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Articles

# Glucose metabolism of human morula and blastocyst-stage embryos and its relationship to viability after transfer

Dr Gayle Jones

Gayle M Jones received her PhD in 1984 from the Faculty of Medicine, University of Melbourne. After a short post-doctoral position investigating the ultrastructure of human oocytes and preimplantation embryos she began her career in clinical IVF as an embryologist and later laboratory manager. In 1995 she returned to research when she accepted a position as research fellow with Alan Trounson at the Monash Institute, Australia. Current research interests are aimed toward developing and/or optimising laboratory protocols to improve assisted reproduction outcomes for infertile patients and include: blastocyst culture, preimplantation genetic diagnosis, laser-assisted hatching, immature oocyte maturation and the development of improved culture systems and culture media to optimise the viability of in-vitro produced human embryos. She is co-ordinator of a core unit in the Monash University, Masters of Clinical Embryology degree. She is also an active committee representative to Scientists in Reproductive Technology and is the elected scientific representative to The Fertility Society of Australia.

Gayle M Jones[1,3], Alan O Trounson[1], Pauline J Vella[2], George A Thouas[1], Nick Lolatgis[2], Carl Wood[2]
[1]Centre for Early Human Development, Monash Institute of Reproduction & Development, Monash University, Melbourne, Australia
[2]Monash IVF, Melbourne, Australia
[3]Correspondence: Centre for Early Human Development, Monash Institute of Reproduction & Development, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, Australia 3168. e-mail: gayle.jones@med.monash.edu.au

## Abstract

The pregnancy rate and implantation rate following blastocyst transfer in the human have been reported to be high; however, it has remained necessary to transfer 2–3 blastocysts to achieve these rates. Morphological criteria are currently used to select blastocysts for transfer and have some limited correlation with ongoing viability. Glucose metabolism of 189 human morula to blastocyst stage embryos was analysed using a non-invasive ultramicrofluorescence technique to determine if this could be used to predict viability. There was a linear trend to increased glucose uptake with progression from the morula to the hatching/hatched blastocyst stage of development, whereas glycolytic activity did not vary. There was no consistent difference in glucose uptake or glycolytic activity for embryos at the various morphological stages on day 5 compared to day 6 *in vitro*. Glucose uptake and glycolytic activity of the nine embryos positively identified as having implanted following transfer varied and were apparently not different from the values for embryos that failed to implant. In addition, viability was demonstrated to be compatible with high glycolytic activity, with four of nine implanted embryos having a glycolytic activity in the highest 15% of the population of embryos studied. Glucose uptake and glycolytic activity of male and female embryos did not appear to be different. Glucose metabolism cannot be used prospectively to select viable human morula or blastocyst stage embryos for transfer and it is also unlikely to be a useful tool to predict the sex of the embryo.

**Keywords**: *blastocyst, glucose, glycolysis, metabolism, viability*

## Introduction

Recent improvements in culture media, in particular the development of sequential media, and improvements in culture conditions have lead to a resurgence in interest in extending culture of human embryos *in vitro* beyond the 4- to 8-cell stage on days 2–3 after insemination to the blastocyst stage of development on days 5–7. Approximately half of all human zygotes produced *in vitro* can now be successfully cultured to the blastocyst stage of development in culture medium in the absence of somatic cell co-culture (Jones *et al*., 1998a,b). Furthermore, blastocysts grown in culture medium alone are viable, as demonstrated by the reports of high pregnancy and implantation rates following transfer to the uteri of patients (Gardner *et al*., 1998, 2000; Milki *et al*., 1999, 2000; Schoolcraft *et al*., 1999; Khorram *et al*., 2000). As a consequence of the high implantation rates following blastocyst transfer, multiple pregnancy rates are also high. In order to reduce the multiple pregnancy rate while maintaining high pregnancy rates, suitable predictive indicators of viability need to

be developed in order to be able to select the single, most viable blastocyst from the developing cohort for transfer to patients.

Morphological criteria are currently used for the selection of blastocysts for transfer and this has been demonstrated to have some limited correlation with ongoing viability (Jones *et al*., 1998b; Balaban *et al*., 2000; Gardner *et al*., 2000). Dokras and co-workers (Dokras *et al*., 1991, 1993) further demonstrated a correlation between the morphological grade of a blastocyst and its developmental potential as assessed by the production of human chorionic gonadotrophin (HCG) *in vitro*. However, these two measurements cannot be combined to use as a predictive indicator of viability as HCG production *in vitro* cannot be detected until day 8 and does not peak until day 10 *in vitro*, which is too late to be useful for selecting blastocysts for transfer.

Retrospective studies have suggested that non-invasive measurement of nutrient uptake may be a useful marker of viability. Hardy and co-workers (Hardy *et al*., 1989) demonstrated that human embryos that arrest *in vitro* consume less pyruvate than embryos which progress in culture to the blastocyst stage of development. Gott *et al*. (1990) confirmed this finding and further demonstrated that human embryos that reach the blastocyst stage produce significantly more lactate than those which arrest in development. Glucose uptake by day 10 bovine blastocysts (Renard *et al*., 1980) and day 4 mouse blastocysts (Gardner and Leese, 1987) *in vitro* has also been positively correlated with viability after transfer to recipients. More recently, Lane and Gardner (1996) used both glucose uptake and its conversion to lactate (glycolytic activity) as a prospective indicator of mouse blastocyst viability. These authors demonstrated that selection of mouse blastocysts for transfer to recipients on the basis of the blastocyst's glycolytic activity could increase the pregnancy rate four-fold over random selection of blastocysts for transfer. In addition, glycolytic activity could be used to predict the re-expansion of bovine blastocysts immediately after thawing (Gardner *et al*., 1996).

The present study was undertaken to determine if the non-invasive measurement of glucose uptake and its conversion to lactate (glycolytic activity) in human morula to blastocyst stage embryos could be used as a non-invasive and prospective indicator of further developmental competence after transfer to patients. Furthermore, glucose metabolism was also investigated with respect to the sex of the child that resulted from the blastocyst transfer, as it has been reported that the metabolic activity of male embryos is significantly higher than female embryos (Ray *et al*., 1995). This study was supported as a competitive research grant by Monash IVF Pty Ltd.

# Materials and methods

## Patient recruitment and stimulation

Forty-four patients admitted to our IVF programme were recruited for this study. All patients gave informed consent for the extended culture period and embryo transfer on day 5 or day 6 post-insemination (day 0 = day of insemination). Patients also consented to the various culture media used throughout development and metabolic incubation. Specific informed consent was not obtained for the metabolic analysis as this was deemed a non-invasive test on 'spent' culture medium.

All patients received human menopausal gonadotrophin (HMG) stimulation (Metrodin HP; Serono, Frenchs Forest, N.S.W., Australia) or human recombinant FSH stimulation (Gonal-F; Serono) in doses ranging from 100 to 600 IU daily for up to 12 days. Gonadotrophin-releasing hormone (GnRH) agonists nafarelin acetate (Synarel; Searle, Delpharn, France) or leuprorelin acetate (Lucrin; Abbott, Kurnell, N.S.W., Australia) were administered in either the long (down regulation) or short (flare) protocols. Follicle growth was monitored using ultrasonography and measurement of peripheral plasma oestradiol 17β concentrations. A dose of 10,000 IU of HCG (Profasi; Serono, Frenchs Forest, N.S.W., Australia) was administered when there were more than three follicles measuring 16 mm and the oestradiol concentration was 545 pg/ml (2000 pmol/l). Oocyte retrieval was scheduled 36 h after HCG injection. Oocytes were recovered transvaginally under ultrasound guidance. The luteal phase was supported by one of three regimens: 16 days of progesterone beginning the day of oocyte retrieval, administered vaginally in a dose of 400 mg/day, or vaginal progesterone at the same dose from the day of retrieval to day 4 in combination with 1000 IU HCG on days 4, 7, 10 and 13 after oocyte retrieval, or HCG only at a dose of 2000 IU on days 3, 6 and 9 after oocyte retrieval.

## Embryo culture

Embryo culture was performed according to the protocol described by Jones *et al*. (1998a). Briefly, two pronucleate zygotes were cultured in groups of two to three in 20 µl microdrops of pre-equilibrated IVF-50 culture medium (Scandinavian IVF Science AB, Gothenburg, Sweden) or, G1 medium (Barnes *et al*., 1995) supplemented with 2 mg/ml human serum albumin (HSA) (Scandinavian IVF Science AB) under oil (OVOIL-150; Scandinavian IVF Science AB). Sixty-five to 76 h after insemination, embryos were transferred to 50 µl microdrops of pre-equilibrated G2 medium (Barnes *et al*., 1995) supplemented with 2 mg/ml HSA under oil. Embryos were regrouped according to morphology and culture continued in groups of two to three embryos of similar morphology. Embryos requiring culture beyond day 5 were transferred to fresh pre-equilibrated 50 µl microdrops of G2 medium supplemented with 2 mg/ml HSA under oil. All embryo cultures were performed in individual sealed chambers (modular incubator chamber; Billups-Rothenberg Inc., Del Mar, CA, USA) at 37ºC and an atmosphere of 5% $CO_2$, 5% $O_2$, 90% $N_2$.

## Analysis of the glycolytic activity of individual embryos

The amount of glucose consumed and lactate generated by individual embryos at different stages of development was quantified by ultramicrofluorescence microscopy. Quantitative analysis of these two substrates was based on the generation of NADPH or NADH (Gardner and Leese, 1990, 1993). Metabolic analysis was performed on either day 5 or day 6 of in-vitro culture, the timing usually dictated by the schedule for embryo transfer. Prior to analysis the morphology of embryos was assessed using an Olympus inverted microscope fitted with Nomarski differential interference contrast optics. Individual

embryos were washed several times in a modification of G2 medium (GM) that contained 0.5 mmol glucose as the sole energy source (no pyruvate, lactate or amino acids). Individual embryos were then incubated for 3 h in a coded 1 µl microdrop of GM under oil in sealed chambers at 37ºC and an atmosphere of 5% $CO_2$, 5% $O_2$, 90% $N_2$. A 1 µl microdrop GM medium incubated adjacent to the microdrops that contained an embryo was used as a control. At the completion of the incubation period, embryos were carefully removed from the incubation drop in a minimum of medium using a finely pulled Pasteur pipette then washed briefly in G2 medium before being placed individually in coded 20 µl microdrops of pre-equilibrated G2 medium under oil. Embryos remained in these drops until the time of embryo transfer.

## Ultramicrofluorescence assays

A 1 nl sample was removed from the control and each of the incubation microdroplets and assayed. Assay reactions were housed in 10 nl drops of reagent under heavy mineral oil (Sigma Chemical Co., St Louis, MO, USA) on siliconized microscope slides. Glucose consumption and lactate production was determined for each embryo. The percentage of glucose metabolized by glycolysis was calculated on the basis that 1 mol glucose produces 2 mol lactate. Therefore if only some of the glucose consumed is converted to lactate the glycolytic activity would be <100%, if all of the glucose consumed was converted to lactate the glycolytic activity would be 100%, whereas if the glycolytic activity is >100%, lactate is being produced from endogenous sources.

## Embryo transfer

Embryos were assessed for blastocyst development early on the morning of day 5 post-insemination. If insufficient numbers of blastocysts with an advanced morphology were available for embryo transfer at this time, the embryos were cultured for a further day and embryo transfer was postponed to day 6.

Morula or blastocyst stage embryos were selected for transfer on the basis of morphology rather than on the basis of metabolic activity. The numbers for transfer being determined by the availability of embryos for transfer and the patient's age and previous clinical history. If the patient was >35 years of age or if the patient had failed to achieve a pregnancy after three or more previous IVF cycles, then consideration was given to transferring three blastocysts if available rather than only one or two blastocysts which would be the usual recommendation to patients of a younger age or with a limited IVF history. One to three blastocysts were transferred to each patient. Occasionally morulae or cavitating morulae were transferred together with at least one blastocyst if there were not sufficient numbers of blastocysts available to complete the recommended number of embryos for transfer.

Embryos were transferred on either day 5 or day 6, the day of transfer being determined by the degree of expansion of the blastocysts. Preference was given to the most advanced blastocyst. The priority was: hatched blastocyst > hatching blastocyst > expanded blastocyst > expanding blastocyst > early blastocyst > cavitating morula > morula (Jones *et al*., 1998a). Within any particular morphological classification, preference for transfer was given to the blastocyst showing the most prominent inner cell mass. Embryos were transferred zona free after a brief exposure to 0.2% Pronase (Sigma Chemical Co.) in HEPES buffered G2 medium. Embryos were transferred in pre-equilibrated G2 medium supplemented with 8 mg/ml HSA, using a Cook Pivet Laboratory Embryo Transfer Set (#K-PETS-2031-Monash, Cook Australia, Eight Mile Plains, Qld, Australia).

Blastocysts in excess of those required for transfer were cryopreserved using a slow cooling protocol with glycerol as the cryoprotectant (Jones *et al*., 1998a).

## Statistical analysis

Glucose uptake and glycolytic activity were analysed using a Kruskal–Wallis non-parametric analysis of variance (ANOVA) test. The individual morphological stages of embryo development (morula, cavitating morula, early blastocyst, expanding blastocyst, expanded blastocyst and hatching/hatched blastocyst) were assigned ordinal values of 1–6 to reflect the progression in development from morula to hatching/hatched blastocyst stages. Post-tests for linear trend were performed relative to the ordinal value representing the morphological stage of development only when the Kruskal–Wallis test demonstrated a significant difference at the level of $P < 0.05$. Pairwise comparisons of glucose uptake and glycolytic activity of embryos at each of the morphological stages relative to the day of metabolic assessment or the selection of embryos for transfer were analysed using a Mann–Whitney U-test.

# Results

A total of 44 non-consecutive patients were recruited from our blastocyst transfer programme for this study, with 43 patients having a transfer of at least one blastocyst. The remaining patient had only morulae on day 5 that were assayed for glycolytic activity and the morulae did not develop further with continued culture. A total of 189 embryos were assayed for glycolytic activity: 30 morulae, 14 cavitating morulae, 37 early blastocysts, 29 expanding blastocysts, 49 expanded blastocysts and 30 hatching/hatched blastocysts. Ninety-seven embryos were transferred: nine morulae, six cavitating morulae, nine early blastocysts, 17 expanding blastocysts, 33 expanded blastocysts and 23 hatching/hatched blastocysts. Nine patients became pregnant with at least one fetal heart (clinical pregnancy rate per transfer of 21%) and another four patients had an elevated serum βHCG on day 16, which over subsequent days returned to negative values (biochemical pregnancy). A total of 22 embryos were transferred to the nine pregnant patients. Nine of these embryos could be positively identified as having implanted. That is, the number of embryos transferred was equal to the number of implantations, with an additional five embryos that implanted when the number of embryos transferred was greater than the number of implantations. From the nine embryos that could be positively identified as having implanted, two females and four males were born. From the remaining five implantations, one female and three males were born.

## Analysis of glucose uptake and glycolytic activity of individual embryos

Glucose uptake by individual embryos varied from 4.6

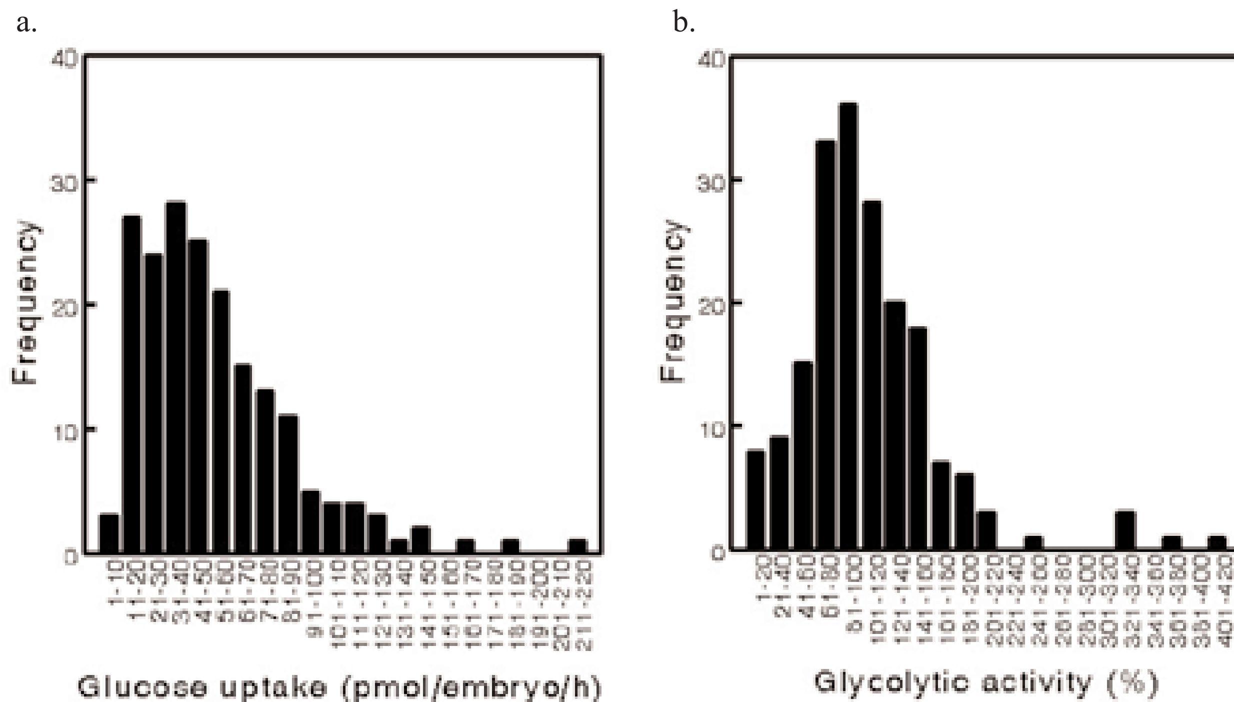


**Figure 1.** (**a**) Distribution of glucose uptake by individual human embryos at the morula to blastocyst stage of development ($n$ = 189). (**b**) Distribution of glycolytic activity by individual human embryos at the morula to blastocyst stage of development ($n$ = 189).

pmol/embryo/h to 216.3 pmol/embryo/h (mean uptake 52.3 pmol/embryo/h, standard deviation (SD) of 34.6 pmol/embryo/h, median uptake 45.7 pmol/embryo/h). The glycolytic activity of individual embryos varied from 7.6 to 416.7% (mean 107.2%, SD 61.6%, median 96.5%). The results for glucose uptake are illustrated in **Figure 1a** and for glycolytic activity in **Figure 1b**.

## Glucose uptake and glycolytic activity of individual embryos with respect to morphological development

On the day of transfer there is often a very heterogeneous population of human embryos displaying morphologies that vary from arrested cleavage stages to morulae through to completely hatched blastocysts. For this reason, glucose uptake and glycolytic activity were analysed with respect to the morphology of the embryo at the time of metabolic incubation to determine if it were possible to compare all developmental stages at the time of transfer. Morphology was classified according to the degree of expansion of the blastocoel cavity with or without evidence of hatching (Jones *et al*., 1998a). Glucose uptake by individual embryos within each of the morphological categories was variable. Furthermore glucose uptake was significantly different for morulae, cavitating morulae, early blastocysts, expanding blastocysts, expanded blastocysts and hatching/hatched blastocysts with a mean and SD of 34.8 ± 30.7, 26.3 ± 14.7, 50.4 ± 37.6, 60.0 ± 43.0, 61.7 ± 29.8 and 61.4 ± 28.0 pmol/embryo/h (median of 21.3, 22.3, 33.7, 48.1, 56.3, 53.6 pmol/embryo/h) respectively (KW = 40.502, $P < 0.0001$). Glucose uptake increased significantly with increasing development from the morula to the hatching/hatched blastocyst stage ($r^2 = 0.1076$, $P < 0.0001$). Glycolytic activity of individual embryos within each of the morphological categories was variable. However, glycolytic activity did not differ significantly for morulae, cavitating morulae, early blastocysts, expanding blastocysts, expanded blastocysts and hatching/hatched blastocysts with a mean of 81.9 ± 46.4%, 133.9 ± 93.2%, 114.9 ± 81.7%, 105.5 ± 41.1%, 107.8 ± 57.9%, 111.2 ± 44.1% (median of 79.4, 94.3, 115.2, 108, 92.8, 107.4%) respectively (KW = 8.5, $P = 0.1308$). The results for glucose uptake are illustrated in **Figure 2a** and for glycolytic activity in **Figure 2b**.

## Glucose uptake and glycolytic activity of individual embryos with respect to morphological development and the day of metabolic assessment

The majority of embryo transfers following blastocyst culture in our programme are performed late morning to early afternoon on day 6 of in-vitro culture. Occasionally embryo transfer is performed late afternoon on day 5 if sufficient numbers of blastocysts have developed to satisfy the numbers required for embryo transfer. Very occasionally the embryo transfer is scheduled for early morning of day 6, which would prohibit metabolic assessment due to the prolonged time required for metabolic incubation and subsequent analysis. For these reasons metabolic assessments were performed on either day 5 or day 6. The results were analysed to determine whether embryos reaching a particular morphological stage on day 5 *in vitro* had a significantly different glucose metabolism profile to embryos at the same morphological stage on day 6 *in vitro*.

The median value for glucose uptake by embryos on day 5 was 14.7, 17.6, 128.1, 199.4 and 75.4 pmol/embryo/h and on day 6 was 36.6, 28.0, 32.5, 46.9 and 56.0 pmol/embryo/h for morulae, cavitating morulae, early blastocysts, expanding blastocysts and expanded blastocysts respectively. The median value for glucose uptake by hatching/hatched blastocysts on day 6 was 53.6 pmol/embryo/h. There were no hatching/hatched blastocysts analysed for glucose uptake on day 5 from this

**Figures 2–4.** Graphical representation of glucose metabolism by individual human embryos at the morula (A), cavitating morula (B), early blastocyst (C), expanding blastocyst (D), expanded blastocyst (E) and hatching/hatched blastocyst (F) stage of development. The box represents the 25th to the 75th percentile of the data and is transected by a line representing the median value. The error bars reflect the 5th and 95th percentile of the data. Glucose uptake is represented in graphs (**a**) and glycolytic activity in graphs (**b**).

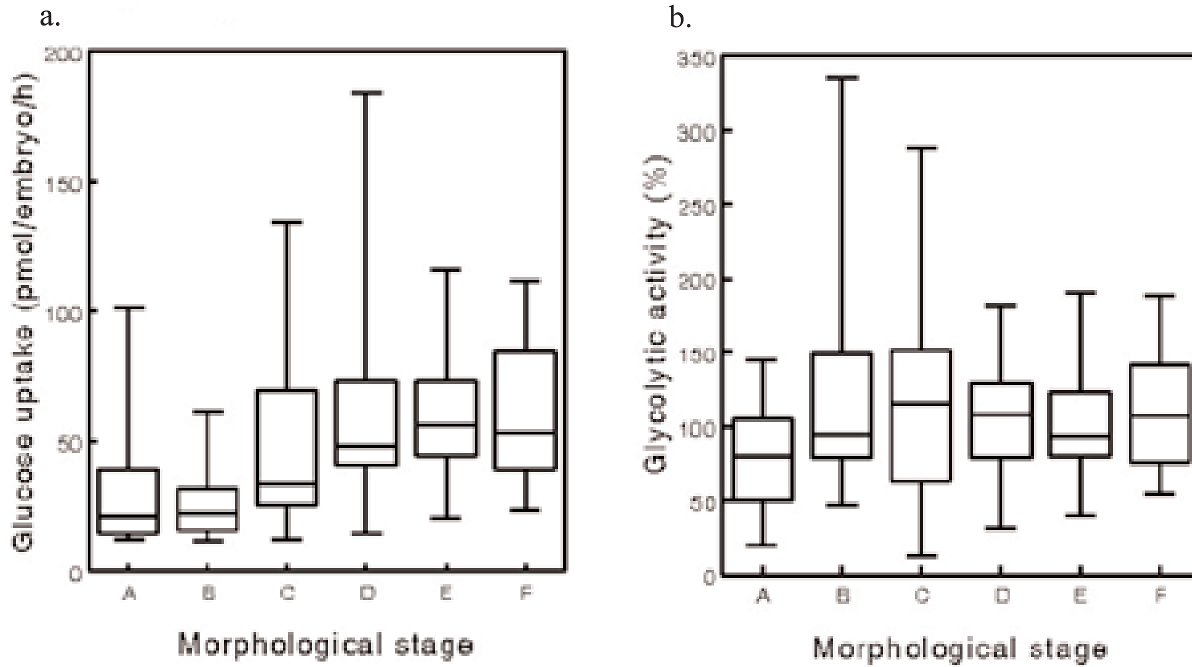


**Figure 2.** Glucose metabolism of human embryos at the morula to blastocyst stage of development.

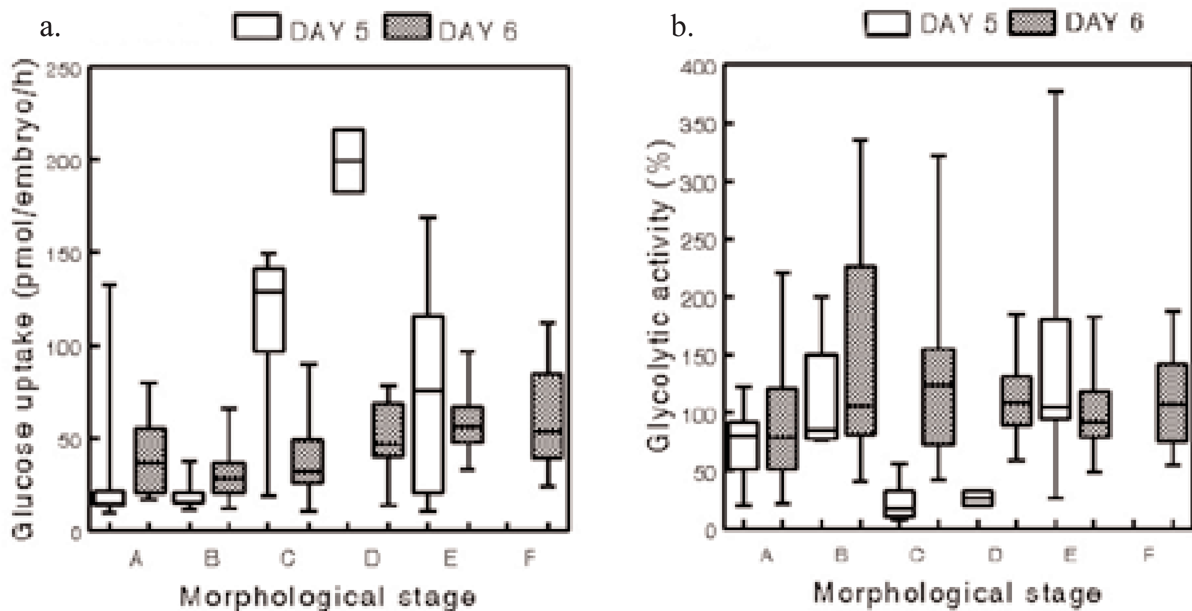


**Figure 3.** Glucose metabolism of human embryos at the morula to blastocyst stage of development on day 5 *in vitro* (open box) or day 6 *in vitro* (shaded box).

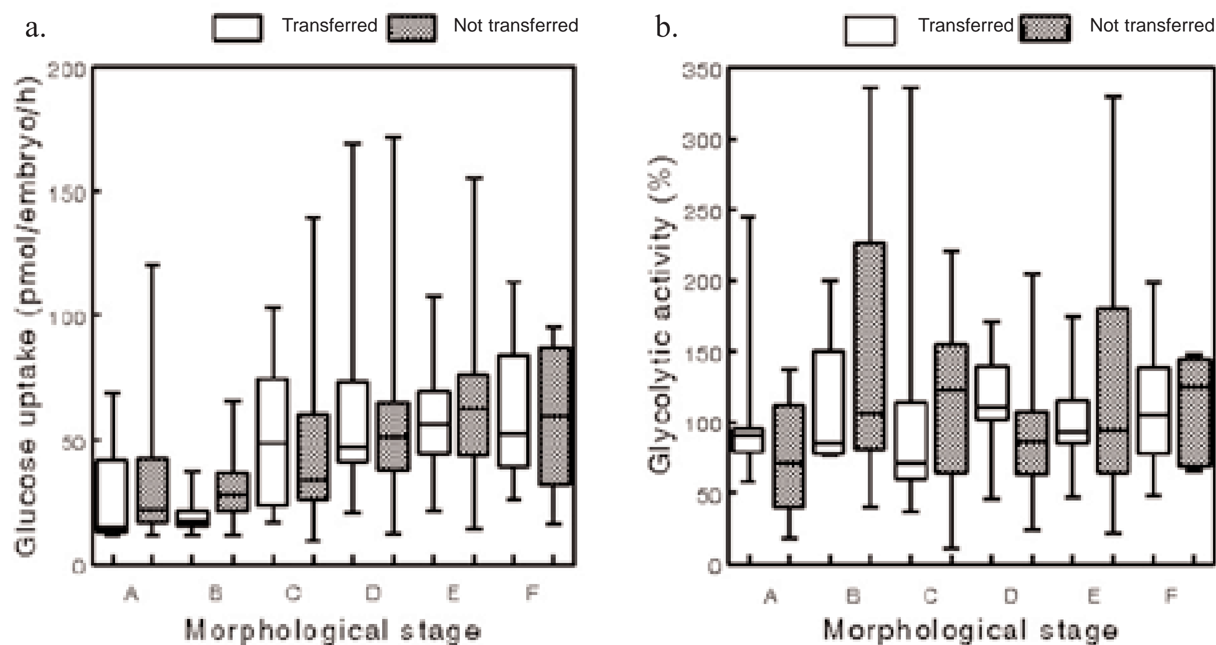


**Figure 4.** Glucose metabolism of human embryos at the morula to blastocyst stage of development that were selected for transfer to the patient's uterus (open box) or not transferred (shaded box).

series of patients. The difference in median values for glucose uptake by the various morphological stages on day 5 compared to day 6 was significant for morulae, early blastocysts and expanding blastocysts only. Glucose uptake was lower for morulae on day 5 ($P = 0.008$), higher for early blastocysts on day 5 ($P = 0.02$) and higher for expanding blastocysts on day 5 ($P = 0.005$). The results for glucose uptake on day 5 compared to day 6 for each of the morphological classifications are illustrated in **Figure 3a**.

The median value for glycolytic activity of embryos on day 5 was 79.6, 85.4, 16.3, 26.1 and 105.4% and on day 6 was 79.1, 106.2, 122.9, 109.1 and 91.8% for morulae, cavitating morulae, early blastocysts, expanding blastocysts and expanded blastocysts respectively and 107.4% for hatching/hatched blastocysts on day 6. There was no difference in glycolytic activity on day 5 compared to day 6 with the exception of early blastocysts and expanding blastocysts on day 5 that showed a significantly lower glycolytic activity ($P < 0.0001$ and $P = 0.0049$ respectively). The results for glycolytic activity on day 5 compared to day 6 for each of the morphological classifications are illustrated in **Figure 3b**.

## Glucose uptake and glycolytic activity of individual embryos with respect to implantation outcome following embryo transfer

Human morula to hatching/hatched blastocyst stage embryos were selected for transfer on the basis of morphological criteria, preference for transfer being given to embryos of a more advanced stage showing prominent inner cell masses. **Figures 4a** and **4b** illustrates the glucose uptake and glycolytic activity of embryos selected for transfer compared to those that were not transferred. Overall, there was no significant difference in glucose uptake or glycolytic activity of embryos that were selected for transfer on the basis of morphology compared to those embryos that were not transferred, indicating that glucose metabolism was not related to the criteria used to select embryos for transfer. Furthermore, embryos that were transferred were representative of the entire population with the exception perhaps of expanding blastocysts whose glycolytic activity was slightly higher than for those that were not transferred ($P = 0.03$).

Only nine of 97 embryos transferred could be positively identified to have implanted. In addition there were five implantations whose origins could not be positively identified resulting in an overall implantation rate of 14%. **Figure 5a** illustrates the glucose uptake and **Figure 5b** illustrates the glycolytic activity of embryos that were transferred and either implanted or failed to implant (additional transferred embryos from which a positive identification of the origin of the implantations are not represented). There was a wide variation in glucose uptake by embryos that implanted (33.5–216.3 pmol/embryo/h) as there was for embryos that failed to implant (11.4–117.7 pmol/embryo/h). Due to the small sample size statistical analysis was not performed. Similarly there was a wide variation in glycolytic activity of embryos that implanted (32.3–187.5%) as there was for embryos that failed to implant (37.2–245%). Indeed, four of the embryos that implanted had a glycolytic activity that fell in the top 15% for the entire population of embryos analysed and four of the embryos that implanted had a glycolytic activity from the bottom 15%.

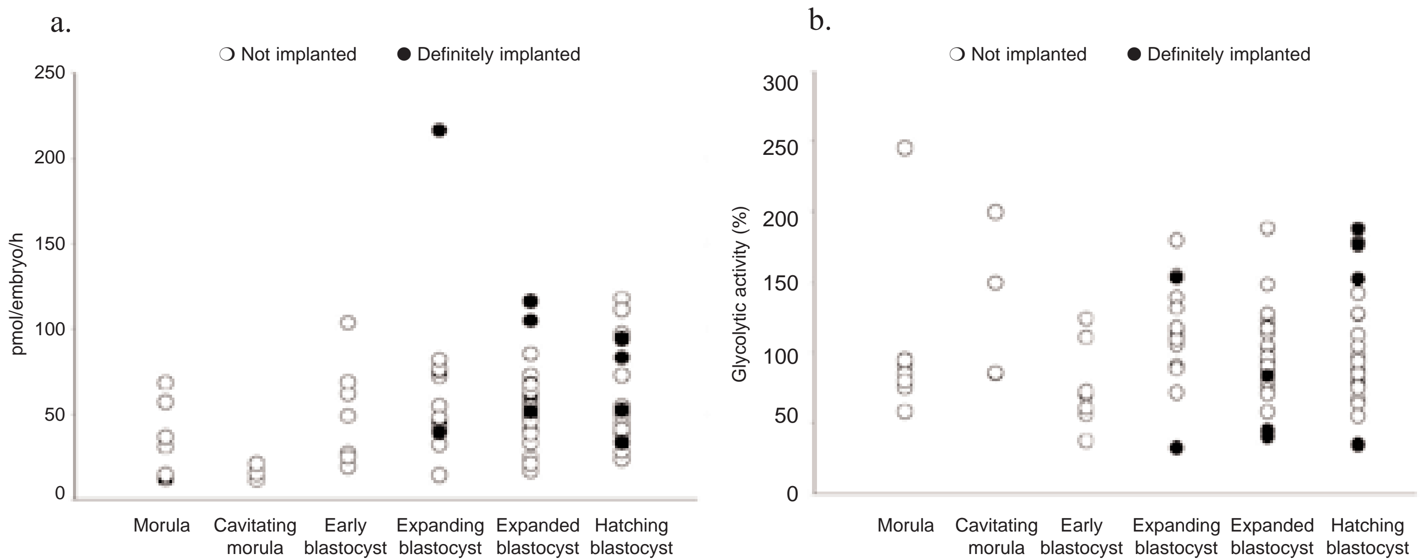


**Figure 5.** Glucose uptake (**a**) and glycolytic activity, (**b**) of individual human embryos transferred to the patient's uterus at the morula, cavitating morula, early blastocyst, expanding blastocyst, expanded blastocyst and hatching/hatched blastocyst stage of development. The open circles represent those embryos known to have failed to implant and the closed circles represent those embryos known to have implanted.

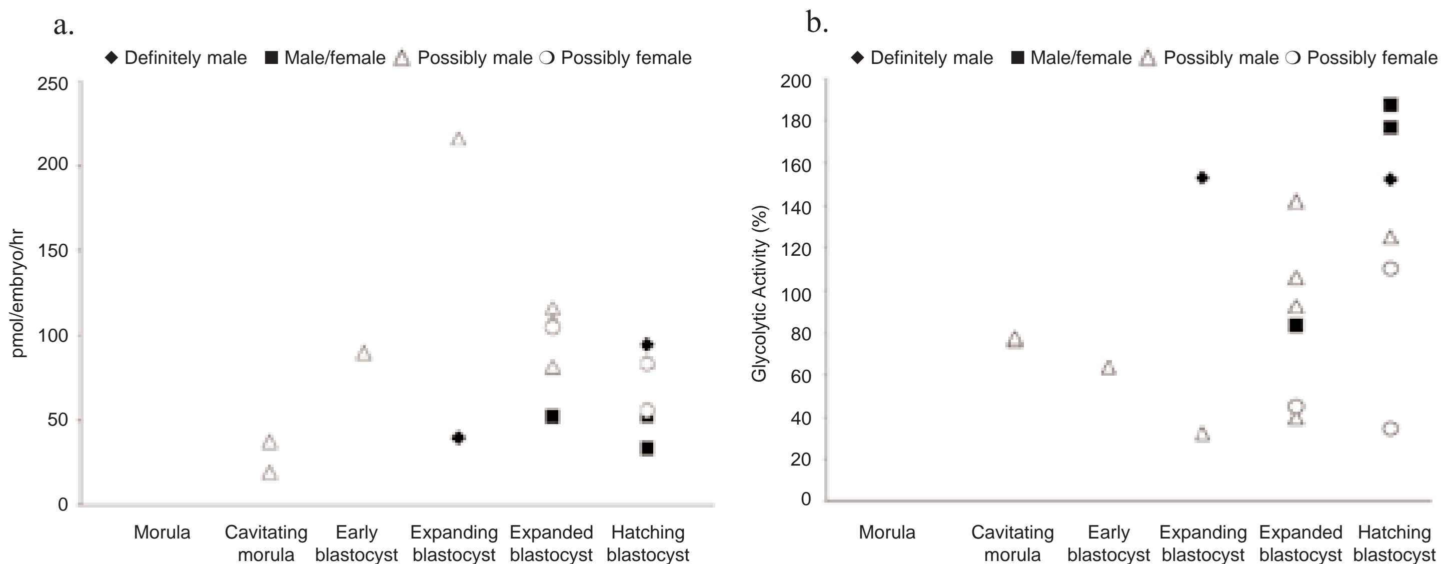


**Figure 6.** Glucose uptake (**a**) and glycolytic activity (**b**) of individual human embryos at the morula, cavitating morula, early blastocyst, expanding blastocyst, expanded blastocyst and hatching/hatched blastocyst stage of development that when transferred to the patient's uterus, either singly or as part of a multiple embryo transfer, resulted in a birth. The closed diamonds represent two embryos that resulted in the birth of two boys, the open triangles represent nine embryos that were transferred as part of multiple embryo transfers that resulted in the birth of four boys, the open circles represent three embryos that were transferred as part of a multiple embryo transfer that resulted in the birth of two girls and the closed boxes represent three embryos transferred as part of a multiple embryo transfer that resulted in the birth of a girl and a boy.

## Glucose uptake and glycolytic activity of individual embryos with respect to sex of resultant birth

Only two of the 10 births could be positively traced back to the originating embryos. Both were males. Of the remaining births a male and a female child were delivered following the transfer of three embryos, four males were delivered from the transfer of nine embryos and two females were delivered from the transfer of three embryos.

The glucose uptake and glycolytic activity of these transferred embryos are depicted in **Figure 6a** and **6b**. Although numbers are extremely small and positive identification of the sex of the transferred embryo could only be confirmed in two instances, it appears unlikely that either glucose uptake or glycolytic activity could be used as a parameter for sex selection.

# Discussion

This study has shown that glucose uptake and glycolytic activity of human morula and blastocyst stage embryos cannot be used as a marker of viability and are also unlikely to be useful parameters for gender selection in the human. Lane and Gardner (1996) reported in the mouse, transfer of blastocysts selected for having a glycolytic activity of <88% (those embryos from the lowest 15% of the glycolytic distribution and with a metabolic profile similar to in-vivo derived blastocysts) resulted in a 4-fold increase in fetal development compared to transfer of blastocysts selected for having a glycolytic activity in the highest 15% of the population (glycolytic activity >160%). The selected blastocysts also had a higher implantation rate than the blastocysts selected at random. In the current study, which used very similar metabolic incubation conditions to those used by Lane and Gardner (1996), human blastocysts having a glycolytic activity in both the lowest 15% of the population (glycolytic activity <58%) and highest 15% of the population (glycolytic activity >152%) were demonstrated to be viable by implanting in the uterus following embryo transfer.

An interesting finding of the present study was that human blastocysts that produce more lactate than can be explained by metabolism of exogenous glucose (glycolytic activity >100%), are developmentally competent. The source of additional lactate is presumably due to oxidation of endogenous reserves of amino acids or from endogenous protein breakdown rather than from glycogen (Leese *et al*., 1993), as the activity of glycogen phosphorylase is undetectable in preimplantation human embryos (Martin *et al*., 1993). In the present study, human embryos were cultured in an optimized system (Jones *et al*., 1998a) in a complex medium containing Eagle's 20 amino acids from days 3–6 *in vitro*. Less than half the morula to blastocyst stage embryos cultured in this system produce lactate in addition to that explained by glucose uptake and metabolism. Additional lactate production is probably due to the oxidation of endogenous reserves of amino acids rather than from breakdown of endogenous proteins, and may in part explain why glycolytic activity cannot be used as a marker of viability using the culture conditions employed in this study.
Lane and Gardner (1996) reported that mouse blastocysts having the highest glucose uptake from within the population of embryos having the lowest 15% of glycolytic activity correlated with a further increase in fetal development. Unlike the mouse, however, human embryos develop asynchronously so that by days 5–6 in vitro, embryos from a heterogeneous population of morphologies are available for selection for transfer. Glucose uptake by human blastocysts in this study was shown to vary with morphology of the blastocyst, increasing in uptake from the morula to the hatching/hatched blastocyst stage of development. Selection of human embryos for transfer on the basis of glucose uptake would therefore also need to account for morphology. Within any particular morphological classification however, glucose uptake of embryos with demonstrated viability was variable and had values both above and below the median values for the particular morphological classification.

An average glucose uptake of 52.3 pmol/embryo/h by blastocysts in the present study was similar to a previous report of 42 pmol/embryo/h (Gott *et al*., 1990) and higher than the 24 pmol/embryo/h reported by Hardy *et al*. (1989) for human blastocysts. Continuous assays for pyruvate and glucose uptake throughout preimplantation development was the objective in both these studies. Blastocyst development in the earlier study was achieved in the metabolic medium, modified T6 medium containing 0.47 mmol/l pyruvate, 1 mmol/l glucose, 5 mmol/l lactate and 4 mg/ml BSA and in the later study in the same medium but in the absence of lactate. The higher glucose uptake reported in the present study may be explained by the absence of exogenous substrates other than glucose in the metabolic medium as concentrations of these substrates may affect uptake (Gardner and Leese, 1990).

Many IVF centres routinely transfer blastocysts on day 5 (Scholtes and Zeilmaker, 1996; Fong *et al*., 1998; Gardner *et al*., 1998). The majority of blastocyst transfers are performed in our IVF programme on day 6 of in-vitro culture but very occasionally blastocyst transfers are performed on day 5 of in-vitro culture (Jones *et al*., 1998a). It was important to determine if metabolic assays could be performed on either day and directly compared and also determine if embryos developing at a faster rate have a different glucose metabolic profile to embryos developing at a slower rate. Although significant differences were detected for glucose uptake by morulae, early blastocysts and expanding blastocysts and for glycolytic activity by early blastocysts and expanding blastocysts on day 5 compared to day 6, the findings were not consistently higher or lower for day 5 embryos. Faster developing embryos therefore do not have a better glucose metabolic profile to embryos developing at a slower rate, at least by day 5 of in-vitro culture.
Gardner and Leese (1987) were the first to report a possible relationship between glucose metabolism at the blastocyst stage and gender of the embryo. These authors demonstrated that in the mouse, male embryos took up slightly less glucose than female embryos although for the small numbers studied this difference was not significant. Ray *et al*. (1995) demonstrated that in the human, glucose uptake and lactate production by male embryos was significantly higher than for female embryos on days 4–5 but that this difference was not significant by days 5–6. Although gender in this study could only be definitively identified to the original transferred embryos for two births, the overlap in glucose metabolic profiles of those embryos that

were potentially male or potentially female indicate that it is unlikely that glucose metabolic profiles can be used to accurately predict the sex of offspring, confirming the findings of Ray *et al*. (1995).

Finally, the pregnancy and implantation rates following metabolic incubation and transfer at the morula to blastocyst stage of development are lower than has been previously reported using identical culture conditions up to the point of metabolic incubation (Jones *et al*., 1998a,b). The metabolic incubation utilised in this study has been reported to have no effect on viability (Gardner and Leese, 1987), although a more recent report by Lane and Gardner (1998) has shown that culture of mouse blastocysts, for periods of 3 h or longer, in media lacking amino acids and vitamins results in significant perturbations in metabolism and a resultant loss in viability following transfer when compared to in-vivo derived controls. Inclusion of amino acids and vitamins to the culture medium prevented any loss in viability for incubation periods up to 6 h. To investigate whether metabolic incubation had a negative impact on blastocyst viability in the present study, 37 cases of blastocyst transfer in our IVF programme that occurred in the absence of metabolic incubation (cases immediately prior to and immediately after a case/s of blastocyst transfer that included metabolic incubation) were retrospectively analysed. The results for the 37 cases revealed a pregnancy rate of 19% and an implantation rate of 11%, which was not different to that reported in the present study. It therefore appears that the low pregnancy and implantation rates reported in the present study were not due to the brief exposure to amino acid deficient metabolic medium but rather due to factors not specific to the present study.

In conclusion, non-invasive measurement of glucose uptake and its conversion to lactate (glycolytic activity) cannot be used as a biomarker of viability to select human blastocysts for transfer. Furthermore, these parameters cannot be used in conjunction with existing selection criteria, such as morphological parameters, to improve further the prediction of viability.

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## References

Balaban B, Urman B, Sertac A *et al*. 2000 Blastocyst quality affects the success of blastocyst-stage embryo transfer. *Fertility and Sterility* **74**, 282–287.

Barnes FL, Crombie A, Gardner DK *et al*. 1995 Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Human Reproduction* **10**, 3243–3247.

Dokras A, Sargent IL, Ross C *et al*. 1991 The human blastocyst: morphology and human chorionic gonadotrophin secretion *in vitro*. *Human Reproduction* **6**, 1143–1151.

Dokras A, Sargent IL, Barlow DH 1993 Human blastocyst grading: an indicator of developmental potential? *Human Reproduction* **8**, 2119–2127.

Fong C-Y, Bongso A, Ng S-C *et al*. 1998 Blastocyst transfer after enzymatic treatment of the zona pellucida: improving in-vitro fertilization and understanding implantation. *Human Reproduction* **13**, 2926–2932.

Gardner DK, Leese HJ 1987 Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *Journal of Experimental Zoology* **242**, 103–105.

Gardner DK, Leese HJ 1990 Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism *in vitro*. *Journal of Reproduction and Fertility* **88**, 361–368.

Gardner DK, Leese HJ 1993 Assessment of embryo metabolism and viability. In: Trounson AO, Gardner DK (eds) *Handbook of In vitro Fertilization*, CRC Press Inc., Boca Raton, Florida, pp. 195–211.

Gardner DK, Pawelczynski M, Trounson AO 1996 Nutrient uptake and utilization can be used to select viable day 7 bovine blastocysts after cryopreservation. *Molecular Reproduction and Development* **44**, 472–475.

Gardner DK, Schoolcraft WB, Wagley L *et al*. 1998 A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Human Reproduction* **13**, 3434–3440.

Gardner DK, Lane M, Stevens J *et al*. 2000 Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertility and Sterility* **73**, 1155–1158.

Gott AL, Hardy K, Winston RML *et al*. 1990 Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Human Reproduction* **5**, 104–108.

Hardy K, Hooper MAK, Handyside AH *et al*. 1989 Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Human Reproduction* **4**, 188–191.

Jones GM, Trounson AO, Gardner DK *et al*. 1998a Evolution of a culture protocol for successful blastocyst development and pregnancy. *Human Reproduction* **13**, 169–177.

Jones GM, Trounson AO, Lolatgis N *et al*. 1998b Factors affecting the success of human blastocyst development and pregnancy following *in vitro* fertilization and embryo transfer. *Fertility and Sterility* **70**, 1022–1029.

Khorram O, Shapiro SS, Jones JM 2000 Transfer of nonassisted hatched and hatching human blastocysts after *in vitro* fertilization. *Fertility and Sterility* **74**, 163–165.

Lane M, Gardner DK 1996 Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Human Reproduction* **11**, 1975–1978.

Lane M, Gardner DK 1998 Amino acids and vitamins prevent culture-induced perturbations and associated loss of viability of mouse blastocysts. *Human Reproduction* **13**, 991–997.

Leese HJ, Conaghan J, Martin KL *et al*. 1993 Early human embryo metabolism. *Bioessays* **15**, 259–264.

Martin KL, Hardy K, Winston RM *et al*. 1993 Activity of enzymes of energy metabolism in single human preimplantation embryos. *Journal of Reproduction and Fertility* **99**, 259–266.

Milki AA, Fisch JD, Behr B 1999 Two-blastocyst transfer has similar pregnancy rates and a decreased multiple gestation rate compared with three-blastocyst transfer. *Fertility and Sterility* **72**, 225–228.

Milki AA, Hinckley MD, Fisch JD *et al*. 2000 Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations. *Fertility and Sterility* **73**, 126–129.

Ray PF, Conaghan J, Winston RML *et al*. 1995 Increased number of cells and metabolic activity in male human preimplantation embryos following *in vitro* fertilization. *Journal of Reproduction and Fertility* **104**, 165–171.

Renard JP, Philipon A, Menezo Y 1980 *In vitro* uptake of glucose by bovine blastocysts. *Journal of Reproduction and Fertility* **58**, 161–164.

Scholtes MCW, Zeilmaker GH 1996 A prospective, randomized study of embryo transfer results after 3 or 5 days of embryo culture in *in vitro* fertilization. *Fertility and Sterility* **65**, 1245–1248.

Schoolcraft WB, Gardner DK, Lane M *et al*. 1999 Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two *in vitro* fertilization programs. *Fertility and Sterility* **72**, 604–609.