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Culture of *In Vitro* Mouse Embryos with Vitamin E Improves Development

Hirotsada TSUJII¹⁾, Masae MURANAKA¹⁾ and Koh-ichi HAMANO¹⁾

¹⁾Faculty of Agriculture, Shinshu University, Minamiminowa-mura, Nagano 399-4598, Japan

Abstract. To investigate the effects of vitamin E on the *in vitro* development of mouse embryos from 1-cell to blastocysts, embryos were cultured in two media, Brinster's BMOCIII and BMOCIII supplemented with 100 μ M vitamin E (BMOCIIIvitE). The incorporation and oxidation of glucose were also compared between the embryos cultured in these media. Both the number of embryos that developed to the 1-cell stage and to the blastocyst stage were much larger for BMOCIIIvitE than BMOCIII. No significant differences in the incorporation of ¹⁴C-glucose or in the oxidation of ¹⁴C-glucose were found between the 2-cell embryos incubated in BMOCIIIvitE and BMOCIII. On the other hand, the incorporation and oxidation rates of ¹⁴C-glucose were significantly higher ($p < 0.05$) in the embryos cultured in BMOCIIIvitE compared with those in BMOCIII at the blastocyst stage. These findings indicate that vitamin E has a beneficial effect on embryo development in mice, perhaps through protecting the cells from oxygen radicals *in vitro*.

Key words: Vitamin E, Mouse embryo, Incorporation of glucose, Oxidation of glucose
(J. Reprod. Dev. 48: 25–29, 2002)

Embryos are able to use endogenous sources of energy or take up nutrients from the medium in which they are suspended. Evidence from a number of sources indicates that early mammalian embryos carry out oxidative phosphorylation. Glycolysis becomes important in some species in the later preimplantation stages. Evidence for aerobic metabolism is as follows. First, the preimplantation mouse embryo failed to develop in the absence of oxygen [1], or in the presence of cyanide or 2,4-dinitrophenol, both of which inhibit oxidative metabolism [2]. Second, the measurement of oxygen partial pressure in the oviduct and uterine fluids [3, 4] indicates that the preimplantation embryo inhabits an aerobic environment. Third, Mills and Brinster [5] showed that early mouse embryos consumed oxygen *in vitro*; the rate of consumption was low up to the 8-cell stage, then increased dramatically. Early

mammalian embryos increase the production of oxygen free radicals when cultured *in vitro* [6].

The free radical nitric oxide (NO) is synthesized during the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS have been identified as endothelial (e)NOS, inducible (i) NOS, and neuronal (n)NOS [7–9]. Nitric oxide is known to mediate a wide variety of physiological functions, including neurotransmission and immune cell cytotoxicity [9].

Vitamin E (α -tocopherol and derivatives), the predominant lipid-soluble antioxidant in animal cells, protects cells from oxygen radical damage *in vivo* [10, 11] and *in vitro*, and is believed to be the primary free radical scavenger in mammalian cell membranes [12]. Olson and Seidel [13] reported that the development of bovine embryos *in vitro* improved with vitamin E addition.

This study describes the beneficial effects of vitamin E on *in vitro* embryo development during

the early preimplantation stages in the mouse, and on the incorporation and oxidation of glucose by embryos at these stages.

Materials and Methods

ICR strain mice 6–7 weeks old were used. Mice were maintained and used in accordance with the Guidelines for Regulation of Animal Experimentation (Faculty of Agriculture, Shinshu University). They were kept under controlled temperature (20 ± 1 °C) and lighting (12 h dark and 12 h light) conditions, and allowed free access to commercial solid food and tap water. Female mice were injected ip. with 5 IU pregnant mare serum gonadotrophin (Peamex, Sankyo-zoki K.K.), followed by 5 IU human chorionic gonadotrophin (hCG: Puberogen, Sankyo-zoki K.K.) 48 h later for the induction of superovulation. After hCG injection, the females were placed with a fertile male overnight and checked for the presence of copulation plugs the following morning. One to two-cell embryos and blastocysts were collected from the mice that had vaginal plugs by breaking the oviducts or by flushing the uteri at 20, 30 and 95 h after hCG injection.

Vitamin E ($[\pm]$ - α -tocopherol; Sigma T-3251) is hydrophobic. It was first dissolved in 95% ethanol as a 2000-strength stock solution, stored in the dark at 4 °C, and then (18–20 h prior to embryo culture) diluted in Brinster's BMOCIII medium to a final concentration of 10, 100 or 200 μ M vitamin E and 0.05% ethanol. Bovine serum albumin (Fraction V; sigma) at 5 mg/ml was added to help maintain the vitamin E in aqueous solution and facilitate delivery to cell plasma membranes with minimal compromise to the nature of the medium. Preliminary experiments indicated that 0.05% ethanol had no effect on mouse embryo development *in vitro* (data not shown).

Experiment 1: This experiment was designed to examine the effects of vitamin E on the *in vitro* development of preimplantation mouse embryos. The basic culture medium used was BMOCIII. Two-cell embryos were collected and washed three times with the same medium and transferred (10 embryos each) to a drop (50 μ l) of one of the following media: BMOCIII medium or BMOCIII medium supplemented with 10, 100 or 200 μ M vitamin E. The drops were covered with paraffin

oil and cultured at 37 °C, 5% CO₂ in air. Development of the embryos was evaluated with phase contrast microscopy (IMT-2, Olympus).

Experiment 2: This experiment was designed to examine the effect of 100 μ M vitamin E on the *in vitro* development of 1-cell stage embryos cultured as above.

Experiment 3: The rates of incorporation and oxidation of ¹⁴C-glucose were used for embryos at the two-cell and blastocyst stages according to the method of Brinster [15]. Ten embryos at each stage were transferred to a small test tube containing BMOCIII medium or BMOCIII medium supplemented with 100 μ M vitamin E (BMOCIIIvit E) and ¹⁴C-glucose (18.5 KBq; Specific activity: 1.85 MBq/M). One milliliter of hyamine was placed in another test tube and incubated for 4 h at 37 °C, 5% CO₂ in air. At the end of incubation, cold PCA was added to a final concentration of 5%. The acid insoluble materials were separated and washed with PCA as described above.

Radioactivity of the acid insoluble materials and hyamine were determined by a scintillation counter (LS-6500: Beckman Instruments, Inc. USA) using a scintillation cocktail (toluene: 1000ml, POPOP: 300mg, PPO: 4 g). Eight samples were used for each observation.

The obtained data were statistically analyzed by Fischer's exact probability test in Experiments 1 and 2 and by Student's *t*-test in Experiment 3.

Results

Experiment 1: The results of Experiment 1 are shown in Table 1. BMOCIII supplemented with 100 μ M vitamin E had a significantly higher percentage of embryos that developed to the morula and blastocyst stages than the other treatments ($p < 0.05$). There was no effect of 10 μ M vitamin E.

Experiment 2: The results of Experiment 2 are shown in Table 2. Embryos cultured *in vitro* with vitamin E showed a significantly higher percentage of embryos developing to morula and blastocyst stages ($p < 0.05$).

Experiment 3: Results are shown in Tables 3 and 4. Both the incorporation and oxidation of ¹⁴C-glucose were significantly higher in BMOCIIIvitE than BMOCIII at the blastocyst stage ($p < 0.05$). The incorporation and oxidation of ¹⁴C-glucose were significantly higher at the blastocyst stage than at

Table 1. Development of morula and blastocyst stage mouse 2-cell embryos cultured in medium with or without vitamin E

Concentrations of vitamine E (μM)	Number of embryos cultured	Number of embryos developed (%)	
		morula	blastocyst
0	53	27 (50.9) ^b	21 (39.6) ^y
10	54	18 (33.3) ^c	12 (22.2) ^z
100	53	33 (62.3) ^a	23 (43.4) ^x
200	53	24 (45.3) ^b	20 (37.7) ^y

^{a-c}, ^{x-z}: Values are highly significantly different ($p < 0.05$).

Table 2. Effect of vitamin E on development of 1-cell mouse embryos

Treatment	Number of embryos cultured	Number of embryos developed (%)			
		2-cell	4-cell	morula	blastocyst
None	120	102 (85.0)	73 (60.8)	58 (48.8)	39 (32.5)
Vitamin E, 100 μM	121	111 (91.7) [*]	81 (72.9) [*]	66 (54.5) [*]	63 (52.1) [*]

*: Percentages with different superscripts within rows differ significantly ($P < 0.05$).

Table 3. Effect of vitamin E on incorporation of ^{14}C -glucose in 2-cell and blastocyst mouse embryos (Mean $\pm$ SEM)

Developmental stage	Vitamin E	n	Incorporation ^{14}C -glucose (CPM/embryo)
2-cell	-	8	33.4 $\pm$ 2.3
	+	8	36.9 $\pm$ 2.1
Blastocyst	-	8	42.8 $\pm$ 1.4 ^a
	+	8	54.1 $\pm$ 2.4 ^c

^{a-c}: $P < 0.05$ for treatments with different superscripts.

Table 4. Effect of vitamin E on oxidation of ^{14}C -glucose in 2-cell and blastocyst mouse embryos (Mean $\pm$ SEM)

Developmental stage	vitamin E	n	CO_2 produced (CPM/embryo)
2-cell	-	8	302.3 $\pm$ 12.0 ^a
	+	8	390.8 $\pm$ 20.8 ^b
Blastocyst	-	8	356.9 $\pm$ 15.8 ^c
	+	8	451.0 $\pm$ 22.0 ^d

^{a-b,c-d}: $P < 0.05$ for treatments with different superscripts.

2-cell stage ($p < 0.05$) in BMOCIII and BMOCIII vitE. There were no significant effects of vitamin E on the incorporation of glucose at the 2-cell stage.

Discussion

In our culture experiment, the rate of mouse embryo development from one-cell to blastocyst stage was higher in the medium supplemented with vitamin E than in the control medium. These findings suggest that the antioxidant vitamin E protects *in vitro* embryos from oxidative damage in 5% CO_2 in air. Olson and Seidel [13] reported that the protective effects of antioxidants with 5% O_2 , 5% CO_2 and 90% N_2 were better than with 5% CO_2 in air.

More embryos developed to blastocysts when vitamin E was added at 100 μM in Brinster's BMOCIII than at other concentrations of vitamin E and control. Vitamin E concentrations were based on approximate physiological levels; normal human plasma ascorbate values are 30–150 μM [16]. The concentration of vitamin E used was ~4–5 times that of normal human adult plasma levels [17] and in the range of concentration found effective for *in vitro* protection of rat hepatocytes (25 μM α -tocopheryl succinate) [18], rat conceptuses (94 $\mu\text{g}/\text{ml}$ or 218 μM α -tocopherol) [19], and mouse embryos (25, 250 μM α -tocopheryl succinate) [20]. Because vitamin E has poor solubility in aqueous solution, and was likely mostly bound to BSA in this system, we assumed that the actual concentration available to the cells was lower than

the amount added.

Vitamin E was first discovered as a dietary, lipid-soluble factor that could prevent fetal death and resorption in rats [21]. In other studies, vitamin E partially protected early murine embryos from the effects of heat shock [20] and a cytotoxic event mediated by free radicals [22]. Although the rate of blastocyst development under heat shock conditions was not affected by culture with vitamin E (α -tocopheryl succinate), the viability as determined by vital dye exclusion was improved [20]. Survival and development of rat conceptuses explanted on Day 8 of gestation were improved by culture with vitamin E [19]. These studies suggest a direct protective and developmental effect on the embryo *in vitro*, which may reflect a similar function *in vivo*.

The main biological function of vitamin E is probably to protect polyunsaturated fatty acids in cell membranes. Peroxidation of these membrane lipids can lead to structural damage, affecting function and permeability of membranes, eventually resulting in irreversible cell injury and death.

In vitro, vitamin E molecules have been subjected to free radical attack [23]. Deleterious effects of oxygen radicals on *in vitro* development of embryos have been reported in various laboratory and domestic animals such as the mouse [24], rabbit [25] and pig [26]. The authors speculate that superoxide radicals are involved in the mouse 2-cell developmental block, because the addition of free radical scavengers such as thioredoxin [27] or SOD [28] to culture medium increased the number of embryos that developed beyond the block.

It is known that the oxygen concentration within the reproductive tract of mammals is lower than

that in air. For instance, the O₂ concentration within the tract of rabbits [29] and rhesus monkeys [30] was reported to be 10 to 60 mmHg, an O₂ concentration of about 5% [31]. Furthermore, the cleaving mouse embryo has a maximal oxygen requirement *in vitro* of approximately 5%, and blastocysts developed under 20% O₂ had fewer cells than those developed under 5% O₂ [32].

In the mouse, pyruvate is essential for the survival of unfertilized and fertilized oocytes and for the first cleavage. Lactate acts synergistically with pyruvate and, as the sole substrate, lactate can support the cleavage of 2-cell mouse embryos. Glucose as the sole substrate cannot support development until the 4- and 8-cell stages.

Schini and Bavister [33] and Seshagiri and Bavister [34, 35] postulated that glucose inhibits TCA cycle activity through a phenomenon known as the *Crabtree effect*. This effect is the result of depletion of the inorganic phosphate pool by glycolytic uptake [36]. An alternative explanation lies in the allosteric inhibition of glycolytic enzymes, such as phosphofructokinase and hexokinase, observed in the *Pasteur effect* [27]. This is the proposed mechanism for the glycolytic block in precompaction mouse embryos [38, 39].

The findings obtained in Experiment 3 demonstrate that the kinetics of glucose use change during preimplantation development. This may reflect differences in either uptake mechanisms or metabolic enzyme activities, or both. Nevertheless, it is of interest that even though the relative use of glucose is much higher in blastocysts than in 2-cell embryos, a higher concentration of exogenous glucose is required for the saturation of the pathways in 8-cell embryos.

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