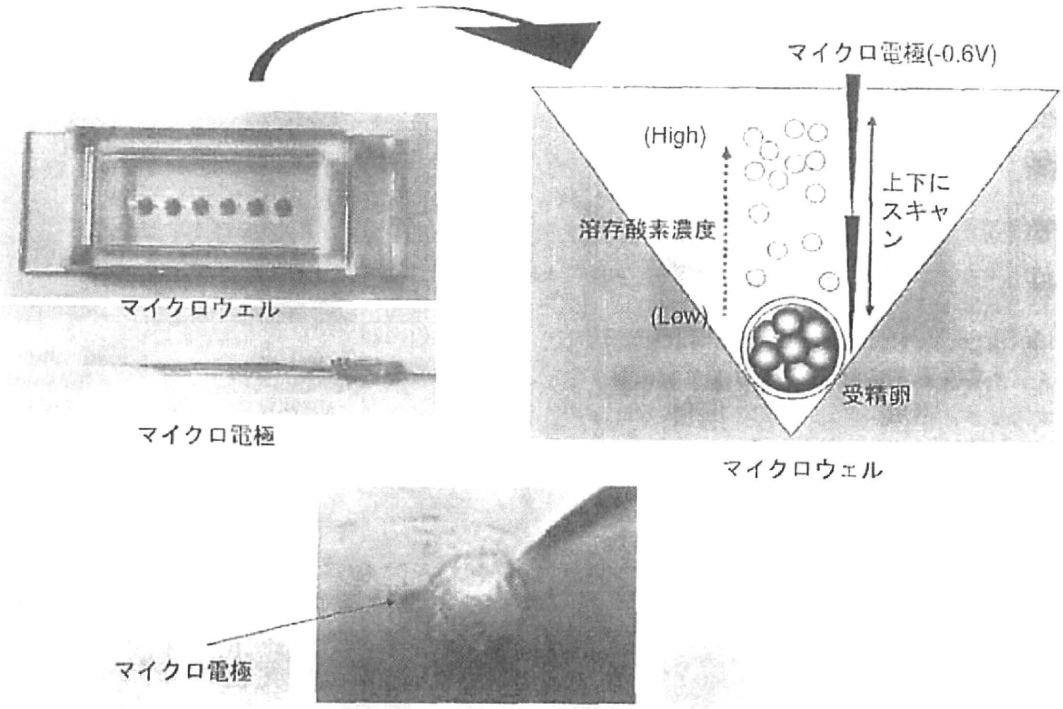

図1　SECMを用いた呼吸量測定

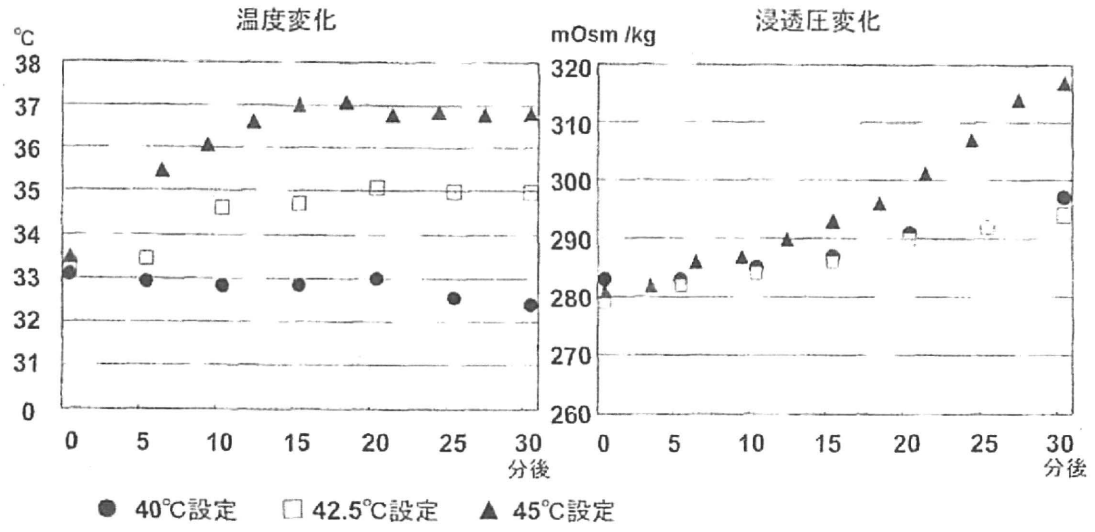

図2　顕微鏡ステージ温度における培養液変化

考　察

胚移植にあたり，最も良好な胚を選別し，1個の移植をすることは多胎率の低下のために不可欠である。これまで，分割期胚の段階でクオリティー良好胚を選別するための形態的判断基準として多くの施設でVeeckの分類[9]が使用されている。さらに，よりクオリティー良好な胚を選択するために様々な判定基準について研究が進められてきた。Zollnerら[10]は前核の接着や前核数の評価に基づく，スコアリングシステムを開発・提案している。

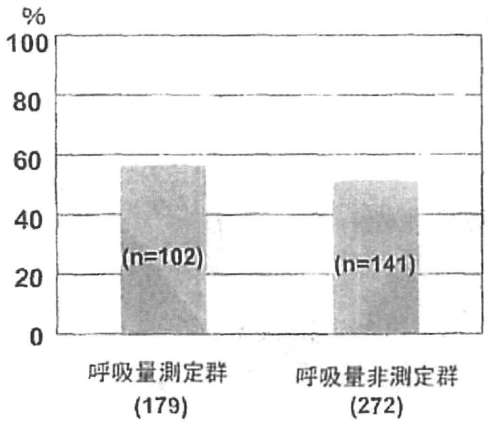

図3　呼吸量測定，非測定群における胚盤胞発生率

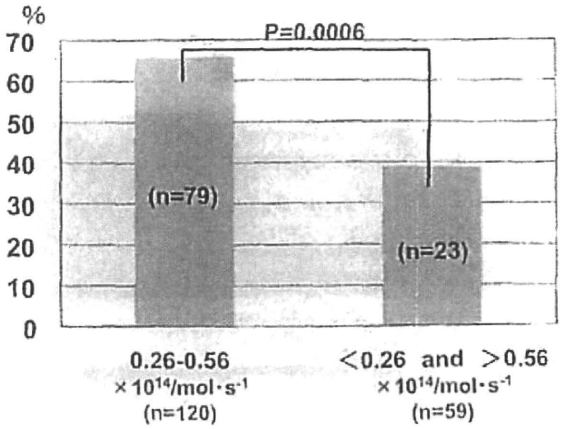

図5　ヒト胚の呼吸能と胚盤胞発生率の関係

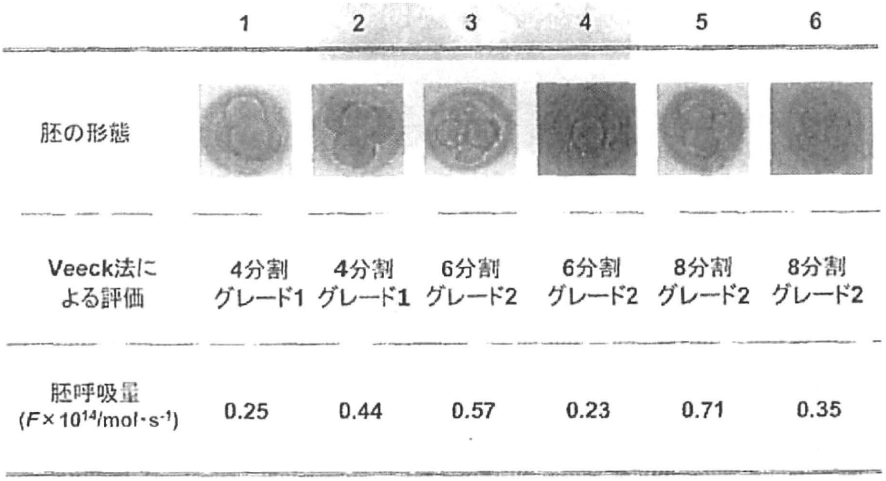

図4　ヒト体外受精胚の形態と呼吸量

表1　異なる分割期におけるヒト胚の酸素消費量（呼吸量）

分割期	測定胚数	呼吸量 ($F \times 10^{14}$/mol·s^{-1})
4細胞	8	0.34 ± 0.1
5細胞	15	0.45 ± 0.2
6細胞	39	0.37 ± 0.1
7細胞	51	0.39 ± 0.2
8細胞	50	0.40 ± 0.2
9細胞	12	0.40 ± 0.1
10細胞	12	0.50 ± 0.2

N.S

Scottら[6]，Tesarik and Greco[7]や公文ら[8]は，前核や核小体の形態による判定を提案している。また，Cirayら[9]は，2細胞期のそれぞれの細胞の核の局在の判定と早期分割による胚発生能の評価法を提案している。しかし，いずれも形態的特徴の観察による評価法であるため，判定結果が観察者の主観によって影響を受ける可能性は否定できない。1998年頃からは，培養期間が長くなることにより移植胚の選別が容易になると考えられ，胚盤胞期移植が広まった。しかし，一卵性双胎が増加することや，培養期間が長くなるため，胚移植キャンセルになる可能性が高くなるという欠点も持ち合わせている。そのため，大多数の施設ではDay 2もしくはDay 3の分割期胚を移植しているのが現状である。分割期胚移植と胚盤胞移植の比較検討としては，Gardnerら[10]やScholtesら[11]が，胚

盤胞移植を行うことにより着床率は分割期胚移植に比べて有意に上昇したと報告をしている。しかし，Coskunら[12]，Karakiら[13]やUtsunomiyaら[14]により，胚盤胞まで発育が進まず移植キャンセルとなった症例を含めた上での妊娠率は分割期胚移植と有意差はないという報告もなされている。分割期胚移植の検討としては，受精確認からDay 2移植もしくはDay 3移植の妊娠率の比較を行った結果，Carilloら[15]の妊娠率に差があるという報告の一方，Lavergeら[16]は妊娠率に差はないという報告をした。

阿部らは，胚のクオリティーに関連する微細構造の解析を行い，ミトコンドリアが胚のクオリティーに密接に関係していることを発見している[17]。ミトコンドリアは酸化的リン酸化反応（呼吸）により細胞活動に必要なエネルギー（ATP）を産生することから，ミトコンドリアが発達している胚は呼吸活性が高く，一方の不良胚ではミトコンドリアの呼吸代謝能が低いと考えられる。この研究成果を基に阿部らは，高感度・非侵襲的に細胞の呼吸を検出できる電気化学計測技術に着目し，この計測技術の中心であるSECMをベースとする受精卵呼吸測定装置を開発した[1, 18, 19]。これまでに，ウシ，ブタ，マウスの胚の無侵襲的呼吸能解析に成功しており，呼吸能を指標とする胚クオリティー評価法の有効性を示している[2]。最近，電気化学的呼吸計測技術のヒト胚への応用を目的に，ヒト余剰胚の呼吸量測定を行っている。体外受精後の全ての発生ステージにおいて胚の呼吸量測定に成功するとともに，ミトコンドリアの発達と呼吸量の増加が一致することを明らかにした[3]。また，以前に我々はヒト胚の形態と呼吸能との関連性を調べた結果，胚の呼吸能とVeeckの分類法による形態的評価は必ずしも一致することはないことを示し，電気化学計測法は，形態観察では評価できない胚の呼吸能をモニタする可能性を報告した[20]。

今回我々はこの呼吸計測技術が胚へ及ぼす影響を調査するとともに胚評価の可能性についても検討を行った。呼吸量計測に用いるマイクロ電極はピコアンペアレベルの電流の変化を検出できる。この微小電極の周りに生じる電場は電圧が0.1 μV以下電流は1 nA以下であり，細胞の膜電位（60-90 mv）の60万分の1以下のため胚への影響は無いと思われる。測定時の温度や浸透圧変化を考え測定プレートを保温する顕微鏡ステージの最適温度についても検討した。40度，42.5度，45度で検討を行ったが，40度では測定プレート内温度の低下が見られ，45度では温度に問題はないものの，浸透圧の上昇が早くなった。m-HFFの指示する浸透圧は275-295 mOsm/kgである。そこで我々は42.5度に設定し呼吸量測定を行った。1個の胚に対し測定に要する時間は30秒だが，マイクロ電極を胚の透明帯近傍に静置すること，次の胚へ電極を移動する動作も含めると1個の胚への所要時間は1分以内である。また，1回の測定で測る胚の個数も少数に制限している。呼吸測定を行った胚への影響は胚盤胞の発生率を以って評価したが，呼吸量測定群，非測定群で差は認められなかった。

胚呼吸量と胚発育の関係では呼吸量0.26-0.56を示す胚で胚盤胞発生率がそれ以外と比較して高くなる結果を示した。これはミトコンドリアによる呼吸活性と胚のクオリティーが密接に関係している可能性がある。呼吸量が0.26より低い胚は胚盤胞への発育が低い。これはミトコンドリアにおける活性が低いことを示しており，胚発育へ影響を与えていることが考えられる。呼吸量が0.56より高い胚でも胚盤胞への発育が低い。阿部らはクオリティー良好胚では，桑実胚から胚盤胞期にかけて多くのミトコンドリアにおいてサイズの増加やクリステの拡張が認められ，呼吸量が増加していくことを報告している[21]。このことから，胚発育が低い原因としてDay 3時の早い時期にミトコンドリアが発達し過ぎていることも考えられ，胚盤胞へ到達する前に活性が終息してしまうかもしれない。その結果，呼吸活性が低すぎても高すぎても胚盤胞発育が低くなる結果を示したのではないかと思われる。

本研究では，電気化学的呼吸測定技術を応用した新しいヒト胚クオリティー評価の安全性と可能性が示された。胚の呼吸測定と形態的評価を併用することで，より厳密に胚のクオリティーを評価できる可能性がある。今後は妊娠率を含めた臨床的有用性を検討していく必要があると思われる

文　献

1) Abe, H., Shiku, H., Aoyagi, S., Hoshi, H.: In vitro culture and evaluation of embryos for production of high quality bovine embryos. J Mamm Ova Res., 21: 22-30, 2004.
2) Abe, H., Shiku, H., Yokoo, M., Aoyagi, S., Moriyasu, S., Minamihashi, A., Matsue, T., Hoshi, H.: Evaluating the quality of individual embryos with a non-invasive and highly sensitive measurement of oxygen consumption by scanning electrochemical microscopy. J Reprod Dev., 52 (Suppl): S55-S64, 2006.
3) 阿部宏之・保尾正樹・荒木康久・他：電気化学的イメージング法を応用した単一ヒト胚の呼吸能解析．産婦人科の実際．56: 2053-2057, 2007.
4) Veeck, L.L.: Atlas of the human oocyte and early conceptus. Vol. 2. Williams & Wilkins, Baltimore, 1991.
5) Zollner, U., Zollner, K.P., Hartl, G., Diell, J., Steck, T.: The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: the German experience. Hum. Reprod., 17: 1327-1333, 2002.

6) Scott, L., Alvero, R., Leondires, M., Miller, B.: The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum Reprod., 15: 2394–2403, 2000.
7) Tesarik, J., Greco, E.: The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum. Reprod., 14: 1318–1323, 1999.
8) 公文麻美・大津英子・長本美幸・他：前核期胚における形態的評価の検討．第58回日本不妊学会九州支部会抄録集，2001.
9) Ciray, H.N., Karaqenc, L., Uluq, U., Bener, F., Bahceci, M.: Early cleavage morphology affects the quality and implantation potential of day 3 embryos. Fertil. Steril., 85: 358–365, 2006.
10) Gardner, D.K., Schoolcraft, W.B., Wargley, L., Schlenker, T., Stevens, J., Hesla, J.: A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. Fertil. Steril., 13: 3434–3440, 1998.
11) Scholtes, M.C., Zeilmaker, G.H.: Blastocyst transfer in day-5 embryo transfer depends primarily on the number of oocytes retrieved and not on age. Fertil. Steril., 69: 78–83, 1998.
12) Coskun, S., Hollanders, J., Al-Hassan, S., Al-Sufyan, H., Al-Mayman, H., Jaroud, K.: Day-5 versus day-3 embryo transfer; a controlled randomized trial. Hum. Reprod., 15: 1947–1952, 2000.
13) Karaki, R.Z., Samarraie, S.S., Younis, N.A., Lahloub, T.M., Ibrahim, M.H.: Blastocyst culture and transfer; a step toward improved in vitro fertilization outcome. Fertil. Steril., 77: 114–118, 2002.
14) Utsunomiya, T., Ito, H., Nagaki, M., Sato, J.: A Prospective, Randomized Study: Day-3 versus Hatching Blastocyst Stage. Hum. Reprod., 19: 1598–1603, 2004.
15) Currillo, A.J., Lane, B., Pridham, D.D., Risch, P.P., Pool, T.B., Silverman, I.H., Cook, C.L.: Improved clinical outcomes for in vitro fertilization with delay of embryo transfer from 48 to 72 hours after oocyte retrieval; use of glucose- and phosphate-free media. Fertil. Steril., 69: 329–334, 1998.
16) Laverge, H., De Sutter, P., Van der Elst, J., Dhont, M.: A prospective , randomized study comparing day 2 and day 3 embryo transfer in human IVF. Hum. Reprod., 16: 476–480, 2001.
17) Abe, H., Matsuzaki, S., Hoshi, H.: Ultrastructural differences in bovine morulae classified as high and low qualities by morphological evaluation. Theriogenology, 57: 1273–1283, 2002.
18) Shiku, H., Shiraishi, T., Ohya, H., Matsue, T., Abe, H., Hoshi, H., Kobayashi, M.: Oxygen consumption of single bovine embryos probed with scanning electrochemical microscopy. Anal Chem,73: 3751–3758, 2001.
19) Shiku, H., Shiraishi, T., Aoyagi, S., Utsumi, Y., Matsudaira, M., Abe, H., Hoshi, H., Kasai, S., Ohya, H., Matsue, T.: Respiration activity of single bovine embryos entrapped in a cone-shaped microwell monitored by scanning electrochemical microscopy. Anal Chim Acta, 522: 51–58, 2004.
20) 那須　恵・後藤香里・熊迫陽子・宇津宮隆史・荒木康久・横尾正樹・阿部宏之：電気化学的呼吸計測によるヒト胚のクオリティー評価．産婦人科の実際，57: 289–294, 2008.
21) 阿部宏之：電気化学的イメージング技術を応用した胚のクオリティ　評価．産婦人科の実際．55: 207–216, 2006.

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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