

Valine Metabolism of Mongolian Gerbil (*Meriones unguiculatus*) Embryos

Ryuichiro Obata and Hirotada Tsujii*

Laboratory of Animal Biotechnology, Interdisciplinary Graduate School of Science and Technology, Shinshu University, Nagano 399-4598, Japan

Abstract: Mongolian gerbil 1-cell embryos do not develop into blastocysts *in vitro* because of 8-cell block. Recently, we reported that 2-cell embryos of Mongolian gerbils developed into blastocysts *in vitro* in mM16 supplemented with valine. However, the role of valine in the *in vitro* development of Mongolian gerbil embryos is unclear. Thus, the objective of this study was to investigate of the role of valine in the *in vitro* development of Mongolian gerbil embryos. Mongolian gerbil embryos at the 1-cell, 2-cell, 8-cell, morula, and blastocyst stages were cultured in mM16 medium containing ^{14}C -valine or ^{14}C -glucose. A 2.5 mM NaOH solution was used as a trap for the evolved $^{14}\text{CO}_2$. After incubation, all samples were analyzed using a liquid scintillation counter. The incorporation of ^{14}C -valine significantly increased at the 8-cell stage. The oxidation of ^{14}C -valine significantly increased at the 8-cell and blastocyst stages. The incorporation of ^{14}C -glucose in mM16+valine was significantly higher than that in mM16 at the blastocyst stage. The oxidation of ^{14}C -glucose in mM16 was significantly higher than that in mM16+valine at the 8-cell stage, although that in mM16+valine was significantly higher than that in mM16 at the blastocyst stage. The carbon skeleton of ^{14}C -valine was metabolized into lipid. Our results suggest that valine was used and metabolized as an energy source in preimplantation Mongolian gerbil embryos.

Key words: Valine, Metabolism, Embryo, Mongolian gerbil

Introduction

Our ability to develop embryos *in vitro* has greatly improved over the period of nearly 50 years since Whitten first cultured eight-cell mouse embryos into

blastocysts in a simple defined medium [1]. As media were subsequently improved and it became possible to culture embryos from earlier stages, it became clear that embryos derived from females of most types of mice would not develop from fertilized eggs past the two-cell stage *in vitro*, although development to the two-cell stage was apparently unimpeded and two-cell embryos produced *in vivo* developed easily into blastocysts *in vitro* [2]. This phenomenon of a specific *in vitro* developmental block at the two-cell stage became known as the two-cell block. The two-cell-block phenomenon was defined functionally as the inability of fertilized eggs to develop *in vitro* past the two-cell stage in media that nonetheless supported both development up to the two-cell stage and development of two-cell embryos produced *in vivo* to blastocysts [2]. Extensive studies of the hamster [3] have demonstrated that the inclusion of certain amino acids (asparagine, aspartate, glycine, histidine, serine, and taurine) in the culture medium stimulates the development of 1-cell embryos to the morula/blastocyst stage *in vitro*. In contrast, other amino acids (cysteine, isoleucine, leucine, phenylalanine, threonine, and valine) strongly inhibit development. Studies of glutamine have revealed that this amino acid can stimulate oocyte maturation in hamsters [4] and rabbits [5] and alleviate the culture-induced 2-cell block in embryos produced from random breeding of mice [6]. Recent experiments on hamster embryos in culture have shown that glutamine can serve as the sole nitrogen source for the development of 1-cell embryos to the blastocyst stage [7].

Martin *et al.* reported [8] that the majority of cells exhibited the 2-cell block at the late 2-cell stage owing to inhibition of processes such as cellular and nuclear division, DNA synthesis, activation of the embryonic genome, qualitative and quantitative changes in amino acid uptake, polypeptide synthesis, and morphological maturation of organelles. These observations are

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*To whom correspondence should be addressed.

e-mail: htsujii@shinshu-u.ac.jp

compatible with the notion that maternally inherited developmental information plays an important role in controlling early cleavage of the mouse embryo.

Mongolian gerbil 1-cell embryos do not develop into blastocysts because of 8-cell block [9]. Recently, we found that Mongolian gerbil 2-cell embryos developed into blastocysts *in vitro* in mM16 supplemented with valine [10]. However, the role of valine in the *in vitro* development of Mongolian gerbil embryos is unclear. Thus, the objectives of this study were as follows: i) analysis of ^{14}C -valine incorporation and oxidation, ii) analysis of the effect of valine on ^{14}C -glucose incorporation and oxidation, and iii) analysis of valine metabolism.

Materials and Methods

Animals

Ten- to fourteen-week-old adult virgin female Mongolian gerbils were used at random times in their estrous cycles. The animals were maintained and used in accordance with the Guidelines for Regulation of Animal Experimentation, Faculty of Agriculture, Shinshu University. They were maintained under controlled light conditions (12 h light:12 h darkness) and allowed free access to a pellet diet and tap water. Mongolian gerbils received 20 IU of pregnant mare serum gonadotropin (Peamex, Sankyo Ltd., Japan) followed after 54 h by 20 IU human chorionic gonadotropin (hCG; Puberogen, Sankyo Ltd., Japan). After hCG injection, the animals were mated overnight with fertile males from the same colony.

Embryo culture medium

The basic medium used in this study was mM16, which was modified by adding 2 mM glucose, removing KH_2PO_4 , adjusting the osmolarity to 290 mOsm using 99 mM NaCl (mM16: 99 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl_2 , 1.19 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25 mM NaHCO_3 , 0.33 mM Na-pyruvate, 23.3 mM Na-lactate, 4 mg/ml bovine serum albumin-V; Sigma, St.Louis, MO, USA), 100 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 100 μM ethylene diamine tetra-acetic acid. Osmolarity was determined by freezing point depression using an Advanced micro-osmometer (Advanced Instruments Inc., Norwood, MA, USA).

Collection of embryos

Embryos at the 1-cell, 2-cell, 8-cell, morula, and blastocyst stages were collected at 15, 43, 73, 113, and 133 h, respectively, after the hCG injection. These

embryos were flushed out from the oviductal tubes or uterine horn.

Incorporation and oxidation of radiolabeled valine and glucose

The experiment was initiated with ^{14}C -valine at 18.5 kBq/0.1 mol (specific activity 6.7–8.1 MBq/mol) or ^{14}C -glucose at 18.5 kBq/0.1 mol (specific activity 8.8–13.32 MBq/mol; Moravek Biochemicals, Inc., CA, USA). Five embryos at the 1-cell, 2-cell, 8-cell, morula, and blastocyst stages were transferred to a microtube containing 100 μl of mM16 medium with ^{14}C -valine or ^{14}C -glucose, and then overlaid with mineral oil. One milliliter of 2.5 mM NaOH solution was transferred to a 1.5 mL microtube as a trap for the evolved $^{14}\text{CO}_2$. Both microtubes containing NaOH and ^{14}C -valine or ^{14}C -glucose and embryos were confined in a scintillation vial using a rubber stopper. The scintillation vials were incubated for 2 h in an incubator at 37°C. After incubation, the metabolic reactions of the embryos were stopped with an injection of 100 μl of 10% trichloroacetic acid (TCA; ^{14}C -valine) or 10% perchloric acid (PCA; ^{14}C -glucose) and kept at room temperature for 24 h. The acid insoluble materials were carefully washed by millipore filtration (8.0 μM white SCWP, 47 mm; Millipore Corporation, Bedford, MA, USA) with 5% TCA (^{14}C -glucose) or 5% PCA (^{14}C -glucose), and the filter papers were kept overnight. After drying, the filter papers were transferred into scintillation vials and analyzed for ^{14}C incorporation. The incorporation blank was a scintillation vial containing only filter paper. The NaOH solution was transferred into a new scintillation vial by washing 3–4 times with a cocktail (0.5% PPO + 0.03% POPOP solution in toluene) and analyzed for oxidation. The oxidation blank was a scintillation vial containing only NaOH. All the scintillation vials with 2 ml of cocktail were set in a liquid scintillation counter (LS-6500, Beckman Instruments, Inc., USA) to determine the levels of radioactivity. Values of incorporation and oxidation were calculated as the difference of the measured value from the blank value. This experiment was conducted three times to improve its accuracy. The values of incorporation and oxidation were expressed directly as disintegrations per minute (dpm).

Lipid analysis

The experiment was initiated with ^{14}C -valine at 18.5 kBq/0.1 mol (specific activity 6.7–8.1 MBq/mol). Twenty blastocysts were transferred to a microtube containing 100 μl of mM16 medium with ^{14}C -valine. The microtubes containing ^{14}C -valine and blastocysts were confined in

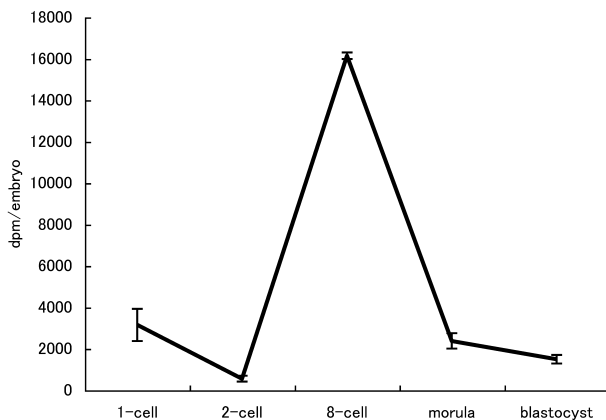


Fig. 1. Incorporation of radiolabeled valine by Mongolian gerbil preimplantation embryos. Data are expressed as mean \pm S.E. (n=3). Different superscripts indicate significant differences ($P < 0.05$).

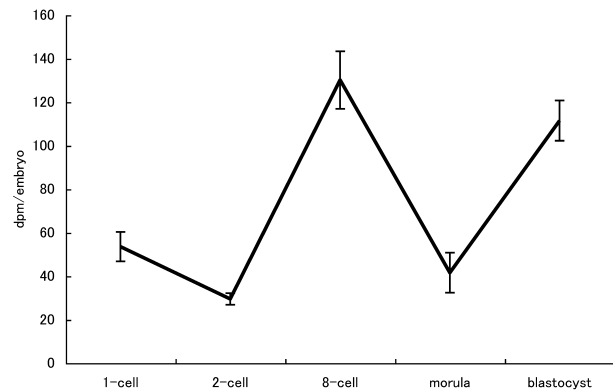


Fig. 2. Oxidation of radiolabeled valine by Mongolian gerbil preimplantation embryos. Data are expressed as mean \pm S.E. (n=3). Different superscripts indicate significant differences ($P < 0.05$).

a scintillation vial using a rubber stopper. The scintillation vials were incubated for 5 h in an incubator at 37°C. After incubation, the blastocysts were collected and washed 3–4 times with mM16 medium. Lipid was extracted from the blastocyst using chloroform: methanol (2:1 v/v). All the samples were analyzed with a liquid scintillation counter. This experiment was conducted three times. The values were expressed directly as disintegrations per minute (dpm).

Statistical analysis

The results are presented as mean \pm S.E. of values. Data of incorporation and oxidation of ^{14}C -valine were analyzed with one-way ANOVA followed by the Tukey-Kramer method as a post hoc test. Data of incorporation and oxidation of ^{14}C -glucose were analyzed with Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

The incorporation and oxidation of ^{14}C -valine by Mongolian gerbil preimplantation embryos are shown in Figs. 1 and 2, respectively. The incorporation of ^{14}C -valine significantly increased at the 8-cell stage. The oxidation of ^{14}C -valine significantly increased at the 8-cell and blastocyst stages. The incorporation and oxidation of ^{14}C -glucose by Mongolian gerbil preimplantation embryos are shown in Figs. 3 and 4, respectively. The incorporation of ^{14}C -glucose in mM16+valine was significantly higher than that in mM16

at the blastocyst stage. The oxidation of ^{14}C -glucose in mM16 was significantly higher than that in mM16+valine at the 8-cell stage, although that in mM16+valine was significantly higher than that in mM16 at the blastocyst stage.

The metabolism of ^{14}C -valine is shown in Table 1. The carbon skeleton of ^{14}C -valine was metabolized into lipid.

Discussion

We examined how valine was utilized and metabolized in early Mongolian gerbil embryos using radioisotopes. We reported that Mongolian gerbil 2-cell embryos can develop to the blastocyst stage with supplementation of valine [10]. In this study, the incorporation and oxidation of valine significantly increased at the 8-cell stage at which cell block normally occurs. Moreover, valine improved the incorporation and oxidation of glucose at the blastocyst stage. These results support our previous results [10]. It has been reported that amino acids are used as energy sources in the early embryos of various other species. The removal of glutamine or glucose from the culture medium suppressed blastocyst formation and significantly reduced cell numbers in embryos. However, the removal of both glutamine and glucose had no further negative effects on blastocyst development. These data indicate that amino acids and carbohydrates may be able to substitute for each other. It has also been reported that the uptake of glutamine by mouse embryos is reduced in the presence of

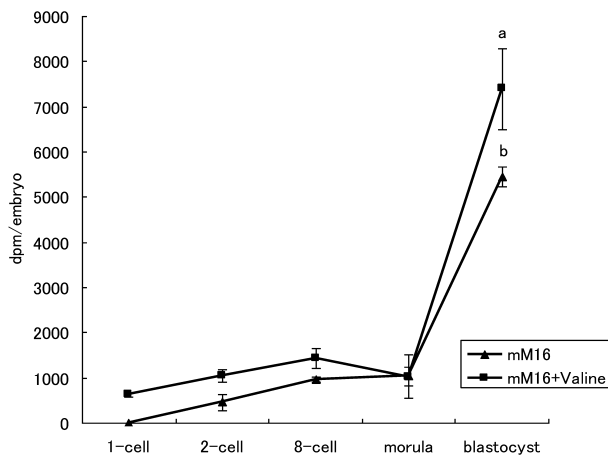


Fig. 3. Effect of valine on incorporation of radiolabeled glucose by Mongolian gerbil preimplantation embryos. Data are expressed as mean \pm S.E. (n=3). Different superscripts indicate significant differences ($P < 0.05$).

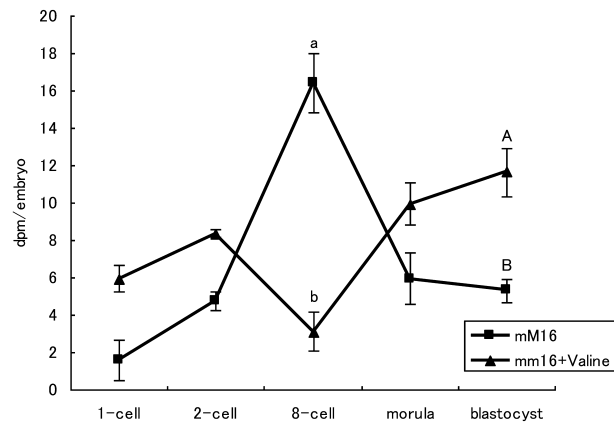


Fig. 4. Effect of valine on oxidation of radiolabeled glucose by Mongolian gerbil preimplantation embryos. Data are expressed as mean \pm S.E. (n=3). Different superscripts indicate significant differences ($P < 0.05$).

Table 1. Metabolism of ^{14}C -valine

	Incorporation	Oxidation	Lipid extract
dpm/embryo	1,495.6 \pm 209.2	111.4 \pm 9.3	12.0 \pm 3.2
Rate (per incorporation) %	100	7.4	0.8

Data are expressed as mean \pm S.E. (n=3).

glucose [11] and that glutamine is oxidized by embryos, indicating its use as an energy source [6]. The ability of glutamine and glucose to interact as energy sources in somatic cells has been documented [12].

Studies of mammalian embryos from several species have revealed that the amino acids, which are present at high levels within the oviduct, oocyte, and early embryo (alanine, aspartate, glycine, glutamate, glutamine, and taurine) [13–15] are important regulators of early embryo development in culture. This group of amino acids has a striking resemblance to Eagle's non-essential amino acids and glutamine [16], with the exception of taurine [17, 18], which is not present in tissue culture media. Eagle's non-essential amino acids and glutamine have been shown to decrease the time taken for mouse embryos to complete the first three cell cycles [19], increase blastocyst formation of the zygotes of mice [20], hamsters [3], and sheep [21], and increase mouse embryo viability [22]. Furthermore, glycine, when present as the sole amino acid, was found to facilitate mouse embryo development at increased osmotic pressure [23]. However, it has also been determined that amino acids are metabolized by the

embryo and spontaneously break down in the culture medium to produce amounts of ammonium that are toxic to the embryo [20–22].

Barnett and Bavister [24] demonstrated that the inhibitory effects of glucose and phosphate on the development of 2-cell hamster embryos in culture are significantly diminished in the presence of amino acids, which supports the hypothesis that amino acids are important regulators of embryo development. It is therefore probable that, during this short collection period when amino acids are absent, there is a sudden efflux of intracellular amino acids [13], leading to intracellular stress such as changes in intracellular pH (pHi) or osmotic stress. In support of this hypothesis, Van Winkle and Dickinson [25] demonstrated that the embryo's intracellular pool of amino acids was affected by the medium used for culture. Amino acids have been identified as regulators of pHi [3] and as organic osmolytes [23, 26, 27], which serve to maintain cellular functions [28]. That a period of 5 min of exposure of the zygote to a medium lacking amino acids has such a detrimental effect on subsequent embryo development is very important, because human IVF oocytes are

routinely collected and held in media lacking amino acids. In our recent study, Mongolian gerbil 2-cell embryos developed to the blastocyst stage after supplementation of the culture. Moreover, threonine, aspartic acid and glutamine improved development to the 4-cell stage [10].

The branched-chain amino acids valine, isoleucine, and leucine are structurally similar, but may not share the same metabolic fate. Valine and isoleucine may be converted into glucose, whereas leucine cannot undergo this conversion. The first two enzymes in the catabolism of branched-chain amino acids are common to all three of these amino acids [29]. The first enzyme is a reversible aminotransferase, which converts the branched-chain amino acids into branched-chain 2-oxo acids. The second enzyme is an irreversible branched-chain 2-oxo acid dehydrogenase. It has been suggested that branched-chain amino acid metabolism proceeds only as far as the 2-oxo acid in muscle, and that these 2-oxo acids leave muscle and are further metabolized in the liver [30–32]. This assertion was originally based on the fact that, in muscle, the activity of the branched-chain amino acid aminotransferase is high relative to that of the branched-chain 2-oxo acid dehydrogenase. In contrast, in liver, the activity of the branched-chain 2-oxo acid dehydrogenase is high compared with that of the aminotransferase. This suggests that the gluconeogenic potential of valine and isoleucine, which are deaminated in muscle, would be conserved because the irreversible branched-chain 2-oxo acid dehydrogenase would only be active in the liver. Valine, leucine, and isoleucine are used by cells for protein synthesis or are catabolized into sources for lipid and glucose production [33]. In Mongolian gerbil blastocysts, valine was metabolized into lipid extract. This result may show that there is a lipid metabolic pathway for the blastocyst formation in Mongolian gerbil embryos.

In this study, the incorporation and oxidation of valine significantly increased at the 8-cell stage at which cell block normally occurs. Moreover, valine improved the incorporation and oxidation of glucose at the blastocyst stage. These results suggest that valine is used in energy metabolism.

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