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Glucose 6-Phosphate Dehydrogenase Activity of Bovine Embryos Produced *in vitro*

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소 체외수정란의 Glucose 6-Phosphate Dehydrogenase 활성

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= 국문초록 =

소 체외수정란에 있어서 pentose phosphate pathway (PPP)를 연구하기 위해서, 한개의 체외수정란으로부터 glucose 6-phosphate dehydrogenase (G6PDH)의 활성을 효소증폭방법으로 측정하였다. Glucose 6-phosphate (G6P) 기질을 처리하지 않은 2, 4, 8세포기, 상실배 및 배반포기 수정란에서의 G6PDH 활성치는 각각 25.5±3.3, 27.8±3.4, 40.9±6.2, 34.9±3.6 및 52.9±2.5×10^{-8} mol/embryo/h 을 나타내었다. 즉, 8 세포기 이후 수정란들은 2 세포기나 4 세포기보다도 높은 효소활성치를 보여주었다 ($P<0.01$). 그리고 G6P 기질을 첨가한 2, 4, 8 세포기, 상실배기 및 배반포기 수정란의 G6PDH 활성치는 각각 32.3±3.9, 29.4±1.8, 51.9±4.2, 42.6±2.7 및 52.9±2.5×10^{-8} mol/embryo/h 로서 기질 무처리구와 마찬가지로 유의성이 인정되었다 ($P<0.01$). 전반적으로 수정란의 발달단계에 있어서 G6P 첨가한 수정란들에 G6PDH의 효소활성치가 기질을 처리하지 수정란들의 것보다도 높은 경향을 보였다. 한편, 소 체외수정란의 G6PDH 효소활성치와 발생능과의 관계를 알아보기 위하여, 4 세포기 수정란들을 효소활성치의 정량적 수준 (low, middle, high)에 따라 3 군으로 분류한 다음 38.5℃, 5% CO_2에서 5일간 난구세포들과 공동배양을 실시하였다. 그 결과, G6PDH 효소활성치 차이에 따른 수정란들의 체외발달율에는 유의성이 인정되지 않았다. 본 실험의 결과를 종합하여 볼 때, 소 체외수정란에 있어서 PPP 대사는 8세포기 이후부터 활발히 이루어지고 있음을 알 수 있었다.

INTRODUCTION

In general, pentose phosphate pathway (PPP) plays an important role in the generation of metabolic energy: NADPH and ribose 5-phosphate. NADPH is a carrier of chemical energy, whereas D-ribose is used in the biosynthesis of nucleic acids. It is known that glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) catalyzes the dehydration of glucose 6-phosphate (G6P) in the first step of the PPP. Several studies have qualitatively estimated the PPP activity of embryos in rabbits (Friedhandler, 1961; Brinster, 1968), mice (Brinster, 1967; Clough & Whittingham, 1983) and rats (Sugawara & Takeuchi, 1973) using glucose radiolabeled at the first and sixth positions of its carbons; the ratio of C-1 and C-6 was determined. O'Fallon & Wright (1968) quantitatively determined the PPP activity in murine embryos by measuring C-1, C-6 and to-

tal glucose metabolism in a mathematical way. The procedure has been applied to fresh (in vivo) preimplantation embryos of cattle (Tiffin et al., 1990; Javed & Wright, 1991) and pigs (Flood & Wiebold, 1988; Selgrath et al., 1989) to measure glucose metabolism via both glycolysis (Embden-Meyerhof pathway; EMP) and PPP. An enzymatic cycling method (Lowry & Passoneau, 1972), in which an oxidized or reduced form of the nucleotide produced by the enzymatic reaction is amplified and multiplied by the cyclic process, has been applied to measure the enzyme activity in a fresh single embryo (Brinster, 1971; Barbehenn et al., 1978). A few studies examined subsequent development of the embryos after measurement of the PPP activity. Recently, Koike et al. (1990) reported that Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline, PBS(-), could be used as a reaction solution to replace the Tris-HCl buffer and that, by doing so, the embryos could be maintained viable after the measurement, leading to subsequent development. Until now, there was a few of reports on measurement of G6PDH activity from single bovine embryos. Therefore, in this study, the activity of G 6PDH was measured from single bovine embryos at various developmental stages. In addition, it was investigated whether the level of G6PDH activity could affect subsequent development of IVF-derived bovine embryos.

MATERIALS AND METHODS

In Vitro Maturation (IVM) and Fertilization (IVF) of Bovine Oocytes

The bovine ovaries were collected at a local slaughterhouse and promptly transported to the laboratory under warm conditions (about 33℃). The oocytes were aspirated from small follicles (2 to 5 mm in diameter) with an 18-gauge needle attached to a 10 ml syringe. Morphologically normal oocytes with dense layers of cumulus cells were used in the subsequent experiment. 25 to 30 oocytes were cultured for 22 hours in a 350μl droplet of TCM-199 medium supplemented with 10% fetal calf serum (FCS: Gibco Lab., USA) under paraffin oil at 38.5℃, 5% CO_2 in air. After in vitro maturation, the oocytes were inseminated with sperm suspension ($5x10^6$ cells/ml) in BO medium (Bracket and Oliphant, 1975) containing 10 mM caffeine and 1% BSA. The sperm suspension was prepared by the following procedure. Frozen semen was thawed in 33℃ water, suspended in 5ml of BO medium containing 10 mM caffeine and heparin (10 μl/ml), and then washed three times in BO medium by centrifugation at 450 x g for 8 min. All ova were washed with TCM-199 medium at 6 hours after insemination and co-cultured with a monolayer of cumulus cells in droplets of TCM-199 medium containing 5% FCS for 1 to 7 days at 38.5℃, 5% CO_2 in air.

Measurement of G6PDH Activity in Bovine Embryos

Individual embryos were assayed for G 6PDH at different developmental stages by an enzyme cycling method (Koike et al., 1990). The reaction mixture for G6PDH consisted of 1.5 mM $NADP^+$, 1 mM EDTA, 0.04% (w/v) BSA and 3 μl/ml G6PDH in PBS(-) free of Ca^{++} and Mg^{++} ions, pH 7.3. Individual embryos were transferred into 1 μl drop of the reaction mixture under paraffin oil and then incubated at 37℃ for 20 min. After removal of the embryos, 1 μl of 0.2 M NaOH was immediately added to each reaction mixture to destroy the unreacted $NADP^+$. Two μl of this reaction mixture was mixed in 1 μl of the cycling reaction solution and incubated at 37℃

for 1 hour. The cycling reaction mixture consisted of 5 mM α-ketoglutarate, 1 mM G6P, 25 mM ammonium acetate, 0.1 mM ADP, 0.02 % BSA, 7.88 μl/ml G6PDH and 103 μl/ml glutamate dehydrogenase (GLDH; EC 1.4.13) in 100 mM Tris-HCl buffer, pH 8.0. After adding indicator mixture to oxidize 6-phosphogluconate produced from the above reaction, the fluorecence of each sample was measured with CAM-220 (Fluorometer, UV Flour x 20, Nikon, Japan). The G6PDH activity indicates the yield of NADPH in mol/embryo/hour. After measurement of G6PDH activity, 4-cell embryos were classified into 3 groups (low, middle and high) by the activity level and then further co-cultured with cumulus cells for 5 days at 38.5℃, 5% CO_2 in air.

Statistical Analysis

The statistical significance of G6PDH activity in various cell stages was analyzed by Student's t-test. Chi-square analysis was used for the evaluation of development rate of 4-cell bovine embryos after measurement of G 6PDH activity.

RESULTS

In the preliminary study, the enzymatic reaction time sufficient for measuring G6PDH activity in a single bovine embryo was 20 minutes. Table 1 shows G6PDH activity of bovine embryos measured without treatment of substrate. G6PDH activity of 2-, 4-, 8-cell embryos, morulae and blastocysts produced in vitro was 25.5 ± 3.3, 27.8 ± 3.4, 40.9 ± 6.2, 34.9 ± 3.6 and $46.9\pm3.4\times10^{-8}$ mol/embryo/h, respectively. Thus, there was significant difference in G6PDH activity between 2- or 4-cell embryos and more advanced embryos ($P<0.01$). When measured by adding 1.5 mM glucose 6-phosphate (G6P) as a substrate, G 6PDH activity of 2-, 4-, 8-cell embryos, morulae and blastocysts was 32.3 ± 3.9, 29.4 ± 1.8, 51.9 ± 4.2, 42.6 ± 2.7 and $52.8\pm2.5\times10^{-8}$ mol/embryo/h, respectively. G6PDH activity of embryos later than 8-cell stage in the group of substrate treatment was also significantly higher compared to that of 2- or 4-cell embryos ($P<0.01$). As shown in Tables 1 & 2, G6PDH activity of bovine embryos treated with substrate was higher in each developmental stage than that without substrate, but this difference was not statistically significant.

It was investigated whether level of G6PDH activity could affect the subsequent development of IVF-derived bovine embryos (Table 3). G6PDH activity of individual 4-cell embryos after treatment of G6P ranged from 15.1 to 55.1 x 10^{-8} mol/embryo/h. The embryos were classified into low (15.1-24.6), middle (25.1-34.8) and high (35.7-55.1 x 10^{-8} mol/embryo/h) groups by the level of G6PDH activity and then further cultured for 5 days at 38.5℃, 5% CO_2 in air. Although frequency of 4-cell embryos with the middle level of activity (48%) was slightly higher than those of low (30%) and high (22%) level, there was no difference in preimplantation development of the three groups of embryos to blastocysts. In addition, the overall developmental rate (22%, 11/50) of the 4-cell bovine embryos subjected to the G6PDH assay was similar to that (31%, 11/36) of control embryos. This result indicated that the method used for measuring G 6PDH activity had no detrimental effect on the subsequent development of IVF-derived bovine embryos.

DISCUSSION

Table 1. G6PDH activity of IVF-derived bovine embryos without substrate

Developmental Stage	No. of embryos Measured	G6PDH activity[1,2] ($\times 10^{-8}$ mol/embryo/h)
2-cell	8	25.5 ± 3.3^{a}
4-cell	8	27.8 ± 3.4^{a}
8-cell	8	$40.9 \pm 6.2^{b,c}$
Morula	8	34.9×3.6^{b}
Blastocyst	8	46.9 ± 3.4^{c}

[1]Values are expressed as mean ± S.E.
[2]a vs b, b, vs c, a vs c; at least $P<0.01$ by a Student's t-test.

Table 2. G6PDH activity of IVF-derived bovine embryos treated with 1.5 mM substrate (glucose 6-phosphate)

Developmental Stage	G6P conc. (mM)	No. of embryos Measured	G6PDH activity[1,2] ($\times 10^{-8}$ mol/embryo/h)
2-cell	1.5	7	32.3 ± 3.9^{a}
4-cell	1.5	8	29.4 ± 1.8^{a}
8-cell	1.5	10	$51.9 \pm 4.2^{b,c}$
Morula	1.5	10	42.6 ± 2.7^{b}
Blastocyst	1.5	8	52.8 ± 2.5^{c}

[1]Values are expressed as mean ± S.E.
[2]a vs b, b, vs c, a vs c; at least $P<0.01$ by a Student's t-test.

Table 3. Preimplantation development of 4-cell bovine embryos produced in vitro according to the level of G6PDH activity

G6PDH activity[1]	No. embryos cultured	Frequency (%)	No. embryos developing to			Developmental rate to blastocysts (%)[2]
			8-cell	morula	blastocyst	
Low	15	30	8	5	3	20
Middle	24	48	16	9	6	25
High	11	22	9	3	2	18
Total	50	100	33	17	11	22
Control	36		24	17	11	31

[1]Embryos were classified into 3 groups depending on the level of G6PDH activity; low (15.1~24.6), middle (25.1~34.8) and high ($35.7 \sim 55.1 \times 10^{-8}$ mol/embryo/h).
[2]No. blastocysts/No. embryos × 100

To evaluate the role of PPP during early development, G6PDH activity of IVF-derived bovine embryos was measured by the enzyme cycling method. It was known through a quantitative estimation of the PPP in murine embryos that the peak of PPP activity occurred at both 2-cell and compact morula stages and that late blastocysts showed the lowest activity (O'Fallon & Wright, 1968). Friedhandler (1961) demonstrated that rabbit embryos utilize glucose via the PPP until blastocsyst stage, at which most glucose is utilized by glycolytic breakdown. Javed & Wright (1991) reported that in bovine embryos much of the glucose metabolism before 16-cell stage occurs via the PPP and total glucose utilization increased drastically at the morula stage with a predominance of the glycolytic pathway, e.g. EMP. In this study, 8-cell bovine embryos derived from in vitro fertilized zygotes, re-

gardless of substrate treatment, showed higher G6PDH activity compared to 2- and 4-cell embryos (Tables 1 & 2). The disparity in the developmental stages showing the peak PPP activity in rabbit and bovine embryos may be partly due to the difference in embryonic potential in different species. Alternatively, the timing of embryonic genome activation may affect the PPP activity during embryogenesis; the activation occurs at 2-cell stage in mice, whereas at 8-cell stage in cattle (Telford et al., 1990).

Rieger (1992) suggested that the onset of glucose metabolism in early embryos is directly related to the synthesis of one or more key glycolytic enzymes. It was reported that glucose is metabolized in bovine blastocysts by the pentose shunt and the Embden-Meyerhof pathway, while glycolysis is blocked because of a lack or inhibition of pyruvate kinase (Rieger & Guay, 1983). In this study, the G 6PDH activity during the early development of IVF-derived bovine embryos was highest in the blastocyst stage (Tables 1 & 2). The pattern of glucose utilization by bovine embryos during preimplantation development is similar to that of porcine (Flood & Wiebold, 1988) and ovine embryos (Thompson et al., 1991).

Since the G6PDH gene is known to be linked to the X chromosome, female and male embryos may have differing developmental potentials in relation to the G6PDH activity prior to the X chromosome inactivation (Tsunoda et al., 1992). Tiffin et al. (1990) tried to identify sex of the bovine embryo by measuring PPP activity, although there was no significant difference in PPP activity between male and female embryos. In our study, no correlation was observed between G6PDH activity and subsequent development of 4-cell bovine embryos (Table 3). However, it needs to be carefully studied in the future whether the sex of bovine embryos could be identified by measuring the level of G6PDH.

In summary, the G6PDH activity of IVF-derived bovine embryos could be measured from a single embryo at various developmental stages by an enzyme cycling method. G6PDH activities were higher later than 8-cell embryos than in 2- or 4-cell embryos. In addition, it was demonstrated that preimplantation development of 4-cell bovine embryos produced in vitro was not significantly different among embryos with varied levels of G6PDH activity.

SUMMARY

To study the pentose phosphate pathway (PPP) in IVF-derived bovine embryos, individual embryos produced in vitro were analyzed for glucose 6-phosphate dehydrogenase (G6PDH) activity using an enzyme cycling method with or without substrate treatment. G6PDH activity of IVF-derived bovine embryos without substrate treatment in 2-, 4-, 8-cell, morula and blastocyst stages was 25.5 ± 3.3, 27.8 ± 3.4, 40.9 ± 6.2, 34.9 ± 3.6 and $46.9\pm3.4\times10^{-8}$ mol/embryo/h, respectively. There was significant difference in G6PDH activity between 2- or 4-cell embryos and embryos later than 8-cell stage ($P<0.01$). G6PDH activity of 2-, 4-, 8-cell embryos, morulae and blastocysts in the presence of glucose 6-phosphate (G6P) was 32.8 ± 3.9, 29.4 ± 1.8, 51.9 ± 4.2, 42.6 ± 2.7 and $52.8\pm2.5\times10^{-8}$ mol/embryo/h, respectively. Difference of G6PDH activity in the group of substrate treatment was also significant between 2- or 4-cell embryos and embryos later than 8-cell stage ($P<0.01$). At each developmental stage, G6PDH activity of IVF-derived bovine embryos treated

with substrate increased compared to that without substrate treatment. Four-cell bovine embryos were classified into 3 different groups (low, middle and high) by the level of G6PDH activity and then further co-cultured with cumulus cells for 5 days at 38.5℃, 5% CO^2 in air. As the result, it was shown that the subsequent development of 4-cell bovine embryos to blastocysts was not affected by level of G6PDH activity. The results suggest that the PPP may be active in bovine embryos in early development, especially in embryos later than 8-cell stage.

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