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Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos

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The consumption of pyruvate and glucose and the production of lactate by 40 single human preimplantation embryos has been measured using a non-invasive technique. Twelve of the embryos showed abnormal fertilization. Of the 28 normally fertilized embryos, nine (32%) developed to the blastocyst stage in culture while the remainder degenerated or arrested during cleavage. In the normal embryos, pyruvate uptake exceeded that of glucose in the early developmental stages (days 2–5 post-insemination) before glucose became the predominant substrate in the blastocyst (day 6). Considerable quantities of lactate were formed throughout development, rising from a value of 43.6 pmol/embryo/h on day 2.5 to 95.4 pmol/embryo/h on day 5.5. The values of pyruvate and glucose uptake and lactate production of those embryos which arrested were below those which developed normally. On the basis that one mole of glucose can give rise to two moles of lactate, only 50% of the lactate produced could be accounted for in terms of glucose uptake from the medium. This figure rose to 90% in the blastocyst. The remaining lactate must be derived from endogenous sources, most probably glycogen. It is proposed that the high production of lactate by human preimplantation embryos *in vitro* is an adaptation to the conditions of culture.

Key words: glucose/human preimplantation embryo/lactate/metabolism/pyruvate

Introduction

Despite the fact that human IVF and embryo culture have been well established for the past decade, little is known about the biochemistry of the human preimplantation embryo. This is partly due to the paucity of experimental material but also because of objections to such research on ethical grounds and the lack of techniques to study single or small groups of embryos. Apart from contributing to our understanding of the biochemistry of early human development, studies on the metabolism of the early human embryo could lead to the development of diagnostic tests of viability. At present, embryos are selected for transfer on the basis of their morphology and rate of development in culture. The obvious need for more reliable indices of embryo health is highlighted by the low success rates in IVF clinics.

Biggers *et al.* (1967) showed that mouse oocytes and early embryos have an absolute requirement for pyruvate rather than glucose as an energy source. Only at the 4- to 8-cell stage can glucose, as the sole substrate, support embryo development in culture. Leese and Barton (1984) developed a non-invasive ultramicrofluorometric technique to study the uptake of nutrients by single mouse embryos. Using this method, Gardner and Leese (1986), confirmed that pyruvate is consumed preferentially by the early cleavage stages of mouse embryos in culture before glucose becomes the predominant energy source in the blastocyst. Hardy *et al.* (1989) applied the same technique to early human embryos and found that their pyruvate and glucose uptake is qualitatively similar to that found in the mouse.

It was also found that human embryos which later arrested in culture consumed ~20% less pyruvate on day 2.5 than those which progressed to the blastocyst stage (Hardy *et al.*, 1989). In a study on glucose uptake by single mouse blastocysts, Gardner and Leese (1987) showed that those embryos which implanted and developed to term after transfer had a significantly higher glucose uptake *in vitro* than those which failed to develop.

These findings give grounds for optimisim that a test for human embryo viability may be devised in terms of nutrient uptake (Leese, 1987). However, a major feature of glucose metabolism in mouse embryos is the production of lactate. The percentage of glucose converted to lactate rises from ~25% at the 2-cell stage to ~50% by the blastocyst (Wales, 1969, 1986; Gardner and Leese, 1988). With a view to assessing the possibility of using lactate formation as an additional parameter of embryo health, this paper reports the first simultaneous measurements of glucose and pyruvate uptake and lactate production by individual fertilized human embryos from day 2 to day 6.

This work has been approved by the Voluntary Licensing Authority for Human In Vitro Fertilization and Embryology of the Medical Research Council and the Royal College of Obstetricians and Gynaecologists, and the Ethics Committees of the collaborating institutions.

Materials and methods

Surplus human embryos were obtained from patients undergoing IVF at Hammersmith Hospital, London. The superovulation regime involved pituitary-gonadal suppression with a luteinizing hormone releasing hormone (LHRH) agonist (Buserelin, Roche) (Rutherford *et al.*, 1988), prior to superovulation with human menopausal gonadotrophin (HMG, Pergonal; Serono). Ovulation was induced with 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl; Organon).

Oocytes were aspirated, preincubated and inseminated (day 0)

and then checked for pronuclei the following day (day 1) as described by Hillier *et al.* (1984). Embryos were cultured in 1 ml of T6 medium (Quinn *et al.*, 1982) or Earle's balanced salt solution (Gibco) containing 10% heat-inactivated maternal serum under a gas phase of 5% CO_2, 5% O_2 and 90% N_2. The Earle's medium was supplemented with 25 mM sodium bicarbonate (BDH, Analar) and 0.47 mM pyruvate (Sigma). On day 2, the embryos were examined and up to three were selected, on the basis of their morphology, for transfer.

After patients' informed consent, surplus embryos after transfer were incubated singly in microdrops (see below). Sixteen hours post-insemination, the embryos were classified prospectively into the following groups according to the number of pronuclei: (i) fertilized embryos having two pronuclei; (ii) polyspermic embryos with three or more pronuclei; (iii) unfertilized oocytes with no pronuclei.

On day 6, the fertilized embryos were further subdivided into those which had developed to the blastocyst stage and those which had either fragmented or arrested during cleavage.

Incubation of surplus embryos

On day 2, any remaining cumulus cells adhering to the zona pellucida of the embryo were removed using a finely pulled Pasteur pipette. Embryos were then washed several times in modified HLT6 (Hardy *et al.*, 1989) containing 4 mg/ml bovine serum albumin (BSA), 1 mM glucose and 0.47 mM pyruvate but no lactate. The embryos were incubated individually in 4 μl drops of this medium under paraffin oil, with a gas phase of 5% CO_2 in air. Each embryo was removed from the droplet after 24 h with the minimum of medium, using a finely pulled, oil-fired Pasteur pipette. They were then washed individually three times in fresh volumes of the modified medium and placed in a further 4 μl droplet of medium for the next 24-h incubation. This process was repeated for each embryo from day 2 to day 6. Droplets of medium alone incubated adjacent to the droplets containing the embryos were used as controls. Spent and control droplets were taken up individually in 10-μl microcaps (Drummond Scientific) which were sealed and stored at −70°C prior to transport on dry ice to the Department of Biology, University of York, for assay. By analysing the difference between substrate levels in control and incubation droplets, consecutive measurements of pyruvate and glucose uptake and lactate production could be made on individual embryos. In this way, a profile of human embryo metabolism from day 2 to day 6 can be produced.

Ultramicrofluorometric methods of analysis

All samples of media were coded before assay to conceal their identity. Analyses were carried out in nanolitre-sized microdrops of reaction mixture under mineral oil on siliconized microscope slides. The details of the fluorescence measurements were as described by Leese and Barton (1984) and Leese (1987). In each assay, 1 nl of sample was added to 10 nl of assay mixture.

Glucose assay

The assay mixture contained 0.4 mM dithiothreitol, 3.07 mM $MgSO_4$, 0.42 mM ATP, 1.25 mM $NADP^+$, 20 U/ml hexokinase/glucose-6-phosphate dehydrogenase (HK/G6PDH) EC 2.7.1.1/1.1.1.49 in EPPS buffer at pH 8.0. The formation of NADPH was monitored using a Leitz Diavert fluorescence microscope with photomultiplier and digital microphotometer attachments. The readings were calibrated against a series of glucose standards.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

Pyruvate assay

The assay mixture contained 0.1 mM NADH and 40 U/ml lactate dehydrogenase (LDH) EC 1.1.1.27 in EPPS buffer, pH 8.0. Changes in fluorescence due to NADH oxidation were monitored as above. The readings were calibrated against a series of pyruvate standards.

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

Lactate assay

The assay mixture contained 40 U/ml LDH, 0.8 mM NAD^+ in a glycine-hydrazine buffer, pH 9.4. Changes in fluorescence due to the reduction of NAD^+ were monitored as above and the readings calibrated against a series of lactate standards.

$$\text{lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

Assays were run in duplicate and results expressed as means ± SEM.

Results

A total of 17 patients donated the 40 surplus embryos used in this study. Of these embryos, 28 were normally fertilized, nine were polyspermic and three contained no pronuclei. All 12 abnormally fertilized embryos arrested during cleavage. Of the 28 normally fertilized embryos, nine (32%) went on to develop to the blastocyst stage; the remainder degenerated or arrested during cleavage stages.

No significant differences in glucose or pyruvate uptake or in lactate production were observed between the three groups of embryos which arrested during development. These groups of embryos were therefore pooled.

Pyruvate uptake (Figure 1)

In the nine embryos which developed to the blastocyst stage, pyruvate uptake increased from 36 pmol/embryo/h on day 2.5 to a maximum of 52.5 pmol/embryo/h on day 4.5 before falling to ~30 pmol/embryo/h on day 5.5. The pyruvate uptake by those embryos which arrested was significantly lower ($P < 0.01$) and remained at ~30 pmol/embryo/h at each time point studied. These embryos also failed to show a significant fall in pyruvate uptake around day 4.5.

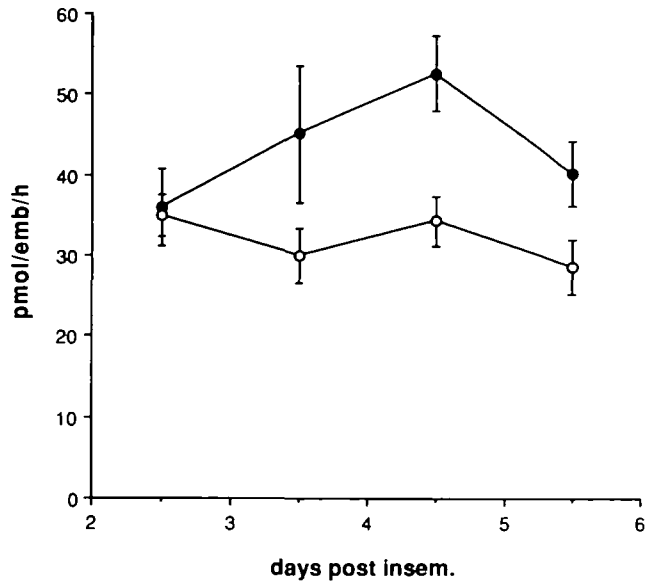

Fig. 1. Pyruvate uptake by fertilized embryos which developed to the blastocyst stage by day 6 (●) and embryos which arrested during cleavage (○).

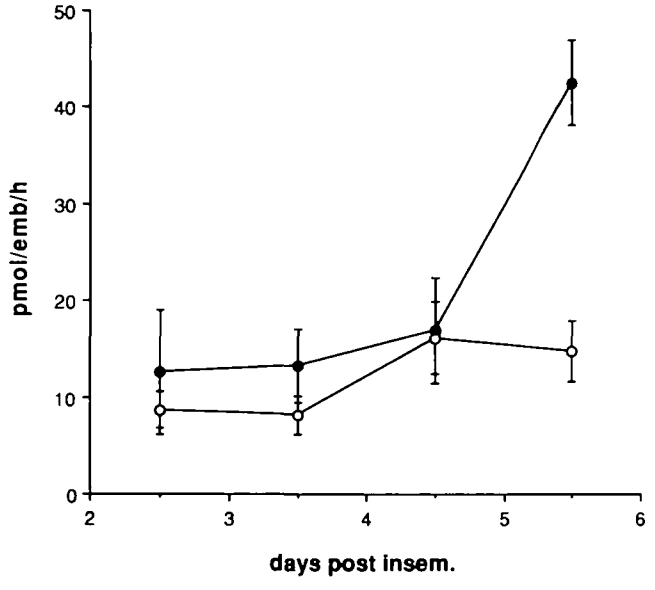

Fig. 2. Glucose uptake by fertilized embryos which developed to the blastocyst stage by day 6 (●) and embryos which arrested during cleavage (○).

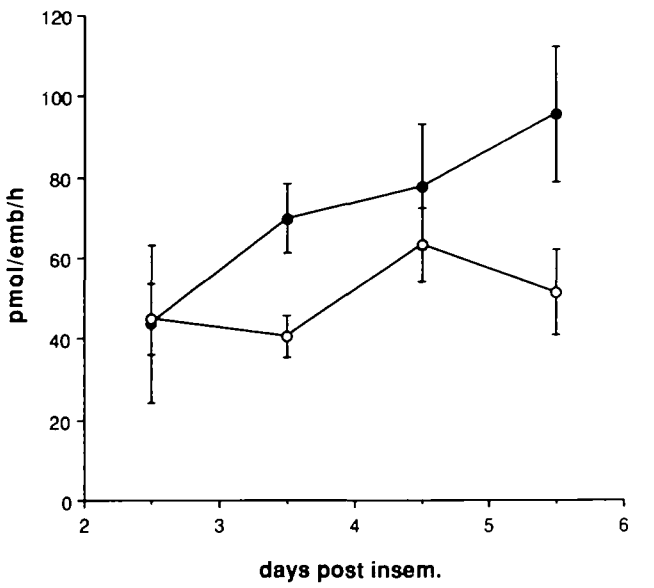

Fig. 3. Lactate production by fertilized embryos which developed to the blastocyst stage by day 6 (●) and embryos which arrested during cleavage (○).

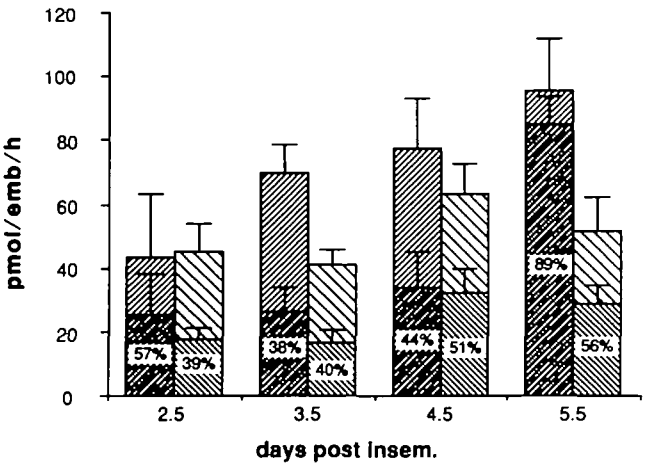

Fig. 4. Lactate production by normal and arrested embryos. The left-hand bar on each day represents normal embryos (▨), the right-hand bar, those which arrested (▧). The proportion of lactate which may be accounted for by glucose taken up from the medium is shown in each case (▨,▧). These figures are expressed as percentages.

Glucose uptake (Figure 2)

Glucose uptake by the embryos which developed to the blastocyst stage, increased from 12.5 pmol/embryo/h on day 2.5 to 16.9 pmol/embryo/h on day 4.5. By day 5.5, their uptake had increased dramatically to 42 pmol/embryo/h. The glucose uptake by the degenerate or arrested embryos was < 15 pmol/embryo/h at each time point. Significantly, these embryos failed to show the dramatic increase in glucose uptake on day 4.5.

Lactate production (Figure 3)

The rate of lactate production in those embryos which developed to the blastocyst stage increased steadily during development, from an initial value of 43.6 pmol/embry/h on day 2.5 to a value of 95.4 pmol/embryo/h on day 5.5. In the arrested embryos, lactate production was lower at each time point.

The proportion of lactate production which may be accounted for by glucose uptake has been calculated assuming that one mole of glucose can be converted into two moles of lactate (Figure 4). This shows that prior to the blastocyst stage, only 50% of the lactate formed can be accounted for by the aerobic glycolysis of the glucose consumed. Upon blastocoel formation, there is a significant increase in lactate formation ($P < 0.01$), 90% of which could be accounted for by the dramatic increase in glucose uptake at this time (Figure 4).

Discussion

The overall pattern of glucose and pyruvate uptake by individual human embryos *in vitro* is similar to that obtained by Hardy *et al.* (1989). The results confirm that pyruvate is consumed in preference to glucose in the early stages of development, before glucose becomes the predominant substrate at the blastocyst stage. It was also notable that the peak value of glucose uptake was 42 pmol/embryo/h, whereas in the earlier study of Hardy *et al.* (1989) it was 28 pmol/embryo/h. The present results were obtained in a lactate-free environment, whereas in the study of Hardy *et al.* (1989) lactate was present at a concentration of 5 mM. It is possible that the presence of lactate inhibits the conversion of glucose to lactate by glycolysis, as has previously been suggested by Leese (1989a). Although the patterns of glucose and pyruvate uptake by human and mouse preimplantation embryos are qualitatively similar, the values for the human are about 15–20 and 10 times greater respectively than the mouse (Gardner and Leese, 1986; Hardy *et al.*, 1989). This difference in uptake cannot be accounted for by the respective volumes of the mouse and human embryo which differ by a factor of ~3.

Pyruvate uptake by the healthy embryos was greater than that of the arrested or degenerated embryos at each stage of development. It was also notable that the arrested embryos were unable to switch to a glucose-based metabolism, as illustrated by their failure to exhibit a surge in glucose uptake on day 4.5. At this stage, glucose will be required in increasing amounts as an energy source for blastocoele formation, as well as a precursor for complex sugars (Pike and Wales, 1982), non-essential amino acids (Schneider *et al.*, 1976), glycerophosphate for lipid synthesis (Flynn and Hillman, 1978) and ribose phosphates for RNA synthesis (Pike *et al.*, 1977), all of which assume greater quantitative significance around this time (Flach *et al.*, 1982).

Lactate production by single human embryos increased throughout development. The absolute amounts formed were up to 80 times greater than those in the mouse embryo (Gardner and Leese, 1988). The large amounts of lactate produced are surprising, since aerobic glycolysis provides 18 times less energy per mole than the complete oxidation of glucose. However, our finding is supported by the work of Wales *et al.* (1987), who measured the metabolism of radiolabelled glucose by single surplus human embryos, and found that 90% of the glucose consumed at the blastocyst stage was converted into lactate, though amounts of lactate produced were significantly lower than those obtained in the present study (Figure 3). Furthermore, Wales *et al.* (1987) failed to observe a significant increase in lactate formation by blastocysts on day 5.5, but obtained values close to those for the degenerate embryos reported in Figure 3. It is possible that this difference could be attributed to differing culture conditions. However, it should be pointed out that the method used by Wales *et al.* (1987) does not measure the total lactate production but only that component derived from glucose taken up from the medium.

Explanations for the high rates of lactate production

Morgan and Faik (1981) confirmed earlier findings by Warburg (1926) and Aisenberg (1961) that when animal cells are placed in culture their oxidative metabolism declines over time and aerobic glycolysis is enhanced. This is shown clearly by cultured liver cells which are initially gluconeogenic, consume lactate (Krebs *et al.*, 1974) and rely solely on oxidative metabolism for their energy requirements (Berry, 1974). After 4 h in culture, lactate uptake by the hepatocytes ceases and aerobic production of lactate begins. A similar process of adaptation to an increasing dependence on aerobic glycolysis occurs in cultured heart cells (Stanisz *et al.*, 1983). The biochemical mechanism by which aerobic glycolysis is enhanced following culture is unclear but has been attributed to a shift in the distribution of hexokinase between the mitochondrial and cytoplasmic compartments (Bustamante and Pederson, 1977).

Gardner and Leese (1990) have recently shown that single mouse blastocysts grown *in vitro* produce almost twice as much lactate as those blastocysts which have been freshly collected. Menke and McLaren (1970) showed that the metabolism of mouse blastocysts cultured from the 8-cell stage also differed from blastocysts flushed directly from the uterus.

It is therefore possible that the preimplantation embryo responds to culture in the same way as many other cell types by increasing lactate production and decreasing oxidative metabolism. It may be notable that certain tumours, namely teratocarcinomas which, like early embryos, are rapidly growing, totipotent cells, exhibit a high rate of aerobic glycolysis (Bustamante and Pederson, 1977; Pederson, 1978).

The high levels of lactate formation in the later stages of development could also be in preparation for the anoxic environment that the embryo may encounter at implantation (Rogers *et al.*, 1983; Gardner, 1987; Leese, 1989b).

Origin of lactate in early human embryos

Studies using radiolabelled glucose showed that 44% of the glucose consumed by mouse blastocysts is converted to lactate (Wales, 1969; Edirisinge *et al.*, 1984). Gardner and Leese (1988) obtained a similar figure by measuring the total lactate output from single mouse blastocysts. The agreement between these studies strongly suggests that all the lactate formed by mouse blastocysts is derived from exogenous glucose.

However, the situation in the early human embryo appears to be different. More lactate is produced than can be accounted for solely by glucose uptake.

One possibility is that a proportion of the lactate produced is derived from the breakdown of endogenous glycogen. Evidence in favour of this possibility is provided by the work of Stern and Biffers (1968) and Ozias and Stern (1973) who showed that glycogen is broken down by mouse embryos *in vivo* from the 2-cell stage onwards. Contrary evidence from Pike and Wales (1982) showed that glycogen stores in cultured mouse embryos were only degraded in the absence of substrates in the culture medium. Further studies by Stern and Biggers (1968) and later by Edirisinghe *et al.* (1984) showed that glycogen is synthesized, rather than degraded, by mouse embryos from the 2-cell stage to the blastocyst. The relative contribution of endogenous and exogenous substrates to the high levels of lactate formed by early human embryos obviously needs further clarification.

Irrespective of the origin of the lactate formed by early human embryos, it was notable that embryos which reached the

blastocyst stage produced significantly more lactate than those which arrested during development (Figure 3). Pyruvate uptake by those embryos which developed to blastocysts was also higher than those which failed to develop. The same was true of glucose uptake at the blastocyst stage.

The potential of these non-invasive studies to provide biochemical criteria of embryo viability is therefore encouraging and is currently under investigation.

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