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Facilitated glucose transporters play a crucial role throughout mouse preimplantation embryo development

Greet Leppens-Luisier, Françoise Urner and Denny Sakkas[1]

Clinic of Sterility, Department of Obstetrics and Gynaecology, University Hospital of Geneva, 1211 Geneva 14, Switzerland

[1]To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar St, PO Box 208063, New Haven, CT 06520–8063, USA. E-mail: Denny.Sakkas@yale.edu

The role of glucose fluctuates during preimplantation mouse embryo development, indicating that a specific interplay exists between glucose metabolism and uptake. In this study, attempts were made to characterize the role of the Na^+-coupled active and the facilitated glucose transporters (GLUT) during preimplantation development by using specific glucose analogues and transport inhibitors and by examining the expression of GLUT1. One-cell outbred mouse embryos were cultured in medium M16 (5.5 mmol/l glucose), M16 without glucose (M16-G), M16-G + 2-deoxyglucose, M16-G + 3-O-methylglucose, M16 + phlorizin and M16 + phloretin and development to the blastocyst stage assessed. The absence of glucose, or the presence of 3-O-methylglucose, which is taken up but not metabolized, did not inhibit blastocyst development. 2-Deoxyglucose, which is phosphorylated but not metabolized, inhibited blastocyst development. Culture in M16 supplemented with phlorizin, an inhibitor of Na^+-coupled active glucose transport did not inhibit blastocyst formation. Phloretin had no effect on the cleavage of two-cell embryos to the four-cell stage, but inhibited the morula/blastocyst transition. Both phloretin and phlorizin inhibited glucose uptake in two-cell embryos. Finally, GLUT1 expression was 10-fold less in blastocysts cultured in M16 compared to in-vivo blastocysts and those cultured in M16-G. The results show that both types of glucose transporters influence preimplantation embryo development and that the embryo has an innate ability to control the uptake of glucose by regulating the expression of GLUT1.

Key words: glucose analogues/glucose transporters/preimplantation mouse embryos

Introduction

Glucose is the main fuel for most cells and its importance as an energy substrate has led to intense research on its actions as a cellular metabolite and into the mechanisms controlling its uptake. Essentially, glucose can be transported across membranes by two different mechanisms: a Na^+-coupled active carrier system, and a growing family of structurally related Na^+-independent glucose transporter glycoproteins (GLUT) (Gould and Holman, 1993; Carayannopoulos *et al.*, 2000).

Whereas a vast number of studies have been conducted on glucose uptake mechanisms in cultured cells, the mechanism of glucose uptake in mammalian preimplantation embryos is still not clear. The GLUT isoforms identified as responsible for glucose uptake in the early mouse embryo are GLUT1, GLUT2, and, more recently, GLUT3 and GLUT8 (Rao *et al.*, 1990; Hogan *et al.*, 1991; Aghayan *et al.*, 1992; Pantaleon *et al.*, 1997; Carayannopoulos *et al.*, 2000). GLUT1, the more ubiquitous transporter, is expressed throughout preimplantation embryo development, while GLUT2 is expressed from the eight-cell stage onwards. GLUT3 expression has been localized on the apical membranes of the polarized cells of the morula and the apical membranes of the trophectoderm cells of the blastocyst (Pantaleon *et al.*, 1997). In contrast, GLUT1 expression was restricted to the basolateral membranes of the outer trophectoderm cells in both morula and blastocysts (Pantaleon *et al.*, 1997). A glucose transporter (GLUT8) responsible for insulin-stimulated glucose uptake in the mouse blastocyst has also been recently identified (Carayannopoulos *et al.*, 2000). The presence of the Na^+-active glucose co-transporter is not as clear. Wiley *et al.* demonstrated that preimplantation mouse embryos contain an antigen that is immunologically and structurally similar to a 75 kDa renal Na^+-active glucose co-transporter (Wiley *et al.*, 1991). In addition, Chi *et al.* proposed that the active hexose transporter could appear in tandem with the glucose transporters on different membranes in mammalian embryos (Chi *et al.*, 1993).

One of the characteristics displayed by preimplantation mouse embryos is the metabolic shift from a dependence on the tricarboxylic acid cycle during the pre-compaction stages to a metabolism based on glycolysis post-compaction (Brinster, 1965; Gardner and Leese, 1986). This change in metabolic preference is coincident with the rapid proliferation that is needed post-compaction. Similar changes in metabolism appear in numerous other cells as they assume a proliferative state.

For example, thymocytes undergo a transition from aerobic to anaerobic metabolism as they undergo mitogen activated proliferation (Bagetto, 1992).

The changes in the role of glucose during preimplantation embryo development indicate that a specific interplay may exist between glucose metabolism and the glucose transporters during different stages of preimplantation embryo development. The importance in examining the changing role of glucose during different embryo stages has also been shown to have relevance to developing new sequential media for human IVF (Gardner and Lane, 1999). In this study, attempts were made to characterize further the role of glucose transporters during preimplantation development by using specific glucose analogues and transport inhibitors during embryo culture and by examining the effect of glucose starvation on expression of the facilitated glucose transporter, GLUT1.

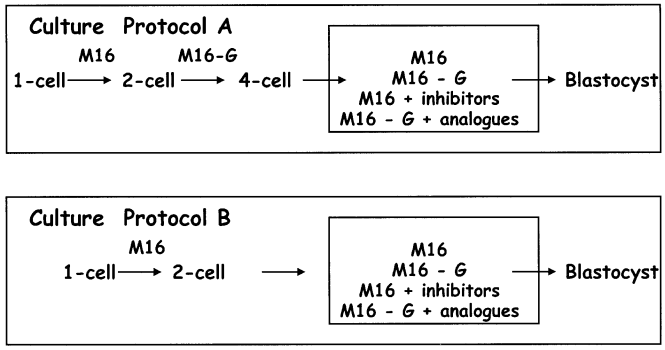


Figure 1. The culture protocols used in assessing development to the blastocyst stage of one-cell outbred OF1 mouse embryos in the presence of glucose analogues and glucose transport inhibitors from (**A**) the four-cell stage and (**B**) the two-cell stage. M16 = embryo culture medium with 5.5 mmol/l glucose, M16-G = M16 without glucose.

Materials and methods

Culture media

The basic embryo culture medium used was M16 (Whittingham, 1971), with (5.5 mmol/l glucose) or without glucose (M16-G), glucose transport inhibitors, and glucose analogues. Two glucose transport inhibitors were used: phloretin (0.1 and 0.5 mmol/l), which inhibits the facilitated diffusion of glucose via the GLUT, and phlorizin (0.5 mmol/l), which inhibits the Na^+-coupled active transport of glucose (Gardner and Leese, 1988). The glucose analogues employed in this study were: (i) 3-O-methylglucose (3OMG) (5.5 mmol/l unless otherwise stated), which interacts with both glucose transport systems but cannot be phosphorylated and generate energy for development, and (ii) 2-deoxy-glucose (2DG) (5.5 mmol/l unless otherwise stated), which interacts with the GLUT and can be phosphorylated, but does not generate adequate energy for development. Antibiotics were added to all culture media (100 IU penicillin G, 0.5 mg/ml streptomycin sulphate) and 4 mg/ml bovine serum albumin (BSA, type V). All chemicals were purchased from Sigma Pharmaceuticals (Buchs, Switzerland), unless otherwise indicated.

Collection of one-cell embryos

In all experiments embryos were recovered from 3–4 week old outbred OF1 female mice, (BRL, Fullinsdorf, Switzerland). These embryos exhibit the two-cell block *in vitro* (Sakkas *et al.*, 1993). Ovulation was induced in the OF1 females and they were mated as previously described (Leppens-Luisier and Sakkas, 1997). Twenty to 22 h after human chorionic gonadotrophin (HCG) injection, the females were killed. One-cell embryos were then collected in HEPES buffered M2 medium (Quinn *et al.*, 1982) and the cumulus cells removed using hyaluronidase (1 mg/ml) in M2 buffer. For Western blot analysis, oocytes were collected from stimulated mice 14–16 h after administration of HCG and the cumulus cells removed using 0.2 mg/ml hyaluronidase (Sigma). In-vivo developed blastocysts were collected 96 h post-HCG after ovulation induction and mating as above. The oocytes and blastocysts were collected in M2 buffer, washed thoroughly and placed in lysis buffer.

Culture of one-cell embryos

To examine the effect of the glucose transport inhibitors and glucose analogues during different cleavage stages, two different culture protocols were examined. The culture protocols used in the different treatment sets are shown in Figure 1A and B. The percentage of embryos reaching the four-cell, morula and blastocyst stage was assessed at 64, 88 and 120 h post-HCG administration respectively. In each individual replicate experiment, one-cell embryos were cultured per individual mouse until the time of distribution to the different culture treatments. Embryos were cultured in groups of 5–15, in 20 µl drops under oil (light white mineral oil; Sigma) at 37°C in 5% CO_2 in air.

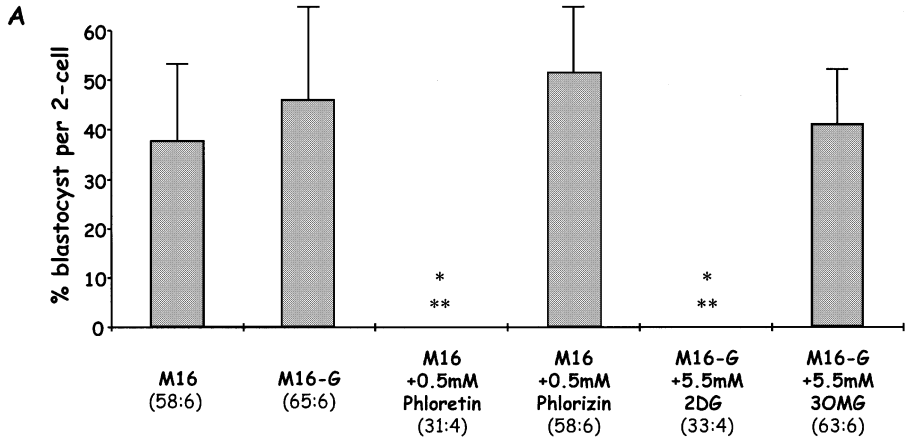
In culture protocol A (Figure 1A) one-cell embryos from each individual mouse were cultured in M16 until the two-cell stage and then placed in M16-G until the four-cell stage, so as to obtain a high number of four-cell embryos. The embryos from each mouse were then randomly allocated to the different culture conditions being tested so that each culture group contained a pool of embryos not biased by any individual mouse (Figure 1A). The number of replicates is shown in Figures 2 and 3. In culture protocol B, one-cell embryos from each mouse were cultured separately from each mouse until the two-cell stage in M16, and then randomly allocated to the different culture conditions as above (Figure 1B).

Differential labelling of trophectoderm and inner cell mass

In both series of experiments individual blastocysts were removed at 120 h after HCG administration and the number of trophectoderm and inner cell mass cells counted. The cell numbers were assessed by the differential labelling technique originally described by Handyside and Hunter using the two polynucleotide specific fluorochromes, propidium iodide and Hoechst 33342 (Sigma) (Handyside and Hunter, 1984).

[^{3}H]3-O-methylglucose uptake in the presence of glucose transport inhibitors

Glucose transporter inhibition studies routinely use 3OMG because it is an effective substrate for the facilitated glucose transporters and also shows a significant affinity for the phlorizin sensitive Na^+-active glucose transporter (Crane, 1960; Crane, 1962; Ulrich *et al.*, 1974; Knight and Sansom, 1977; Turner and Silverman, 1977; Mullin *et al.*, 1992). In this part of the study, a modification of the method previously described by Gardner and Kaye was used to measure the [^{3}H]3OMG uptake of the two-cell embryos (Gardner and Kaye, 1995). Uptake at two time points was measured, in the presence of 25 mmol/l 3OMG and a radioactive trace of [^{3}H]3OMG (5 Ci/mmol/l; Amersham, Dubendorf, Switzerland). Briefly, one-cell OF1 embryos were recovered from individual mice as previously described and cultured overnight in M16. The following morning, two-cell embryos were allocated equally to the following culture groups: (i) M16; (ii) M16 + 0.1 mmol/l phloretin and (iii) M16 + 0.5



B

No. of blastocysts		22	26	---	26	---	19
Mean cell number (±s.d)	TE	31.9±16.3	24.7±9.8*	---	27.4±10.1	---	22.3±10.8*
	ICM	17.3±8.5	16.2±8.4	---	14.5±7.0	---	12.8±8.6
	Total	49.2±23.2	40.9±16.7	---	42.2±14.7	---	35.2±17.6*

Figure 2. (**A**) Development to the blastocyst stage (mean ± SD) of one-cell outbred OF1 mouse embryos using the culture protocol of Figure 1A. Embryos were transferred to M16, M16 without glucose (M16-G), M16 + phloretin, M16 + phlorizin, M16-G + 2-deoxyglucose (2DG) and M16-G + 3-O-methylglucose (3OMG) at the four-cell stage. The number of embryos assessed and replicate experiments performed are shown in parentheses. (**B**) The mean total, inner cell mass (ICM) and trophectodermal (TE) cell numbers (±SD) of blastocysts obtained after culture of one-cell outbred embryos according to the Figure 1A culture protocol. Embryos were transferred to M16, M16-G, M16 + phloretin, M16 + 0.5 mmol/l phlorizin, M16-G + 2DG and M16-G + 3OMG. Cultured blastocysts were assessed at 120 h after human chorionic gonadotrophin (HCG). *Denotes significantly different $P < 0.05$ when compared with M16 and ** denotes significantly different $P < 0.05$ when compared with M16-G.

mmol/l phlorizin. After a 3 h preincubation, the two-cell embryos were transferred to another drop containing M16 + 3OMG (25 mmol/l), a radioactive trace of [^{3}H]3OMG and the glucose transporter inhibitors. After the incubation period (6 or 12 min), embryos were transferred in a minimum volume (3 µl) of medium to 2 ml of ice cold hexose-free M16 medium and quickly washed through a further three changes (2 ml each) of the same medium on ice. Sham preparations, containing the same volume as that used to transfer the embryos, were included in each replicate. Ten two-cell embryos were assayed at each time. Three experiments with five replicates of 10 two-cell embryos were performed. From the last wash drop, 10 two-cell embryos were taken up in 3 µl, lyzed in 100 µl of water and added to 3 ml of scintillation liquid (Lumac; Lumagel, Basel, Switzerland).

GLUT1 expression in blastocysts cultured in the presence and absence of glucose

To investigate whether the presence or absence of glucose could induce changes in GLUT 1 expression, blastocysts that developed *in vivo* and after different periods of culture were compared in the presence or absence of glucose: i.e. (i) from the one-cell stage in M16-G, (ii) from the one-cell to the two-cell stage in M16 (5.5 mmol/l glucose) and then transferred to M16-G, and (iii) embryos recovered from oviducts at the two-cell stage cultured in M16. Western blot analysis was performed using similar procedures to that described by Aghayan *et al.* for the detection of GLUT in preimplantation embryos (Aghayan *et al.*, 1992). Briefly, 60 blastocysts from each group were lyzed in 10 µl of lysis buffer [80 mmol/l Tris–HCl, 5% sodium dodecyl sulphate (SDS), 5 mmol/l EDTA, 0.2 mmol/l PMSF, 2 mmol/l *N*-ethyl-maleimide] and stored at –20°C. Prior to electrophoresis, the samples were diluted in a twice concentrated solution of Laemmli's sample buffer (Laemmli, 1970). Electrophoresis was performed using a 10% polyacrylamide SDS minigel system (BioRad, Zurich, Switzerland) and the proteins transferred to the nitro-cellulose membrane with the BioRad mini-transfer system. After transfer, the membrane was washed and prepared, using a slight modification of the procedure described by Aghayan *et al.* (Aghayan *et al.*, 1992), and the detection of GLUT1 performed using the enhanced chemiluminescence system (ECL) and western blotting detection reagents of Amersham (Amersham Rahn). Erythrocytes were used as a control on each gel. The GLUT1 antibody was a kind gift from Dr Bernard Thorens (Institute of Pharmacology and Toxicology, University of Lausanne, Switzerland) and has previously been used for the detection of GLUT1 on mouse embryos (Hogan *et al.*, 1991). Detection was performed using Hyperfilm ECL (Amersham).

Statistical analysis

The χ^2 test with continuity correction and analysis of variance using the arcsin transformation were used to compare the percentages of embryos reaching the four-cell, morula and blastocyst stage in each experiment. Comparison of replicates within treatment groups was also performed using χ^2 analysis with continuity correction and analysis of variance using the arcsin transformation. No significant differences were found between replicate experiments. Differences between the mean number of cells per blastocyst and the means of [^{3}H]3OMG uptake measurements were compared using one-way analysis of variance and

the Sheffé's *F*-test for multiple comparisons. All statistical evaluations were performed using Statview 4.5 (Abacus Concepts Inc., Cary, NC, USA).

Results

Effect of glucose during preimplantation mouse embryo development

Culture protocol A

Glucose transport inhibitors

When one-cell OF1 embryos were cultured until the four-cell stage and then allocated to M16 or M16-G, approximately 40% of the embryos developed to the blastocyst stage (Figure 2A) but the number of trophectodermal (TE) cells was lower in the absence of glucose (Figure 2B). The addition of 0.5 mmol/l phloretin to M16 completely inhibited blastocyst development, hence in the final two replicates this group was not tested (Figure 2A). In contrast, when the Na^+-coupled active glucose transport inhibitor phlorizin was added to M16 blastocyst development was not inhibited. The total number of cells, the number of ICM and TE cells in individual blastocysts did not differ significantly when comparing M16 and M16 + phlorizin as observed when compared to M16-G (Figure 2B).

To examine further the effects of phloretin, three experiments were performed where one-cell embryos were cultured, according to culture protocol A, but in lower concentrations of phloretin (0.05 mmol/l; 0.1 mmol/l). In these experiments there was no significant difference in the proportion of embryos reaching the morula stage. The percentage of blastocysts developing per morula was, however, significantly lower ($P = 0.02$) in the 0.1 mmol/l phloretin group (8/21, 38.1%) compared to 0.05 mmol/l phloretin (16/24: 66.7%) and to the control without phloretin (18/29, 62.1%).

Glucose analogues

When glucose analogues were assessed we found that addition of 5.5 mmol/l 3OMG to M16-G after the four-cell stage had no effect on the percentage of blastocysts developing (Figure 2A). The number of cells per blastocyst was significantly reduced when compared to M16 (Figure 2B). This reduction in the presence of 3OMG was due to a significantly decreased number of trophectodermal cells (Figure 2B). The addition of 5.5 mmol/l 2DG to M16-G inhibited blastocyst development, and was not tested in the final two replicates (Figure 2A).

Culture protocol B

Glucose transport inhibitors

When one-cell OF1 embryos were cultured until the two-cell stage in M16 and then allocated to M16 or M16-G, a poor rate of cleavage to the four-cell stage was observed in the presence of glucose [(8/60) 13% versus (61/79) 77% respectively]. This observation is similar to our previous studies (Sakkas *et al.*, 1993). In the presence of 0.1 mmol/l phloretin, 38.8% of the two-cell embryos cleaved to the four-cell stage. However, whereas 37% of the four-cells formed morula only two of the compacted embryos went on to form blastocysts (Figure 3A). The presence of phlorizin allowed 36.7% of two-cell embryos to cleave to the four-cell stage and a greater proportion of embryos reached the morula and the blastocyst stage (Figure 3A). The total number of cells and the number of ICM and TE cells in individual blastocysts did not differ when comparing M16-G to M16 + 0.5 mmol/l phlorizin (Figure 3B). To examine further the effects of phloretin, three experiments were performed where one-cell embryos were cultured according to culture protocol B, using 0.05 mmol/l and 0.1 mmol/l phloretin. No blastocysts developed in M16. The percentage of blastocysts developing per two-cell embryo was similar in the 0.1 mmol/l phloretin group (5/43, 11.6%) compared to 0.05 mmol/l phloretin (6/44: 13.6%), while the rate in M16-G remained higher (15/44, 34.1%). The percentage of compacted embryos per two-cell embryo was 40, 47 and 37% for the 0.1 mmol/l, 0.05 mmol/l phloretin and M16-G groups respectively.

Glucose analogues

When the role of the glucose analogues was assessed, a similar pattern was observed to that seen when using culture protocol A. In the presence of 3OMG, embryo development was similar to that in absence of glucose. In the presence of 0.55 mmol/l 2DG, 43.5% of the two-cell embryos cleaved to the four-cell stage (Figure 3A). In two replicate experiments performed with 5.5 mmol/l 2DG and in four replicate experiments using 0.55 mmol/l 2DG, no embryos went on to form blastocysts (Figure 3A). In the three treatment groups that gave rise to blastocysts, there was no significant difference in the cell numbers of the blastocysts that developed (Figure 3B).

Glucose uptake at the two-cell stage in the presence of glucose transporters inhibitors

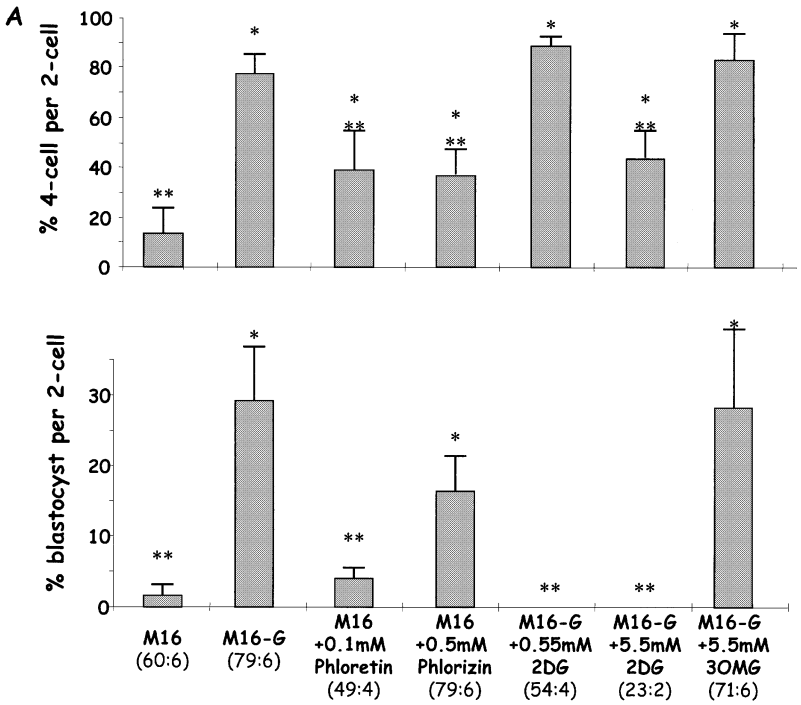
The measurement of [^{3}H]3OMG uptake was performed in the presence of 25 mmol/l of 3OMG and a trace of radiolabelled [^{3}H]3OMG. Groups of 10 two-cell embryos were measured in the presence of 0.1 mmol/l phloretin or 0.5 mmol/l phlorizin. At the two-cell stage, glucose uptake was reduced significantly after 6 min in the presence of both inhibitors (Figure 4). After 12 min, glucose uptake was not significantly different between the groups, suggesting that equilibrium was reached.

GLUT1 expression in blastocysts cultured in the presence and absence of glucose

Western blot analysis revealed a strong presence of GLUT1 in in-vivo blastocysts (Figure 5) This presence was reduced by ~17–30% in blastocysts that had not been exposed to glucose from the one-cell or two-cell stage (Figure 5). In contrast, GLUT1 expression was reduced by 90% in blastocysts developed in the presence of glucose from the two-cell stage compared to those grown *in vivo* (Figure 5). Density analysis, expressed as mean arbitrary units ± SD, of three replicate gels showed that blastocysts that had developed from the two-cell stage in the presence of glucose (2867 ± 344) had a significantly ($P < 0.01$) reduced expression of GLUT1 compared to those grown *in vivo* (29 255 ± 655) and those cultured in the absence of glucose (24 282 ± 3972 and 20 497 ± 3181 for one-cell embryos and two-cell embryos cultured to the blastocyst stage in medium without glucose respectively).

Discussion

In this study, the specific interplay that exists between glucose and the glucose transporters during different stages of preimplantation



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		M16 (60:6)	M16-G (79:6)	M16 +0.1mM Phloretin (49:4)	M16 +0.5mM Phlorizin (79:6)	M16-G +0.55mM 2DG (54:4)	M16-G +5.5mM 2DG (23:2)	M16-G +5.5mM 3OMG (71:6)
No. of blastocysts		---	20	---	10	---	---	20
Mean cell number(±s.d.)	TE	---	24.3±7.1	---	26.5±12.4	---	---	22.3±11.3
	ICM	---	13.6±5.0	---	11.7±8.3	---	---	11.1±6.9
	Total	---	37.9±10.2	---	38.2±18.6	---	---	33.4±15.4

Figure 3. (**A**) Development to the four-cell and blastocyst stage (mean ± SD) of one-cell outbred OF1 mouse embryos using the culture protocol of Figure 1B. Embryos were transferred to M16, M16 without glucose (M16-G), M16 + phloretin, M16 + phlorizin, M16-G + 2-deoxyglucose (2DG) and M16-G + 3-O-methylglucose (3OMG) at the two-cell stage. The number of embryos at the two-cell stage assessed and replicate experiments performed is shown in parentheses. (**B**) The mean total, inner cell mass (ICM) and trophectodermal (TE) cell numbers (±SD) of blastocysts obtained after culture of one-cell outbred embryos according to the Figure 1B culture protocol. Embryos were transferred to M16-G, M16 + phlorizin and M16-G + 3OMG at the two-cell stage. Cultured blastocysts were assessed at 120 h after HCG. *Denotes significantly different $P < 0.05$ when compared with M16 and ** denotes significantly different $P < 0.05$ when compared with M16-G).

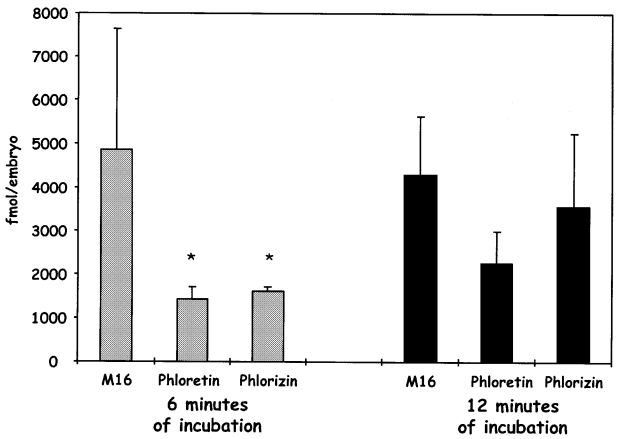

Figure 4. Uptake of (3H)-3-OMG by two-cell embryos in the presence of 25 mmol/l of 3OMG and a trace of radiolabelled [^{3}H]3OMG following the protocol of Gardner and Kaye (Gardner and Kaye, 1995). * Denotes significantly lower $P < 0.05$ when compared to M16.

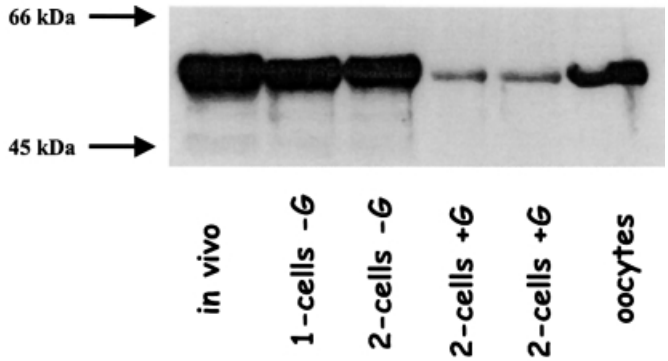

Figure 5. Detection of facilitated glucose transporter (GLUT1) expression in blastocysts developed *in vivo* (lane 1), cultured in the absence of glucose from the one-cell (lane 2) and two-cell (lane 3) stage and cultured in the presence of glucose from the two-cell stage (lanes 4 and 5). Each lane contained 60 blastocysts. The GLUT1 expression of 60 oocytes is shown in lane 6.

embryo development was examined by using glucose analogues and glucose transporter inhibitors.

Development of embryos in glucose free medium and in the presence of glucose analogues

Glucose has been strongly implicated in (i) the cleavage arrest of outbred strain embryos during the two-cell stage *in vitro* (Chatot *et al.*, 1989; Brown and Whittingham, 1991, 1992; Sakkas *et al.*, 1993) and (ii) in allowing embryos to undergo the morula/blastocyst transition (Chatot *et al.*, 1989; Brown and Whittingham, 1991, 1992; Martin and Leese, 1995). Outbred mice represent an interesting model to further our understanding of the effect of glucose on preimplantation embryo development. Importantly, this mouse model has been useful in formulating media that are currently substantially improving outcomes in human IVF (Gardner and Lane, 1999). The present study has confirmed that glucose has an inhibitory effect during the two- to four-cell stage and that absence of glucose reduces blastocyst cell numbers (Sakkas *et al.*, 1993), an occurrence related to a reduction in TE cell number. In addition, the inability of 3OMG to improve development when added to medium devoid of glucose compared to when glucose is present contradicts the proposal that glucose was involved in stimulating a cell surface mediated event or an intracellular signalling process (Chatot *et al.*, 1994) and confirms that glucose needs to be metabolized to improve development. In contrast to 3OMG, 2DG which is phosphorylated on entry into the cell but not metabolized, was completely inhibitory to embryo development at 5.5 mmol/l, while the inhibitory effect was more gradual at 0.55 mmol/l. Barbehenn *et al.* (Barbehenn *et al.*, 1974, 1978) have shown that glucose-6-phosphate concentrations are characteristic at each stage and do not rise dramatically, even when refeeding with glucose. The toxic effect of 2DG was likely to be due to wastage of intracellular ATP in the phosphorylation of 2DG, which would reduce the amount of ATP available for other cellular processes. This accumulation of phosphorylated 2DG would also lead to a disequilibrium in the balance of enzyme activities.

Na^+-coupled active glucose transport

Western analysis has shown the presence of an antigen that was similar to a 75 kDa renal Na^+-glucose cotransporter in preimplantation mouse embryos (Wiley *et al.*, 1991). Indirect immunofluorescence failed to detect the presence of the active transporter in two-cell embryos, while it was observed in some four-cell embryos and in all subsequent stages (Wiley *et al.*, 1991). Phlorizin, a known inhibitor of active glucose transport (Sacktor, 1989), has no effect on glucose uptake by mouse blastocysts (Wiley and Obasaju, 1989). Wiley and Obasaju also showed that varying concentrations of phlorizin had no effect on the cavitation of blastocysts (Wiley and Obasaju, 1989). The ability of some four-cell embryos to develop from the two-cell stage in the presence of M16 + phlorizin indicates that it may suppress the inhibitory effect of glucose during this stage and that the Na^+-coupled active glucose transport may play a role between the two- and four-cell stage. These observations were confirmed by the reduction of [^{3}H]3OMG uptake in two-cell embryos in presence of phlorizin, even though the current uptake results were higher than those reported by Gardner and Kaye (Gardner and Kaye, 1995). Interestingly, the suggested role for the Na^+-active glucose transporter may provide part of the explanation as to why reducing Na^+ levels in culture medium leads to improved development of early cleavage embryos (Lawitts and Biggers, 1991a,b; Summers *et al.*, 1995).

Facilitative glucose transport

GLUT1 is detectable throughout mouse preimplantation development (Hogan *et al.*, 1991; Aghayan *et al.*, 1992; Mullin *et al.*, 1992; Morita *et al.*, 1994). GLUT3 also plays a vital role in the development of the mouse embryo (Pantaleon *et al.*, 1997). In the experiments performed in the current study, it was not possible to detect GLUT3 expression in blastocysts (unpublished observations), although the same antibody was able to detect GLUT3 in mouse sperm (Urner and Sakkas, 1999). In humans, GLUT1 expression, but not GLUT3 expression, has been found in early cleavage stage embryos (Dan-Goor *et al.*, 1997). GLUT3 expression, but not GLUT1 or glucose supply, has been postulated to be essential for blastocyst formation in a manner independent of its function as a glucose transporter (Pantaleon *et al.*, 1997). When different stage embryos were cultured in the presence of phloretin the role of the facilitated glucose transporters was crucial after the morula stage. The role of the facilitated glucose transporters may not, however, be solely in providing glucose to the embryo. If the sole action of the transporters were to provide glucose then we would have expected to see a similar rate of development as that observed in the absence of glucose. The results observed when supplementing media with phloretin indicate that inhibiting the facilitated glucose transporters may have secondary consequences to the embryo. The activity of the facilitated glucose transporters may therefore be involved in promoting secondary factors that are linked to the metabolism of glucose. Another possibility is that phloretin does not act solely on glucose transport, and it is possible that the inhibition of morula/blastocyst transition may have arisen due to another effect of phloretin. It can also activate the Ca^{2+} channels and produce a precocious fusion between cells (Shin *et al.*, 1996), but this is unlikely because no fusion of blastomere membranes was seen in the presence of phloretin.

Further evidence of the requirement for the facilitated glucose transporters is provided by the regulated expression of GLUT1 in blastocysts grown in the absence or presence of glucose. The expression of GLUT1 in blastocysts developed in the absence of glucose from either the one-or two-cell stage is close to the expression of GLUT1 in blastocysts grown *in vivo*. In addition, similar GLUT1 expression was observed when OF1 mouse embryos were cultured from the one-cell stage in synthetic oviductal medium enriched with potassium (KSOM) medium (Summers *et al.*, 1995) containing 0.2 mmol/l or 5.56 mmol/l glucose (data not shown). This was in contrast to blastocysts developing in the presence of glucose from the two-cell stage, which showed vastly diminished GLUT1 expression. Although these differences are relative to the whole blastocyst, it would be of interest to examine if GLUT1 expression differs per cell between different culture groups. Morita *et al.* have shown that GLUT1 mRNA expression of *in vitro* developed blastocysts was less than half that of in-vivo blastocysts (Morita *et al.*, 1994). Moley *et al.* have also shown that in response to maternal

hyperglycaemia, preimplantation mouse embryos experience a decrease in glucose utilization directly related to a decrease in glucose transport at both the mRNA and protein levels (Moley *et al.*, 1998a,b). The high number of GLUT1 transporters *in vivo* may be linked to the embryo's need for extra glucose to be stored as glycogen prior to implantation, when it enters an anoxic environment (Leese, 1991). Unlike their in-vivo counterparts, embryos cultured *in vitro* are constantly in a high glucose environment and may not need to store glycogen prior to implantation.

Significance of glucose transport to glucose metabolism

The metabolic control of preimplantation embryo development proceeds at two levels: (i) control intrinsic to the embryo due to the amounts and activities of enzymes, intracellular mediators and plasma membrane transport systems and (ii) control extrinsic to the embryo mediated via the environment provided by the female reproductive tract (Leese, 1995). The change from the use of pyruvate to glucose as a major energy substrate, observed in preimplantation mouse embryos (Leese and Barton, 1984), is thought to arise due to a specific control at the level of two key enzymes in glycolysis, hexokinase and phosphofructokinase (Barbehenn *et al.*, 1974, 1978). Although previous studies have shown that intrinsic control mechanisms exist at the enzyme level it is also highly feasible, as acknowledged by Barbehenn *et al.*, that the metabolic changes occurring during preimplantation embryo development are partly dependent on glucose uptake (Barbehenn *et al.*, 1978).

The results of this study have confirmed that the effect of glucose changes during preimplantation embryo development, in that high concentrations (5.5 mmol/l) are inhibitory during the early cleavage stages but it is needed to sustain an augmentation in cell numbers post-compaction. An examination of a greater range of glucose concentrations for each embryo stage is, however, still necessary to ascertain if glucose is always inhibitory or whether low concentrations may enhance development. More importantly, the Na^+-active glucose transporter may play a role in glucose uptake during the two- to four-cell transition in the mouse, although its actions do not appear to be crucial, as the presence of phlorizin still allows blastocyst formation. The facilitated glucose transporters do, however, play a crucial role throughout preimplantation embryo development, in particular during the morula/blastocyst transition phase when glucose uptake increases dramatically. This and other studies (Wiley and Obasaju, 1989; Morita *et al.*, 1992) indicate that the numbers of both glucose transporters at the two-cell stage may be important for embryo development. Finally, it is clear that the embryo has an innate ability to control the uptake of glucose by regulating the expression of GLUT1. This study has demonstrated that the glucose transporters play a key role during preimplantation embryo development. To understand the role of glucose during preimplantation further, it is now necessary to examine the interplay between glucose metabolism and the control of regulation and expression of both the Na^+-active and facilitated glucose transporters during the different cleavage stages.

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