Comparison of Development and the Incorporation of Glucose and Methionine between Parthenogenic and Fertilized Mouse Embryos

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Abstract: *In this study, we used $SrCl_2$ treatment to activate mouse oocytes. The objectives of this study were to compare the development of parthenogenic and fertilized mouse embryos, and the effect of insulin-like growth factor II (IGF-II) on their development to blastocysts; glucose and methionine metabolism were also examined. Compared to normal fertilized embryos, parthenogenically developed embryos exhibited delayed development, reduced total cell number, and reduced trophectoderm cell (TC) and inner cell mass (ICM) cell numbers of blastocysts. Supplementation of IGF-II to the culture medium enhanced parthenogenic embryo development, cell number, and TC and ICM cell numbers but not to the same level as that of normal fertilized embryos ($P < 0.05$). Incorporation and oxidation of glucose and protein synthesis from methionine were lower in parthenogenic blastocysts than in fertilized blastocysts ($P < 0.05$).*

Key words: *Mouse, Parthenogenetic embryo, Protein synthesis, Incorporation of glucose, IGF-II*

Introduction

Studies of mice have revealed that the preimplantation developmental potential of parthenogenic embryos is significantly impaired relative to fertilized embryos. In mammalian species, parthenogenic development has been achieved by a variety of methods that elevate Ca^{2+} transiently [1]. Although single Ca^{2+} elevations induced by ethanol, calcium ionophore, or an electrical pulse are not sufficient to promote development, Sr^{2+} treatment significantly enhances the development of oocytes [2–3] by mimicking sperm-induced Ca^{2+} oscillations [2–4]. Sr^{2+} itself does not cause chromosome abnormalities in activated oocytes [5]. Sr^{2+} used to activate cytoplasts during nuclear resulted in produced cloned mice, demonstrating the suitability of Sr^{2+} for promoting full-term development [6, 7]. However, if oocytes activated by Sr^{2+} are allowed to extrude a second polar body, an aneuploid, specifically parthenogenic embryo, a parthenote is induced. Exposure of activated oocytes to cytoskeletal inhibitors, to inhibit polar body extrusion, produces diploid parthenotes. Parthenogenically developed embryos exhibit delayed development, reduced total cell number, and fewer cells in the inner cell mass (ICM) of blastocysts compared with fertilized embryos [5, 8]. Moreover, medium composition (the presence or absence of growth promoting factors) and the presence of damaging substances such as free oxygen radicals play a role in embryo development [9].

The influence of insulin-like growth factor II (IGF-II) on mouse embryo development has been reported by other investigators [10–12]. Thuan *et al.* [13] reported that IGF-II has the protective potential to protect embryos exposed to the inhibiting influence of oxidative stress. Thus, the synergistic effect of Sr^{2+} and IGF-II on the developmental potential of parthenogenic mouse embryos might be beneficial.

The purpose of this study was to compare the development of parthenogenic and fertilized embryos

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and their incorporation of methionine and glucose *in vitro* was also examined.

Materials and Methods

Recovery of in vivo matured oocytes

Embryos were obtained from 7–8 week old female ICR mice. They were given feed with a standard diet and tap water *ad libitum*. Animals were kept in polycarbonate cages with wood shavings under a 12 h light: 12 h dark regimen (light on at 6:00), at a temperature of 20 ± 1°C in accordance with the "Guideline for the Regulation of Animal Experimentation, Faculty of Agriculture, Shinshu University." Mice were induced to superovulate with Pregnant Mare Serum Gonadotropin (PMSG) (5 IU, i.p.) followed 48 h later by human Chorionic Gonadotropin (hCG) (5 IU, i.p.). Both PMSG and hCG used in this study were obtained from Sankyo Chemical Industries Ltd (Tokyo, Japan). The superovulated mice were sacrificed at 16 h after hCG injection and harvested oviductal ampullae were opened in Ca^{2+}-free Chatot-Ziomek-Bavister (CZB) medium to release the cumulus-oocyte complexes.

Oocyte parthenogenic activation and embryonic development

The procedures used for oocyte activation were those reported by Ma *et al.* [14] with slight modifications. The activation medium used was Ca^{2+}-free CZB medium supplemented with 10 mM $SrCl_2$ and 5 mg/ml cytochalasin B. On the release of the cumulus-oocyte complexes, oocytes were stripped of their cumulus cells by pipetting in Ca^{2+}-free CZB medium containing 80 unit/ml hyaluronidase. Oocytes with a homogenous cytoplasm were selected and incubated for 1 h in Ca^{2+}-free CZB medium [15] before treatment for parthenogenic activation. After being washed twice in activation medium, oocytes were incubated in activation medium for 6 h at 37°C in a humidified atmosphere with 5% CO_2 in air. Six hours after the onset of activation treatment, oocytes were examined under a microscope for the evidence of activation. Oocytes were considered activated when each contained two well-developed pronuclei. The Sr^{2+} activated oocytes were cultured for 6 days in KSOM (K^+ Simplex Optimized Medium) at 37.0°C under a humidified atmosphere of 5% CO_2 in air.

Fertilized embryos were collected after induced superovulation as described above. Female mice were caged with fertile ICR males overnight. On the following morning, the presence of a vaginal plag was indicative of successful copulation. Sixteen hours after hCG injection, the oviductal ampullae were opened in KSOM. After release of the cumulus-oocyte complexes, embryos were washed and cultured for 6 days in the KSOM at 37.0°C under humidified atmosphere of 5% CO_2 in air.

Blastocyst trophectoderm cells (TC) and ICM cell numbers were determined by differential nuclear staining as previously described [16] with slight modifications. After culture, embryos were stained with Hoechst 33342 (Sigma-Aldrich Co., USA) and propidium iodide (PI) (Sigma-Aldrich Co., USA). Embryos were stained for 30 sec in PI solution (PI 0.1 mg/ml, and 0.2% Triton X-100 in phosphate-buffered saline: PBS) which included neither calcium nor magnesium, and overnight in Hoechst 33342 solution (25 μg/ml Hoechst 33342, 99.5% ethanol). They were then washed with glycerol, observed under a fluorescence microscope (IXY-70 OLYMPUS Co., Japan), and cell numbers were counted. ICM showed blue fluorescence and TC showed pink fluorescence under 352 nm light.

Incorporation and oxidation of radiolabeled glucose and methionine by parthenogenic and fertilized mouse embryos

All radioactive substrates, ^{14}C-glucose 18.5 kBq/50 μM (spec. act: 9.63 MBq/mM), and ^{14}C-methionine 9.25 kBq/50 μM (spec. act: 2.04 MBq/mM) were obtained from Moravek Biochemicals, Inc., USA. Each of five embryos at a particular stage of development was transferred to a microtube containing 50 μl maturation medium and 9.25 kBq ^{14}C-glucose or ^{14}C-methionine, then overlaid with mineral oil. ^{14}C-glucose and ^{14}C-methionine were incorporated into a glucose or methionine containing maturation medium with embryos. Also, 1 ml of 2.5 mM NaOH solution was put in a 1.5-ml microtube as a trap for the $^{14}CO_2$ emitted. Micro tubes of NaOH and ^{14}C-glucose or ^{14}C-methionine with embryos were sealed in a scintillation vial using a rubber stopper. The scintillation vials were incubated for 2 h in a incubator with a humidified atmosphere of 5% CO_2 in air at 37°C. After completion of the 2-h incubation period, the metabolic reactions of the embryos were stopped with an injection of 100 μl of 10% perchloric acid (PCA) for glucose, or and trichloroacetic acid (TCA) for methionine and kept at room temperature for 24 h. The acid insoluble materials were carefully collected by millipore filtration (8.0 μM white SCWP, 47 mm; Millipore Corporation, Bedford, MA, USA) with 5% PCA or TCA and the filter papers

Table 1. Effect of IGF-II on the development of mouse parthenogenic embryos and cell number per blastocyst

IGF-II	n	Blastocyst (%)	Number of cells/blastocyst		
			ICM	TC	Total
– Part	81	20 (24.7)^{c}	7.8 ± 1.2^{b}	29.8 ± 2.0^{c}	37.5 ± 2.1^{b}
– Fert	100	79 (79.0)^{a}	13.3 ± 1.1^{a}	40.5 ± 2.6^{ab}	53.8 ± 1.9^{a}
+ Part	68	31 (45.6)^{b}	8.0 ± 1.2^{b}	32.3 ± 1.8^{bc}	44.5 ± 3.9^{ab}
+ Fert	83	67 (80.7)^{a}	11.8 ± 1.3^{ab}	43.3 ± 3.1^{a}	52.8 ± 3.8^{a}

Different superscrips differ significantly ($P < 0.05$). Part: Parthenogenic; Fert: Fertile; ICM: Inner cell mass; TC: Trophectoderm cells.

were dried overnight under a lamp. After drying, the filter papers were transferred in scintillation vials. The NaOH solution was put in a new scintillation vial by washing 3–4 times with cocktail (0.5% 2,5-diphenyloxazole + 0.03% 1,4-bis(5-phenyloxazol-2-yl)-benzene solution in toluene). All the scintillation vials with 5 ml of cocktail were set in a liquid scintillation counter (LS-6500, Beckman Instruments, Inc. USA) to determine the levels of radioactivity. This experiment was conducted three times. The values of incorporation and oxidation of glucose and methionine were expressed directly as counts per minute (cpm).

Statistical analysis

The results are given as mean ± SD. Significant differences between groups of cultured embryos were determined by using the Tukey-Kramer test.

Differences in the time log of cleavage and development were determined by using the χ^2 test. Data for incorporation and oxidation of radiolabeled glucose and methionine were analyzed with Student's *t*-test for statistical significance. Differences were considered significant if $P < 0.05$.

Results

The effect of IGF-II on the development of mouse parthenogenic embryos and cell numbers per blastocyst is shown in Table 1. The development rate of parthenogenic blastocysts was significantly lower than that of normal fertilized embryos ($P < 0.05$). Addition of IGF-II to the medium increased the development rate of parthenogenic embryos, it was not enhanced up to the normal fertilization rate. The number of cells per blastocyst in parthenogenic embryos was lower than normal fertilized embryos but this cell number increased when IGF-II was added to the medium ($P < 0.05$).

The effects of IGF-II on the time log of cleavage and the development of mouse parthenogenic embryos are noted in Table 2. There was no significant difference in development between parthenogenic and fertilized embryos at the 1-cell and 2-cell stages. The development rate of parthenogenic embryos was significantly lower than that of fertilized embryos after the 4-cell stage ($P < 0.05$). The addition of IGF-II did not influence the development rate of the fertilized embryos. After the 8-cell stage, the development rate of parthenogenic embryos cultured with IGF-II was significantly higher than those cultured without IGF-II addition ($P < 0.05$).

The results of incorporation and oxidation of glucose by parthenogenic embryos are shown in Fig. 1. Incorporation of glucose into the parthenogenic embryos did not significantly differ between normal fertilized and parthenogenic embryos until the 4-cell stage. The fertilized embryos gradually increased incorporation of glucose from the 1-cell to the blastocyst stages. There was a sudden increase of incorporation of glucose in the parthenogenic embryos at the morula stage, but it decreased at the blastocyst stage ($P < 0.05$). Oxidation of glucose by the parthenogenic embryos was not significantly different from the fertilized embryos until the 4-cell stage. Oxidation of glucose by the parthenogenic embryos was significantly increased at the morula stage ($P < 0.05$). Oxidization of glucose by the parthenogenic and fertilized embryos was low, and no significant difference in oxidation by the parthenogenic and the fertilized embryos at the blastocyst stage was observed.

The results of the incorporation and oxidation of methionine by parthenote embryos is shown in Fig. 2. The incorporation of methionine parthenogenic embryos was higher than that by fertilized embryos at the 1-cell stage ($P < 0.05$), but there were no significant difference at the 2-cell and 4-cell stages of development. The incorporation of methionine on the parthenogenic

Table 2. Effect of IGF-II on time log of cleavage and development of mouse parthenogenic embryos (%)

IGF-II	Period	1-cell	2-cell	4-cell	8-cell	morula	blastocyst
–	Day1	100.0					
		100.0					
	Day2		85.2	1.2			
			84.0	3.0			
	Day3			60.5*	2.5*	0	
				6.0	37.0	43.0*	
	Day4					29.6*	0
						75.0	11.0
	Day5					18.5*	11.1*
							79.0
	Day6						24.7*
							77.0
+	Day1	100.0					
		100.0					
	Day2		75.0				
			94.0				
	Day3			57.4*	10.3*	0	
				8.4	22.9	59.0*	
	Day4					69.1	0
						62.7	14.5
	Day5					27.9*	17.6*
						9.6	71.1
	Day6						45.6*
							80.7

Values of upper lane for each row: Parthenogenic embryo, n = 81; Values of lower lane for each row: Fertilized embryo, n = 100. *: Significant difference ($P < 0.05$).

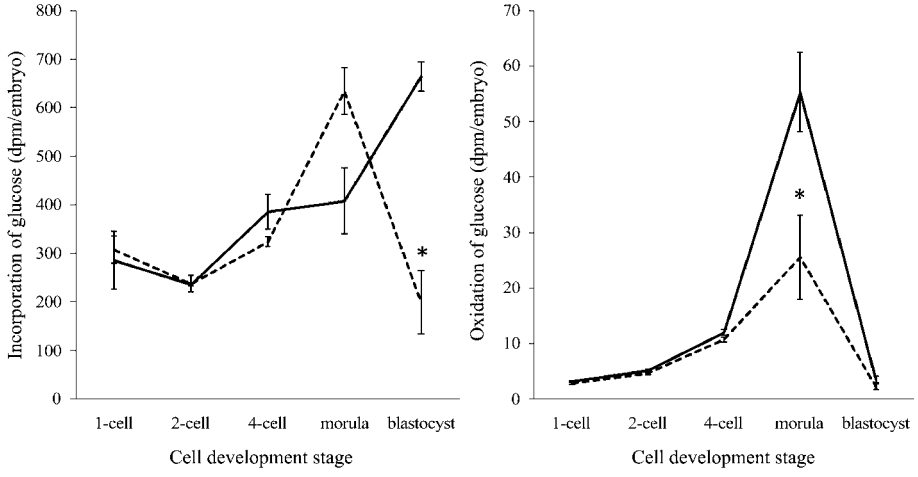

Fig. 1. Incorporation and oxidation of ^{14}C-glucose by parthenogenic mouse embryos. —: Fertilized embryo; - - -: Parthenogenic embryo; *: Significant different ($P < 0.05$).

embryo was lower than the fertilized embryo at morula and blastocyst stage. The oxidation of methionine by parthenogenic embryos was significantly lower than that by fertilized embryos at the 1-cell and blastocyst stages ($P < 0.05$), but, the oxidation of methionine by parthenogenic embryos was significantly higher than that by fertilized embryos at the 2-cell and morula stages ($P < 0.05$). There was no difference in oxidation

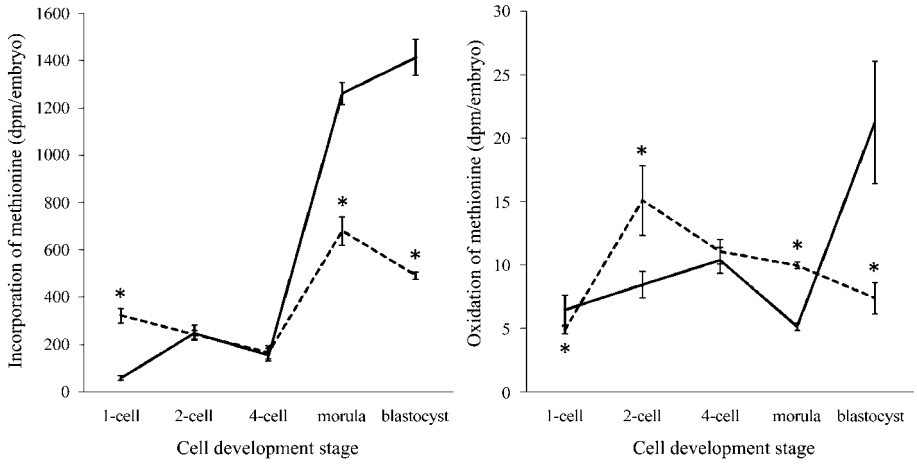

Fig. 2. Incorporation and oxidation of ^{14}C-methionine by parthenogenic mouse embryos. —: Fertilized embryo; - - -: Parthenogenic embryo; *: Significant different ($P < 0.05$).

at the 4-cell stage by both types of embryos.

Discussion

Strontium is the only parthenogenic agent for mouse oocytes that induces repetitive intracellular calcium releases in a fashion similar to those following normal fertilization by spermatozoa [16]. In fact, when we treated oocytes with $SrCl_2$ in Ca^+-free CZB, most of them ceased development at the 2–4-cell stages, and only 24.7% of the oocytes developed into blastocysts.

Embryo cell number has been one of the most useful parameters for evaluating the ability of the different culture conditions to promote development [17]. Haploid parthenogenic embryos were developmentally retarded (most arrested at the morula stage), and parthenogenic embryo (activation with Sr^{2+}) embryos had fewer cells than diploid (activation with Sr^{2+} and cytochalasin B) embryos [18]. Both the total and ICM cell numbers in the diploid parthenotes were significantly lower than those of *in vivo* and *in vitro* developed embryos. Uranga and Arechaga [19] also found that parthenogenic mouse blastocysts had a significantly smaller cell number than fertilized control embryos. Furthermore, the percentage of parthenotes that developed to the blastocyst stage at Day 8, total cell number and the ratio of ICM to TC or ICM to total cells did not differ from that of *in vitro* fertilization (IVF) controls [20]. In our result, there was a significant delay in parthenogenic mouse embryo development which was in agreement with the findings of other reports [5, 8]. We think that the developmental potential of parthenogenic embryos is less than that of normally fertilized embryos. The developmental deficiencies have been hypothesized to result from insufficient parthenogenic activation, suboptimal *in vitro* culture conditions, genomic imprinting, or apoptosis.

The total, TC and ICM cell numbers of parthenogenic embryos were significantly lower than those of the fertilized embryos. However, the percentage of parthenogenic embryos surviving throughout the preimplantation period until the blastocyst stage is always lower than that of fertilized embryos. Similarly, Howlett and Renard *et al.* reported a certain expression of paternal genes before the 8-cell stage seems to be necessary to assure parthenogenic viability [21, 22]. Related to this, in our study, the development rate of parthenogenic embryos cultured with IGF-II after the 8-cell stage was significantly higher than that of those cultured without IGF-II addition. Uranga [19] made a quantitative study of the blastocyst cell number of parthenogenic mouse embryos to evaluate whether genetic imprinting affects cell proliferation during the preimplantation period. Several morphometric parameters such as volume, shape, and coefficient of diversity were measured to complement the cell number data. Classic features of apoptosis, including cytofragmentation, increased levels of reactive oxygen species, caspase activation, and nuclear and DNA fragmentation, have also been identified in preimplantation embryos [23–25]. Apoptosis may also be implicated in embryonic arrest [25]. Thus, cell death could play a role in the elimination of abnormal embryos, including aneuploid embryos with

compromised developmental potential [26].

Parthenogenically developed embryos exhibit delayed development compared to fertilized embryos [5, 8]. The developmental potential of human parthenogenic embryos is also reduced [27]. Moreover, haploid parthenogenic embryos are developmentally delayed compared with diploid parthenogenic embryos in the mouse, pig, and cow [20, 28–31]. These findings are in agreement with our results.

IGF-I, IGF-II and insulin-like peptides are all members of the insulin superfamily of peptide hormones but bind to several distinct classes of membrane receptor. Peptide growth factors have pleiotropic effects on somatic cell function and embryo development, which include increasing the proportion of embryos which reach each stage of development, advancing the timing of differentiation, stimulating mitogenesis and blocking apoptosis [32–34]. The present study demonstrated that IGF-II increases blastocyst formation and the cell numbers of cultured blastocysts. Parthenogenically developed embryos exhibited advanced development because variability of embryonic development is reduced by IGF-I and IGF-II which have positive effects on pre-implantation embryo development under detrimental culture conditions of oxidative stress [13, 35]. Although we did not measure it, we speculated that the beneficial effects of the incorporation of methionine by parthenogenic embryos on apoptosis was probably improved by IGF-II.

The energy metabolism of preimplantation embryos can be used to predict viability and postimplantation development. Ongeri and Krisher [36] reported that there was no significant difference in glucose or pyruvate metabolism between *in vitro* fertilization and parthenogenically activated blastocysts. The present study demonstrated significant differences in the incorporation and oxidation of glucose or methionine between parthenogenic embryos and fertilized embryos. The incorporation of glucose in parthenogenic and the fertilized embryos was small at the 2-cell stage and there was no significant difference. It is known that most types of mice do not develop from the fertilized embryo past the 2-cell stage *in vitro*, although development to the 2-cell stage is apparently unimpeded, and 2-cell embryos produced *in vivo* develop easily to blastocysts *in vitro* [37]. This phenomenon of a specific *in vitro* developmental arrest at the 2-cell stage is known as the 2-cell block. Furthermore, a mechanism facilitating glucose entry into the mouse embryo exists from at least the 2-cell stage onwards [38]. Although glycolytic activity remains low until around the time of compaction [39, 40], glucose is utilized at relatively high rates by the cleavage-stage mouse embryo through the pentose phosphate pathway [41], presumably to fulfill biosynthetic requirements. Mognetti *et al.* [42] reported that mouse preimplantation embryo development is characterized by a switch from dependence on the tricarboxylic acid cycle, pre-compaction, to metabolism based on glycolysis, post-compaction. However, the incorporation of glucose decreased at the blastocyst stage of the parthenogenic embryo in this study.

The incorporation of methionine by parthenogenic embryos was higher than that by fertilized embryos at the 1-cell stage, but there were no significant differences at either the 2-cell or 4-cell stage. The incorporation of methionine by parthenogenic embryo was lower than that by fertilized embryos at the morula and blastocyst stages. Ca^{2+} increase during embryo activation is known to stimulate a number of protein kinases such as calmodulin-dependent protein kinase II and protein kinase C (PKC) [43, 44]. These protein kinases are involved in causing the immediate events of oocyte activation, such as meiotic resumption and cortical granule exocytosis. However, these or other Ca^{2+}-dependent protein kinases may have other substrates that exert a long-term influence. This might involve protein phosphorylation leading to changes in protein synthesis that could have a selective effect upon particular genes during embryonic genome activation. Since each wave of gene expression has an influence upon the next phase [45, 46], any difference in the pattern of gene expression at these earlier stages could have knock-on effects upon the pattern of expression during the mid-preimplantation stage of development [47]. The efficient activation of oocytes in Ca^{2+}-free CZB may also be attributable to protein synthesis being reduced in oocytes cultured in the absence of Ca^{2+} [16].

In conclusion, our results indicate that parthenogenically developed embryos exhibit delayed development, reduced total cell number, and reduced TC and ICM cell numbers of blastocysts. The incorporation and oxidation of glucose and methionine were lower in parthenogenic blastocysts compared with fertilized blastocysts. IGF-II mediates these phenomena and enhances development to the blastocyst as well as increasing the cell numbers.

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