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Selection of viable mouse blastocysts prior to transfer using a metabolic criterion

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The success rate of human in-vitro fertilization (IVF) remains low, with only ~10% of embryos transferred resulting in a term pregnancy. A major contributor to this embryonic loss is poor embryo development *in vitro*. Such poor development can be attributed to both chromosomal and anatomic anomalies in oocytes after ovarian stimulation and to suboptimal embryo culture conditions. The low success rate of IVF is compounded by an inability to select those embryos most likely to implant after transfer (viable). Currently morphology is used almost exclusively as the sole criterion to decide which embryos are replaced. This procedure is not only subjective but has a poor correlation with subsequent developmental competence. Therefore, the development of techniques to quantify embryo viability prior to transfer will significantly increase pregnancy rates. We report here that the non-invasive assessment of glycolytic activity (percentage of glucose converted to lactate) in individual mouse blastocysts prior to transfer can be used successfully to identify viable embryos. Blastocysts with a low glycolytic activity, close to that of in-vivo developed blastocysts, had a significantly higher viability than those with abnormally elevated levels of glycolysis. Using glycolytic activity as a marker of viability resulted in a four fold increase in the pregnancy rate compared with embryos selected at random for transfer. We propose that the success of clinical IVF can be increased significantly by employing quantitative tests for viability.

Key words: fetal development/glucose uptake/glycolysis/embryo/viability

Introduction

Mammalian embryo development is compromised *in vitro*, resulting in low pregnancy rates after transfer. Embryo development *in vitro* is associated with slow cleavage rates and perturbation of metabolic pathway activities, so that by the blastocyst stage embryos are retarded up to 24 h compared with those *in vivo* (Bowman and McLaren, 1970; Streffer *et al.*, 1980) and exhibit abnormally high levels of glycolytic activity (Gardner and Leese, 1990; Gardner and Sakkas, 1993). Previous attempts to increase the success of assisted reproduction by the selection of the most viable embryos within a cohort have been based on nutrient uptake (Renard *et al.*, 1980; Rieger, 1984; Gardner and Leese, 1987). Using this approach it has been shown retrospectively that those embryos with the capacity to develop after transfer to a recipient uterus have a significantly higher glucose uptake at the blastocyst stage of development. Despite these data, there have been no prospective studies on embryo selection.

Therefore we have used both glucose uptake and its conversion to lactate (hence glycolytic activity) to select prospectively mouse blastocysts for transfer.

Materials and methods

Embryo collection

Embryos for culture were collected from 4 week old F_1 hybrid (C57BL/6×CBA/Ca) mice Multiple ovulations were induced by an i.p. injection of 5 IU pregnant mare's serum (Folligon; Intervet; Lyppard, Victoria, Australia), followed 48 h later by an i.p. injection of 5 IU human chorionic gonadotrophin (HCG; Chorulon; Lyppard) Immediately after the second injection, females were placed with males of the same strain overnight and mating was assessed the following morning by the presence of a vaginal plug.

Zygotes were collected 21 h post-HCG in a HEPES-buffered modification of medium DM1 (Lane and Gardner, 1995) Cumulus masses were dispersed by a brief incubation with hyaluronidase (1 mg/ml) (bovine testes; Sigma Chemical Co., St Louis, MO, USA) Denuded zygotes were washed three times in HEPES–DM1 and once in DM1, before being placed into culture.

In-vivo developed control blastocysts were collected from 6–8 week old mice of the albino strain CF1. Blastocysts were flushed from the uterus with HEPES–DM1 immediately prior to transfer at 92 h post-HCG.

Embryo culture

Embryos were cultured in groups of 10 in 20 µl medium DM1 under mineral oil (Sigma Chemical Co.) at 37°C in 5% CO_2 in air (Lane and Gardner, 1992). After 48 h of culture, the medium was renewed to alleviate ammonium toxicity (Gardner and Lane, 1993) After 89 h of culture, morphologically identical blastocysts with equivalent diameters were pooled and their glycolytic rates determined.

Analysis of glycolytic rates in individual blastocysts

Glycolytic rate was assessed using quantitative microfluorescence, based on the generation of either NADH or NADPH (Gardner and Leese, 1990; 1993) Individual blastocysts were washed several times and then subsequently incubated in 35 nl drops of modified medium MTF (Gardner and Lane, 1993) containing 0.5 mM glucose as the sole energy source. Blastocysts were incubated at 37°C for 75 min Serial 1 nl samples of medium were removed at 15 min intervals and analysed fluorometrically Assay reactions were housed in 10 nl drops of reagent on siliconized microscope slides. Linear rates of glucose

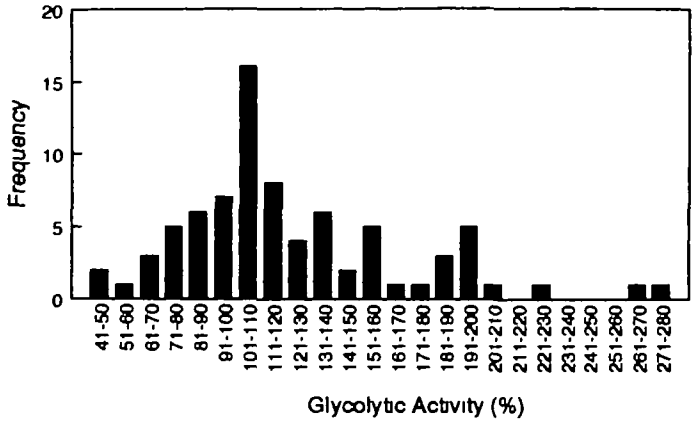

Figure 1. Distribution of glycolytic activity in a population of morphologically similar individual mouse blastocysts cultured in medium DM1 (Lane and Gardner, 1995) (n = 79 blastocysts)

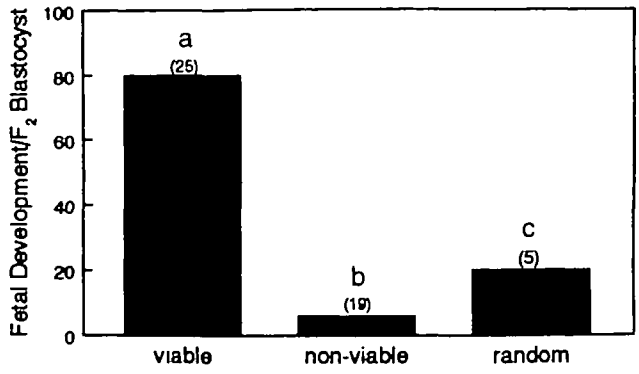

Figure 2. Fetal development of mouse blastocysts selected for transfer using glycolytic activity as a biochemical marker. 'Viable' blastocysts were classified as those with a glycolytic rate in the lowest 15% of the distribution (glycolytic rate <88%), whilst 'non-viable' blastocysts were those with a glycolytic rate in the highest 15% of the distribution (glycolytic rate >160%). On each day of the experiment, a selection of blastocysts were transferred at random. Values in parentheses are numbers of blastocysts transferred. [a,b,c]Different superscripts indicate significantly different populations ($P < 0.01$).

consumption and lactate production were determined for each blastocyst, and the glycolytic activity was calculated on the basis that 1 mol glucose produces 2 mol lactate Glycolytic activity has been expressed as a percentage.

Initially the distribution of glycolytic activity in a population of 79 morphologically similar blastocysts was determined (Figure 1). The range of glycolytic activity varied from 41%, the level observed in in-vivo developed blastocysts (Gardner and Leese, 1990; Gardner and Sakkas, 1993), to up to 280%, and was not normally distributed. In the latter case, this represented lactate formed from energy sources other than the glucose consumed because no other substrates were present in the incubation medium. The observed distribution of glycolytic activity of individual blastocysts cannot be attributed to the allocation of F_2 embryos into subpopulations because the highly polygenic variation in F_2 embryos would preclude any sharp separation. Should the genotype of the embryo be related to metabolism, then a normal distribution of glycolytic activities would have been observed as the two parental genotypes are randomly segregated. Such a distribution was not evident. Even if genotype and metabolic activity of the blastocysts were associated, then the data would reflect the ability to select blastocysts from different strains of mice. However, when blastocysts that had been selected as viable prior to transfer were allowed to litter, both parental phenotypes were present, indicating that viable embryos had indeed been selected irrespective of the genetic background of the parents.

Subsequently, a further 200 blastocysts had their glycolytic activity quantified and were selected prospectively for transfer on the following grounds: embryos in the lowest 15% of the glycolytic distribution (<88% glycolytic activity, i.e. those embryos with a metabolic profile similar to those of in-vivo developed embryos) were predicted to be viable, in contrast, embryos in the top 15% (>160% glycolytic activity, i.e. those embryos apparently prematurely utilizing the endogenous energy reserves required for implantation) were predicted to have little if any viability. On each day of the experiment a selection of blastocysts was transferred at random from the pool of morphologically similar blastocysts. Their metabolism was not determined.

Blastocyst transfers

Blastocyst viability was determined by uterine transfer to pseudopregnant recipients. Recipient females were obtained by placing 8–10 week old F_1 hybrid females with vasectomized males (Hogan *et al.*, 1986) of the same strain. The day of mating was determined to be day 1 of pseudopregnancy. Immediately following metabolic analysis, single F_2 blastocysts were transferred to the left uterine horn of pseudopregnant recipients (day 4 of pregnancy), along with four in-vivo developed CF1 blastocysts (albino strain). A further five CF1 blastocysts were transferred to the right uterine horn. On day 17 of pregnancy, the F_2 fetuses were identified by eye pigment and their sex determined.

Statistical analysis

Data for glycolytic activity were found not to be normally distributed and were analysed using the Mann–Whitney U-test where there were no assumptions made as to the distribution of the data. Glucose uptake and lactate production were normally distributed and analysed using an analysis of variance. Between-treatment differences were determined using Duncan's multiple range test.

Results

Analysis of glycolytic rates of individual blastocysts

Data (Figure 2) indicated that the use of glycolysis for prospective embryo selection was a successful way of predicting pregnancy outcome (number of fetuses/embryos transferred), with blastocysts classified as 'viable' *in vitro* showing a 4-fold increase in pregnancy rate over a randomly selected population (80 versus 20%; $P < 0.01$). In contrast, those blastocysts classified as 'non-viable' resulted in significantly fewer pregnancies than the control group (6%; $P < 0.01$). The observed 20% fetal development of the randomly selected blastocysts compared well with that reported previously for F_2 blastocysts cultured in the same conditions (26% per blastocyst transferred; Lane and Gardner, 1994). An analysis of glycolytic activity required the measurement of glucose uptake of each blastocyst. A retrospective analysis of the glucose uptake of blastocysts within the population of 'viable' blastocysts (i.e. those with a glycolytic rate in the bottom 15%; <88%) revealed an increase in fetal development with increasing glucose consumption (Table I). There was no significant difference in the sex ratio at birth and no difference between

Table I. Glucose uptake by blastocysts classified as viable using glycolytic activity (i.e the lowest 15% of the population distribution with respect to glycolytic activity)

Range in distribution (%)	Range of glucose uptake (pmol/embryo/h)	Fetal development (%)	
		F_2 blastocysts	CF1 blastocysts
31–50	4.0–5.3	0 (0/2)	83 (15/18)
51–70	5 3–6.5	60 (3/5)	78 (35/45)
71–85	6 5–7 2	100 (8/8)	82 (59/72)
86–100	>7.2	100 (10/10)	82 (74/90)

The glucose uptakes of blastocysts classified as viable were grouped according to ranges in the frequency distribution of this subpopulation Values in parentheses indicate the number of fetuses per blastocyst transferred

the sexes in blastocyst glycolytic rate (77.1 ± 3.1 by females, $n = 9$; 73.1 ± 3.8 by males, $n = 13$; values are mean ± SEM). However, female blastocysts did appear to take up 27% more glucose (6.94 ± 0.58 pmol/embryo/h) than the males (5.47 ± 0 71) (not significant; $P<0.07$).

Discussion

This study has shown that it is possible to increase the pregnancy rates associated with blastocyst transfer procedures by using a metabolic criterion, glycolytic activity, to select viable mouse embryos. Such an ability to identify viable embryos prior to transfer should significantly increase the success of human assisted reproductive technologies, such as clinical in-vitro fertilization.

Embryos with a level of glycolysis corresponding to that observed *in vivo* were shown to be highly viable, confirming the importance of maintaining the appropriate levels of specific energy-generating pathways in the embryo Blastocysts with elevated glycolytic activity, i.e. with a lactate production beyond that which can be accounted for by glucose consumption, were presumably utilizing endogenous energy reserves such as glycogen. It is proposed that such blastocysts were unable to implant because of the premature exhaustion of their glycogen stores, which are required at the time of implantation due to the relatively anoxic lumen of the uterus and the absence of appropriate vasculature in the endometrium (Rogers *et al.*, 1982).

It is possible that blastocyst metabolism is related to genotype and that in a population of F_2 embryos there will be a normal distribution of parental genes. However, in this study we did not observe a normal distribution of glycolytic activity, and found that blastocysts selected as viable gave rise to different phenotypes when allowed to go to term. This is therefore indicative that the selection of blastocysts for transfer by glycolytic activity is independent of genotype.

In contrast to our study, Leppens *et al.* (1996) did not find an association between viability and glucose flux through the Embden–Meyerhof pathway, as determined by radiolabelled glucose. The explanation for this apparent discrepancy is due entirely to the techniques employed to assess glucose metabolism by the embryo. In this study both total glucose uptake and the appearance of lactate (an end product of metabolism) were quantified. In contrast, the technique employed by Leppens *et al.* (1996) measured total glucose flux through the Embden–Meyerhof pathway and did not determine the subsequent fate of glucose, i.e. its conversion to lactate or its oxidation to CO_2. As such, this highlights the importance of quantifying the end-point of metabolism.

The data presented are also consistent with previous retrospective studies which showed that the increased rates of fetal development after transfer were associated with high levels of glucose uptake at the blastocyst stage in culture (Renard *et al.*, 1980; Gardner and Leese, 1987). Conversely, blastocysts with a low glucose uptake resulted in significantly fewer fetuses (Renard *et al.*, 1980; Gardner and Leese, 1987). A more detailed analysis of the viable group of blastocysts revealed that the rate of glucose uptake was also associated with subsequent fetal development, confirming the importance of this carbohydrate in influencing blastocyst viability. Of further interest was the fact that although there was no difference in glycolytic rate between male and female blastocysts (not statistically significant), glucose uptake was apparently elevated by 27% in female blastocysts, a finding reported previously for the mouse (Gardner and Leese, 1987). Such differences may be attributed to the 2-fold excess of the X-linked enzyme glucose 6-phosphate dehydrogenase present in the mouse blastocyst. This enzyme is rate limiting for the pentose phosphate pathway Indeed, the activity of the pentose phosphate pathway of female cattle blastocysts was found to be greater than that of male blastocysts (Tiffin *et al.*, 1991)

In contrast to the findings of this study, Conaghan *et al.* (1993) reported an inverse relationship between pyruvate uptake in culture and human embryo viability. An analysis of day 2 and 3 (2- to 8-cell stage) human embryos prior to transfer revealed that pyruvate uptake was significantly lower by embryos that subsequently implanted after transfer. However, in the human the embryonic genome is activated at around the third cell cycle, meaning that prior to the 8-cell stage the embryo is dependent on enzymes synthesized by the oocyte. Indeed, Martin *et al.* (1993) reported that the activities of key metabolic enzymes did not change until after the 8-cell stage. It is therefore plausible that prior to genome activation the observed differences in pyruvate uptake reported (Conaghan *et al.*, 1993) may simply reflect the differences inherited from the oocyte, and do not truly represent the physiology of the later stage and implanting embryo.

In conclusion, this study has demonstrated that mouse blastocyst viability can be quantified non-invasively and rapidly prior to transfer, leading to significant increases in pregnancy rates. The successful application of this technology will require the ability to culture successfully embryos to a stage that utilizes embryonic genes, thereby reflecting true developmental potential (Gardner, 1994)

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