Energy metabolism in late preimplantation rat embryos

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Summary. The consumption of pyruvate and glucose, and the production of lactate, by single preimplantation embryos, was measured using a noninvasive technique. Embryos were cultured in 300–500-nl microdrops, for 8–12 h at a time, from Day 4 to Day 6 after mating, when they developed from the 8-cell stage to expanded blastocyst. Pyruvate was the predominant substrate at the 8-cell/morula stage; glucose uptake exceeded that of pyruvate after the onset of blastocoe1 formation. Lactate production increased in parallel with glucose consumption. For most stages, ~100% of the glucose uptake was accountable for by lactate production and in some cases an additional source of lactate must be postulated. Culture in vitro had little effect on lactate production, although a lower level of metabolism was observed compared with fresh blastocysts. Rat embryos were capable of developing to blastocysts in the absence of glucose, when lactate production was greatly reduced.

Keywords: rat; preimplantation; embryo; metabolism; nutrition

Introduction

The mammalian embryo undergoes qualitative and quantitative changes in energy substrate utilization during the preimplantation period. The mouse embryo, the most widely used model, exhibits a characteristic switch in substrate preference; from pyruvate during the early cleavage stages, to glucose after compaction (Brinster, 1973; Leese & Barton, 1984). The same pattern is exhibited, though less markedly, by the human preimplantation embryo (Hardy et al., 1989; Gott et al., 1990). Absolute rates of substrate uptake are about 7–8 times and 2–3 times higher for glucose and pyruvate, respectively, in the human embryo than in mice. Limited information on substrate utilization is available for rabbits (Brinster, 1973) and hamsters (Seshagiri, 1990; and personal communication).

While the rat is a commonly used model in many areas of reproductive biology, studies on the developmental biology of the preimplantation stages are scarce and information on metabolism virtually nonexistent. This is partly due to a 4-cell block to in-vitro development (Mayer & Fritz, 1974; Whittingham, 1975) and to the difficulty in obtaining large numbers of developmentally competent embryos by superovulation (Miller & Armstrong, 1981).

Some success has been reported in culturing rat embryos from the 8-cell to the blastocyst stage (Folstadt et al., 1969; Mayer & Fritz, 1974; Wood & Whittingham, 1981). More recently, Armstrong has developed a successful superovulatory regimen which involves the continuous infusion of follicle-stimulating hormone (FSH; Armstrong & Opavsky, 1988) and has reported success in culturing rat embryos from the 8-cell stage to blastocyst in a chemically defined medium (Zhang & Armstrong, 1990).

There is clearly a need to define the nutritional requirements of the preimplantation rat embryo as a contribution to our understanding of early development in this species and as a prerequisite to the formulation of more physiological culture media. There is also the need to generate comparative data on preimplantation development in a range of species. We therefore studied rat embryo
metabolism using a modification of a method for the noninvasive assessment of single embryos previously applied to mice and humans (Gardner & Leese, 1986; Hardy et al., 1989).

**Materials and Methods**

*Animals and treatment.* Immature (28–30 days old) randomly bred female rats of the Wistar strain were given a single injection of 5 i.u. of pregnant mares' serum gonadotrophin (Folligon: Intervet, Cambridge, UK) to synchronize the timing of ovulation. After 48 h they were placed singly with males and mating was confirmed by the presence of a vaginal plug the following morning (Day 1). The animals were not superovulated, as this proved to be unreliable and to cause degeneration of embryos in the reproductive tract (Miller & Armstrong, 1981). Embryos were recovered by flushing of the oviducts or uteri with a modified T6-Hepes medium (Wood & Whittingham, 1981); 8-cell embryos were recovered early on Day 4 following mating, and expanded blastocysts on Day 5. Stages prior to the 4-cell block were not studied.

*Culture of embryos.* Embryo culture from the 8-cell stage to blastocyst was carried out in a modified T6 medium (Wood & Whittingham, 1981). Cultures were maintained under light paraffin oil (BDH, Poole, Dorset, UK) at 37°C in an atmosphere of 5% CO₂ in air. Preliminary experiments indicated that T6 was superior to other conventional mouse embry or culture media, such as Whittingham's M16 (Whittingham, 1971), Ham's F10 (Ham, 1963), and Brinster's medium (Brinster, 1965), in supporting development. Development was improved at 260 mosmol/kg, rather than the more usual 280–290 mosmol/kg. For measurement of embryo metabolism, concentrations of glucose, pyruvate and lactate in the medium were adjusted to 1.0, 0.25, and 2.0 mmol/l, respectively. These concentrations are close to those found in the lumen of mouse and rabbit oviducts (Gardner & Leese, 1990; Leese & Barton, 1985). In addition, the medium contained 4 mg bovine serum albumin/ml (ICN Immunobiologicals, High Wycombe, Bucks., UK).

*Substrate utilization or production.* Measurements were taken by a modification of the methods of Leese & Barton (1984), Gardner & Leese (1986) and Hardy et al. (1989). The embryos were incubated singly for 8–12 h, in microdrops of 300–500 nl of T6 under oil and then transferred to a fresh microdrop of medium. The use of microdrops in the nanolitre range allows substrate utilization or product formation to be measured over a defined period within a given developmental stage. The spent microdrop was assayed either immediately or after freezing at −20°C. Manipulated control drops were included in each dish to correct for possible loss of substrates to the oil, or the effects of bacterial contamination or freezing. In this way, the consumption of glucose and pyruvate and the production of lactate was measured for the same embryo and a profile was built up over Days 4 and 5 of development. Embryos were scored for the following developmental stages: 8-cell, compacted morula, early blastocyst (blastocoel up to half the volume of the embryo), blastocyst (blastocoel at least half the volume of the embryo) and late blastocyst (blastocoel occupied virtually all of the space inside the zona pellucida, which was expanded to up to 150% of its original diameter).

Embryo-containing microdrops were assayed either by removing 100-nl samples and adding them to 1-μl drops of reagent mixture using a conventional microsyringe, or by taking nanolitre samples with specially constructed and calibrated glass constriction micropipettes (Mroz & Lechene, 1980). Substrates were measured essentially by the method of Gardner & Leese (1990), using an ultramicrofluorometric technique. This is based on conventional methods of enzymatic analysis in which the pyridine nucleotides NADH and NADPH, generated or consumed in coupled reactions, are measured fluorometrically (Lowry & Passonneau, 1972; Bergmeyer & Gawehn, 1974). Changes in fluorescence were quantified using a Leitz Diavert or Fluovert microscope, with photometer and photomultiplier attachments, and, in the case of the Fluovert, interfaced to an IBM-compatible microcomputer.

Values for substrate uptake or product formation are expressed as pmol/embryo/h ± s.e.m. (no. of embryos).

*Statistical analysis.* Results were analysed and tested for significance by one-way analysis of variance, Student's *t* test, and linear regression, as appropriate.

**Results**

Over 80% of 8-cell embryos developed to blastocysts. Pyruvate uptake declined from 5.29 ± 0.59 (n = 13) pmol/embryo/h at the 8-cell/morula stage to barely detectable rates by the blastocyst stage (Fig. 1). In contrast, glucose uptake increased from 1.27 ± 0.44 (n = 21) at the 8-cell/morula stage to 8.18 ± 0.58 (n = 12) pmol/embryo/h at the late blastocyst stage. There was a highly significant negative correlation between glucose and pyruvate uptakes (R² = 0.56, P < 0.001; data not shown), i.e. when glucose uptake was high, that of pyruvate was low, and vice versa.

Lactate production (Fig. 2) rose in parallel with glucose uptake, from 5.21 ± 0.64 (n = 21) at the 8-cell/morula stage to 16.58 ± 1.61 (n = 12) pmol/embryo/h at the late blastocyst stage. The percentage of glucose metabolized by glycolysis was calculated on the basis that 1 mol of glucose formed 2 mol of lactate. The values (Fig. 2) were close to 100%, suggesting that all of the glucose...
taken up was being converted to lactate by glycolysis; the exception being morula-stage embryos, which appeared to produce 44% more lactate than could be accounted for by glucose uptake. There was a highly significant linear relationship between glucose uptake and lactate production for all stages ($R^2 = 0.75, P < 0.001$; data not shown), with a ratio of $\sim 2:1$ between lactate production and glucose uptake.

To examine in more detail the pattern of energy metabolism around the time of hatching from the zona pellucida in vivo, blastocysts were freshly flushed from the uterus on Days 5 and 6 (Fig. 3, groups 2, 3, 4). Three stages were identified: Day 5 expanded blastocyst (immediately prior to hatching), Day 5 hatched blastocyst and Day 6 hatched blastocyst. These were recovered in the late afternoon and early evening on Day 5 and on the morning of Day 6 (i.e. after implantation should have occurred). In agreement with previous observations (Whittingham, 1975), hatching was not observed in vitro in the absence of fetal calf serum. Pyruvate uptake was unchanged over this
period, at $\sim 2-3$ pmol/embryo/h, while glucose uptake increased from $11.06 \pm 0.72$ (n = 18) to $14.92 \pm 0.42$ (n = 6) pmol/embryo/h, and lactate production from $21.28 \pm 1.37$ (n = 19) to $31.01 \pm 1.77$ (n = 6) pmol/embryo/h. For all three stages, close to 100% of glucose was converted to lactate.

![Figure 3](image)

**Fig. 3.** Uptake of pyruvate (■) and glucose (□) and the percentage of glucose uptake accountable for by lactate production (numbers above bars) for the following groups of rat embryos: B/LB = cultured blastocysts/late blastocysts; D5.B/LB = Day 5 freshly flushed blastocysts/late blastocysts; D5.HB = Day 5 freshly flushed hatched blastocysts; and D6.HB = Day 6 freshly flushed hatched blastocysts. Values are means ± s.e.m. of at least 17 determinations, except for the hatched blastocyst values, which are the means of 3 (Day 5), and 6 (Day 6) determinations.

Figure 3 compares the metabolism of blastocysts which were freshly flushed (group 2) with those cultured in vitro from the 8-cell stage (group 1); pyruvate uptake, glucose uptake and lactate production were significantly higher ($P < 0.01$) in the freshly flushed embryos, while glucose to lactate figures were similar in both groups, at $\sim 100\%$.

In one experiment (Fig. 4), glucose was omitted from the culture medium, to examine the effect of the absence of the embryo's prime energy source on the uptake of pyruvate, the production of lactate, and the ability to form a blastocoel. In the absence of glucose, 76% of 8-cell embryos developed normally to blastocysts, i.e. the ability to form a blastocoel was not significantly impaired. Pyruvate uptake was not significantly different from controls incubated with glucose, while lactate production, unsurprisingly was reduced to 1–2 pmol/embryo/h.

**Discussion**

This is the first report of the noninvasive assessment of energy metabolism in preimplantation rat embryos. The system described permits the sensitive, reproducible measurement of up to 3 parameters (pyruvate and glucose uptake and lactate production) on a single embryo. This is of particular value in rats, from which it is difficult to obtain large numbers of embryos.

The pattern of glucose and pyruvate uptake seen in rat embryos is qualitatively similar to that observed in mice (Gardner & Leese, 1986) and, to a lesser extent, in human embryos (Hardy et al., 1989; Gott et al., 1990) and suggests that, at this stage of development, the embryos of all these species have similar physiological needs. In spite of their similarity in size to mouse embryos, rat embryos take up about double the amount of glucose and pyruvate, and produce far more lactate. This may be a species-specific phenomenon, or the result of differences in culture technique. The present measurements on rat embryos were carried out in conventional bicarbonate-buffered
media; in contrast, Gardner & Leese (1986) carried out their measurements on mouse embryos in Hepes-buffered media, which they and others showed was detrimental to embryo development (Farrell & Bavister, 1984) and glucose uptake (Butler et al., 1988).

Sugawara & Takeuchi (1973) showed that rat embryos oxidized pyruvate to CO₂ via the tricarboxylic acid (TCA) cycle at all stages of preimplantation development and that the yield of CO₂ is greater from pyruvate than from glucose. However, their work also showed that the oxidation of glucose to CO₂ increases greatly throughout the preimplantation period. In contrast, the present study shows that rat embryos produce large amounts of lactate, increasing with development. At each stage at least 100% of the glucose consumed is accountable for by lactate formation, which correlates with glucose consumption in a 2:1 ratio. In the absence of an exogenous glucose source, lactate production is reduced by 90–95% at the blastocyst stage (Fig. 4). Taken with the consistency of the ~100% glucose conversion to lactate, this suggests that the major (if not exclusive) pathway of glucose metabolism during the late preimplantation stages is the glycolytic pathway. This pattern differs somewhat from that seen in mice, in which ~40% of glucose is converted to lactate at the blastocyst stage (Gardner & Leese, 1990), but is similar to that seen in man (Gott et al., 1990). Ellington (1987), using Day 9–10 rat embryos showed that 100% of glucose uptake is accountable for by lactate production. Clough & Whittingham (1983), using Day 6–9 postimplantation mouse embryos, have reported similar results. While glycolysis is far less efficient than the TCA cycle in generating adenosine triphosphate (ATP), it has the capacity to yield ATP at a higher rate and is characteristic of rapidly dividing cells in culture, including certain tumour cells (Mandel, 1986). Lactate may also be formed in anticipation of the anoxic conditions encountered in the uterus at implantation (Leese, 1989).

Gardner & Leese (1990) found that the production of lactate in mouse embryos was increased by overnight culture. In the present study conversion of glucose to lactate was ~100% for both cultured and freshly flushed rat blastocysts (Fig. 3), suggesting that this is not an artefact of culture. The overall level of metabolism is, however, significantly reduced by culture (Fig. 3), consistent with the widespread observation of developmental retardation of embryos in vitro as compared with in vivo (Bowman & McLaren, 1970).

Embryos cultured in the absence of glucose (Fig. 4) appeared able to cleave normally and to develop a blastocoel, in spite of the absence of the preferred energy substrate at this stage. This confirms the observations of Whittingham (1975), who also found that rat blastocysts developed in
the complete absence of energy sources and bovine serum albumin (BSA). In mice, embryos can also be cultured from the 8-cell stage to blastocyst in the absence of glucose (Brinster, 1965; Manejwela et al., 1989); however, other workers have found that glucose is essential to blastocoeel development (Chatot et al., 1989). The question arises as to how the embryo satisfies its, presumably high, ATP requirement at this time, even assuming the complete oxidation of the low amounts of pyruvate taken up in the present study.

The work of Sugawara & Umezu (1961) and Boell & Nicholas (1948) suggests that oxygen uptake by rat embryos is considerable during the late preimplantation stages, increasing from 1-06–1-40 nl/embryo/h at the 8-cell/morula stage to 2-0 nl/embryo/h at the blastocyst stage. This is much higher than that of mouse embryos, which rises from 0-191 to 0-461 nl/embryo/h during this period (Mills & Brinster, 1967). However, direct comparisons are of questionable validity, because of variations in measurement technique and culture conditions used (see Biggers & Stern, 1973). Byatt-Smith et al. (1991) have calculated that a mouse embryo cultured under the conventional, static conditions described in this study should be able to satisfy its maximum oxygen requirement of 0-5 nl/h. Application of this model to the rat embryo suggests that the blastocyst might experience some difficulty in satisfying the reported oxygen requirement of 2-0 nl/h (Boell & Nicholas, 1948), but that the much smaller oxygen requirement to oxidize glucose completely at this stage should be easily satisfied. Thus, if oxygen supply is not limiting, it would appear that the conversion of glucose to lactate observed in this study represents genuine aerobic glycolysis.

Nevertheless, it is likely that the rat embryo carries out considerable oxidative metabolism during the late preimplantation stages. Using data from the present study and assuming 100% nonoxidative conversion of glucose to lactate and the complete oxidation of pyruvate, calculation shows that up to 30% only of the oxygen uptake reported by others can be accounted for by pyruvate oxidation. This figure agrees with that of Sugawara & Takeuchi (1973), who found for rat embryos that ~30% of oxygen uptake could be accounted for by oxidation of glucose and pyruvate.

Since it is unlikely that electron transport is uncoupled from ATP generation, it is necessary to postulate the oxidation of further substrates, which would provide the ATP required in large amounts at the blastocyst stage. One possibility might be fatty acids bound to BSA (Kane 1987), although these are unlikely to be of quantitative significance. Possible endogenous substrates include lipid, glycogen and protein. Khurana & Wales (1989) have studied the breakdown of glycogen in mouse preimplantation embryos and, while there is insufficient glycogen to account for more than a few hours of oxygen uptake, this may be the source of the excess lactate produced by morula-stage embryos over and above their glucose uptake (Fig. 2). Lipid is also unlikely to provide oxidizable fuel for more than a few hours. It is possible that the embryo generates ATP from the oxidation of endogenous protein (Leese, in press). While it is unrealistic to suppose that protein accounts for the oxygen requirement throughout the whole of the preimplantation period, it is possible that this source makes up the putative shortfall in ATP at the blastocyst stage.

We would like to thank M. Snelling, A. Haigh and the staff of the Animal Unit for breeding and maintaining the animals used in this study. D. R. Brison is in receipt of a Medical Research Council studentship.

References


Received 2 January 1991