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Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles

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The uptake of pyruvate by human embryos derived from natural cycles in the first 24 h following fertilization was examined. Since only one egg was obtained and therefore only one embryo transferred to the woman, it was possible to relate pyruvate consumption by a particular embryo to the outcome of that cycle (pregnancy or no pregnancy). The results showed that embryos have a wide range of pyruvate uptake values (2–53 pmol/embryo/h) but that this variation was reduced significantly to an intermediate range of values in those embryos that were able to implant (10–30 pmol/embryo/h). An association was found between embryo morphology and pyruvate consumption. Morphologically good embryos were more likely to implant if they demonstrated an intermediate pyruvate uptake. However, poor embryos did not implant even if they had a pyruvate uptake of 10–30 pmol/embryo/h. No relationship was found between the type of infertility and pyruvate consumption of individual embryos. It is suggested that the ability of an embryo to implant is multifactorial and that both morphology and pyruvate uptake may be factors.

Key words: embryo morphology/human embryo/metabolism/natural cycle IVF/pyruvate uptake

Introduction

At present, in human in-vitro fertilization (IVF) programmes, embryo quality and suitability for transfer are based on two main criteria: the relative rate of development and morphology. However, whilst providing some indication of viability, these qualitative assessments are widely acknowledged as being imprecise and highly subjective. This is highlighted by the large embryo wastage that occurs at the embryo transfer stage, since relatively few of the embryos replaced implant successfully. Recent data for the UK showed that the overall pregnancy rate per embryo was 13% for 1991 (Human Fertilisation and Embryology Authority, 1993).

There is therefore a need for quantitative, non-invasive methods to assess the viability of an embryo prior to transfer. Studies in the mouse showed that early cleavage stage embryos have a preference for pyruvate, while glucose becomes the major substrate at the blastocyst stage (Leese and Barton, 1984). Extension of this work to the human showed that those embryos which developed to blastocysts in culture had a higher rate of pyruvate consumption than those that arrested (Hardy *et al.*, 1989). These data suggested that pyruvate uptake had the potential to form the basis of a discriminatory test for embryo quality. Support for this concept comes from the work of Gardner and Leese (1988) who showed that the glucose consumption by mouse blastocysts which gave rise to a pregnancy after transfer was higher than those which did not. However, a study on pyruvate uptake by single human embryos from stimulated cycles (Conaghan *et al.*, 1993) showed that the mean pyruvate uptake was significantly lower by embryos which implanted than by those which failed to implant, following replacement on days 2 and 3 of embryo development. Due to the wide variation in individual results, the authors concluded that pyruvate uptake alone could not predict which embryos would implant following transfer and that morphological grading, although inefficient, remained the most consistent indicator of pregnancy potential.

In stimulated cycles, however, it is common to replace more than one embryo, and unless all the embryos implant, relating pyruvate uptake to embryo viability can be difficult. Embryos derived from natural cycles were therefore used in this study. Since only one embryo is obtained and replaced, any resulting pregnancy may be related unequivocally to that embryo. Thus it is easier to relate factors such as pyruvate uptake, morphological grade and the background infertility of the subject from whom the embryo was derived. It is also possible that embryos derived from natural cycles may have a different metabolism to their stimulated counterparts.

Materials and methods

Source of human embryos and subject monitoring

Couples undergoing natural cycle IVF at the Sheffield Fertility Centre, UK, were recruited to the study once a normally fertilized embryo had been obtained. Informed consent was given to the study in all cases and the approval of the local ethics committees in Sheffield and York had been obtained.

Hormone monitoring was performed from day 9 of the menstrual cycle and involved measurement of serum luteinizing hormone (LH) concentrations every 12 h (at 08.00 and 20.00 h) and oestrogen concentrations every 24 h. The start of the LH surge was defined as a sustained rise in serum LH >10 IU/l and oocyte retrieval was performed by vaginal ultrasound-guided aspiration 30–40 h after the start of the surge.

Culture of oocytes and measurement of pyruvate uptake

Culture was performed in Nunclon 4-well dishes (Gibco, Paisley, UK) in human tubal fluid (HTF) medium containing 0.47 mM pyruvate (Quinn *et al.*, 1985), overlaid with paraffin oil (Boots, Nottingham, UK), at 37°C in an atmosphere of 5% CO_2 in air.

Oocytes were matured in 0.5 ml HTF medium supplemented with 10% donor serum for 3–6 h before insemination. Fertilization checks were performed between 16 and 20 h post-insemination. All cumulus cells were carefully removed from the surface of the egg using a finely drawn Pasteur pipette. Eggs from which it was impossible to remove adherent cumulus cells were excluded from the study. Normally fertilized eggs were then washed through three changes of HTF medium, containing 15% donor serum, before being placed individually in a 4 μl microdrop of the same medium under oil. A control microdrop lacking an embryo was always set up in parallel (modified from Hardy *et al.*, 1989).

Embryo transfer and microdrop collection

After 20–24 h (the exact time being noted), embryos were removed from the microdrops and placed in fresh medium. The control and embryo microdrops were then collected into 5 μl capillaries (Drummond Scientific) and the ends sealed with parafilm (Fisons Scientific Equipment, Loughborough, UK) before storage at −20°C.

Prior to embryo transfer, the embryos were graded on the basis of cell shape, fragmentation and texture as follows: good: uniform blastomeres, <10% fragmentation and clear cytoplasm; reasonable: some unevenness in blastomere shape or size, moderate cell fragmentation (10–30%) and some degree of cytoplasmic granularity; poor: very uneven blastomeres, severe cell fragmentation (>40%) and prominent cytoplasmic granularity. Consideration was also given to the rate of embryo cleavage, with those embryos dividing slowly classified as poor, irrespective of their appearance.

Embryo transfer was performed at 40–50 h post-insemination using Janson-Anderson Bulb tip embryo transfer catheters (WA Cook, Queensland, Australia). Luteal support was given in the form of 10 mg tablets of Duphaston (Duphar Laboratories Ltd, Southampton, UK), three times daily. A blood sample was taken 14 days after egg recovery and assayed for the presence of β-human chorionic gonadotrophin (HCG). If a rise in βHCG was detected, sampling and measurement were repeated on day 18, followed by ultrasound scans for the presence of a sac and fetal heart on days 25 and 30–40 respectively.

Ultramicrofluorometric assay of samples

The stored microdrop samples were transported in dry ice for assay in the Department of Biology, University of York, UK. All samples were coded prior to transport to remove any bias during analysis.

Analysis of the control and embryo samples of media for pyruvate was performed using an ultramicrofluorometric technique (Leese and Barton, 1984; Gardner and Leese, 1986). Analyses were carried out in nanolitre-sized microdrops of reaction mixture under mineral oil on siliconized microscope slides.

Sample medium (0.5 μl) was added to 5 μl of assay mixture, containing 0.1 mM NADH and 40 IU/ml lactate dehydrogenase. Changes in fluorescence due to NADH oxidation were measured using a Leitz Diavert fluorescence microscope with photo-multiplier and photometer attachments and calibrated against a series of pyruvate standards. Pyruvate uptake by a single embryo over the 20–24 h period was calculated and expressed as pmol/embryo/h.

Statistical analysis

The implantation rate was defined as an elevated serum concentration of βHCG on day 14 after egg recovery and expressed per embryo replaced. A clinical pregnancy was defined as the presence of a fetal heart beat on ultrasound scan 30–40 days after oocyte retrieval. The data were tested for normality using probit transformations and the χ^2 test was used to evaluate significant differences between the data sets.

Results

Pyruvate uptake and pregnancy rate

Eighty embryos were obtained from 80 couples undergoing natural cycle IVF. Of these, 18 had elevated serum βHCG on day 14 after egg recovery and all were subsequently found to have a fetal heart beat. This gave both a clinical pregnancy rate per embryo transfer and an implantation rate per embryo of 22.5% (since there were no biochemical pregnancies in this particular study and only one embryo was ever transferred per attempt). Two subjects subsequently aborted with the remaining 16 going to term, thereby giving a live birth rate of 20% per embryo transfer. Of the babies delivered, 10 were male and the remaining six were female. Considering only the pregnancies that went to term, no relationship was found between the sex of the baby and the pyruvate uptake value. The mean age (±SD) of women achieving or not achieving a pregnancy was 32.9 (±4.4) and 33.7 (±4.6) years respectively. These values were not significantly different.

The pyruvate uptake (pmol/embryo/h) by each embryo is shown in Figure 1 for both pregnant and non-pregnant subjects. The mean value (±SD) of those embryos resulting in a pregnancy was 21.5 (±7.2) pmol/embryo/h, and for those not resulting in a pregnancy the corresponding value was 23.5 (±10.9) pmol/embryo/h. There was no significant difference between these mean values.

The data were also analysed with respect to the variances of the pregnant and non-pregnant populations, both of which consisted of normally distributed, independent observations. This demonstrated that the range and variation of pyruvate uptake in the non-pregnant subjects were significantly greater ($P = 0.03$) than in the pregnant women. If those subjects that subsequently aborted were excluded from the pregnant group, this comparison became even more significant ($P = 0.003$). To analyse the data further, a subset of intermediate pyruvate uptake values was defined which incorporated 95% of the clinical pregnancies (between 10 and 30 pmol/embryo/h), as shown in Figure 1. Within this subset the pregnancy rate per embryo was 30% (17/57).

Relationship between infertility and pyruvate uptake

The type of infertility was categorized on the basis of Fallopian tube status. In this study, 46% of women had tubal infertility (more than one non-functional tube) and 54% had non-tubal infertility (two patent tubes). These proportions were similar to the overall distribution of infertility of couples attending the clinic.

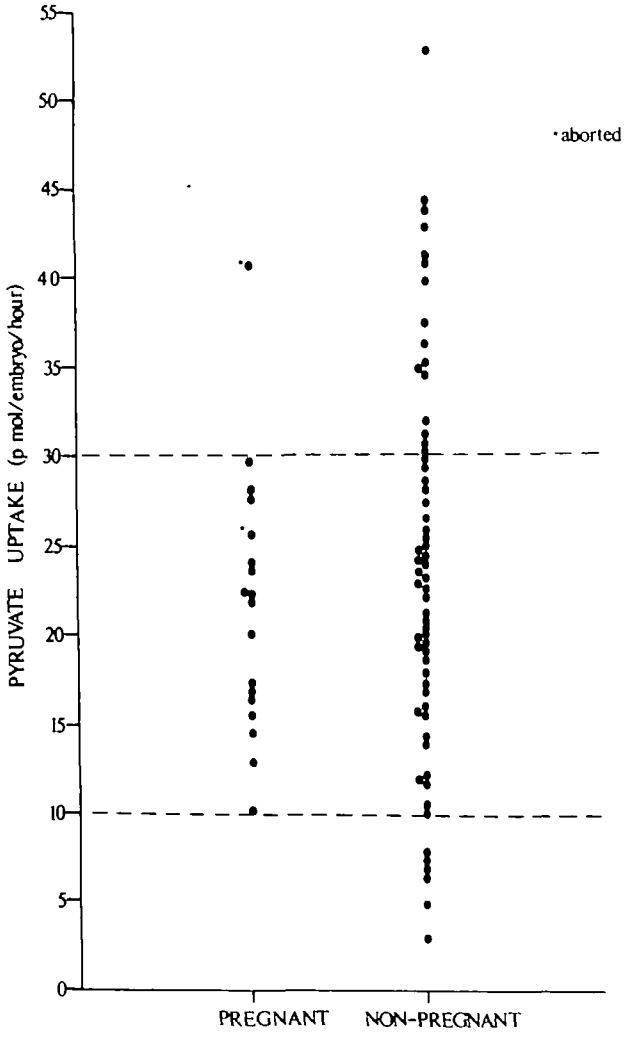

Fig. 1. Pyruvate uptake by single embryos which were able to implant and by those which failed to implant. Each point represents a single embryo.

Male factor infertility was not considered since the spermatozoa of all the subjects involved had demonstrated fertilizing ability by formation of an embryo. The overall pregnancy rates per embryo for tubal and non-tubal subjects were not significantly different at 27% (10/37) and 19% (8/43) respectively. There was no difference in the distribution of pyruvate uptake values by embryos from tubal and non-tubal subjects.

Relationship between embryo grade and pyruvate uptake

All embryos were assigned a grade based on their morphology and rate of cleavage prior to transfer. From Table I, 29% (23/80) of the embryos were graded as good, 47% (38/80) as reasonable and 24% (19/80) as poor. In all, 61% (14/23) of good embryos, 66% (25/38) of reasonable embryos and 95% (18/19) of poor embryos fell within the 10–30 pmol/embryo/h subset of pyruvate uptake values.

Of those embryos that resulted in a pregnancy, 56% (10/18) were defined as good and 44% (8/18) as reasonable, whilst none of the 19 poor embryos implanted (Table I). Thus, of all the good embryos 44% (10/23) resulted in a pregnancy, as compared with 21% (8/38) of all reasonable embryos. Consideration of only embryos which failed to implant showed that the distribution of good and poor embryos differed greatly ($P < 0.00001$), with good embryos being concentrated outside and poor embryos being concentrated within the 10–30 pmol/embryo/h subset.

Relationship between embryo grade and infertility

The relationship between embryo grade and infertility was also examined and the data are shown in Table I. There was no significant difference in the overall distribution of good, reasonable and poor embryos between tubal and non-tubal subjects. However, it appeared that of the embryos resulting in pregnancy, only 40% (4/10) of those from tubal subjects were classified as good quality as opposed to 75% (6/8) from non-tubal subjects.

Discussion

In the first 24 h following fertilization, there is a large variation in the amount of pyruvate consumed by individual human embryos derived from the natural cycle. It is also clear that this variation is much smaller in embryos capable of implanting, with these embryos tending to be clustered in the middle of the

Table I. Distribution of embryos, based on their grade, in relation to pyruvate uptake, pregnancy outcome and infertility status of the patient

	Good	Reasonable	Poor	Total
Pyruvate uptake				
Within 10–30 pmol/embryo/h subset	14	25	18	57
Outside 10–30 pmol/embryo/h subset	9	13	1	23
Total	23	38	19	80
Outcome				
Pregnant	10	8	0	18
Non-pregnant	13	30	19	62
Total	23	38	19	80
Infertility status of patient				
Tubal	8	18	11	37
Non-tubal	15	20	8	43
Total	23	38	19	80

spectrum. Since only one egg and therefore one embryo is obtained in natural cycle IVF, it has been possible to link pyruvate uptake unequivocally to a particular embryo and thus to the outcome of that cycle. By defining a subset of pyruvate consumption values, within which 95% of the pregnancies fell, the pregnancy rate per embryo was increased from 22.5% for the data overall, to 30%. Although an improvement, this increase in pregnancies still means that 70% of embryos within and 96% of embryos outside the subset could not implant, and suggests that pyruvate uptake alone cannot be used as a definitive test to determine which embryos will implant.

The application of pyruvate uptake as a predictor of the ability of embryos to implant after transfer has been examined previously for stimulated IVF by Conaghan *et al.* (1993). As the sole criterion for selection, where there were more than two or three embryos to choose between, pyruvate uptake was found to be of little value in predicting implantation, due to the small differences between embryos derived from an individual subject. In this earlier study, the mean pyruvate uptake values of embryos that implanted (22.9 pmol/embryo/h) and did not implant (27.1 pmol/embryo/h) were of a similar order to those obtained in the present study (21.5 and 23.5 pmol/embryo/h respectively). However, in contrast to the data of Conaghan *et al.* (1993), we did not find a significant difference between the means of these groups, although there was a difference in the population variance. It is encouraging to note that as far as pyruvate consumption is concerned, embryos derived as a result of stimulation do not appear to differ from natural cycle embryos.

Neither pyruvate uptake nor morphology was useful for discriminating between embryos from women with tubal or non-tubal infertility, suggesting that the cause of unexplained infertility is not due to large numbers of poor quality embryos. In fact, 75% of the embryos from non-tubal subjects which implanted were classified as good, as opposed to only 40% of embryos from tubal subjects. This supports the findings of Monks *et al.* (1993), who suggested that the appearance of the embryo in natural cycles may be of clinical relevance in women with unexplained infertility.

Tubal subjects had a higher pregnancy rate (27%) than non-tubal subjects (19%), though the difference was not statistically significant. The implantation rate per embryo for our tubal infertility subjects was 27%, comparable with the 25% reported previously by Dawson *et al.* (1991) for stimulated cycles. The similarity of these figures for transfers of only one embryo compared with two or more embryos does not support the notion that in cases where multiple embryos are transferred, better quality embryos are able to rescue poorer ones (Lane and Gardner, 1992).

There was a relationship between the grade of an embryo and its pyruvate uptake. Figure 2 shows how the pregnancy rate is increased for tubal and non-tubal infertility after selection on the basis of these two parameters. In this series, for tubal and non-tubal infertility respectively, 50 and 40% of those embryos graded as good and 33 and 10% of those graded as reasonable, implanted. None of the embryos graded as poor implanted. Those embryos graded as good but not resulting in a pregnancy tended to lie outside the subset where 95% of the pregnancies occurred, such that consideration of those embryos graded as good which lay within the 10–30 pmol/embryo/h subset gave a combined pregnancy rate per embryo of 71% (10/14).

Conversely, poor embryos, with one exception, were concentrated within the 10–30 pmol/embryo/h subset. Therefore, it may be that those embryos with the greatest chance of giving rise to a pregnancy need to have both good morphology and an intermediate pyruvate consumption, in the region of 10–30 pmol/embryo/h. Thus, good embryos which do not have, and poor embryos which do have, this level of consumption are less likely to implant because only one critical factor is present.

The relationship between the replacement of morphologically good embryos and the establishment of pregnancy is well documented. The pregnancy rate, implantation rate and incidence of multiple pregnancies have all been shown to increase significantly with the number of good quality embryos transferred (Staessen *et al.*, 1992; Visser and Fourie, 1993). Poor quality embryos have been associated with reduced embryo potential, although they may still establish a pregnancy, albeit at a lower rate (Veeck, 1987). Poor quality embryos have also been observed to occur *in vivo*, which suggests that they may be a normal feature of human reproduction rather than a culture-induced phenomenon (Buster *et al.*, 1985; Sauer *et al.*, 1987).

Failure of embryos to implant may be due to other factors that are not reflected in either their morphology or pyruvate consumption in the first 24 h following fertilization. For example, it is estimated that as many as 29% of embryos produced by IVF have an abnormal karyotype (Plachot *et al.*, 1988). Moreover, pregnancy failure could also be due to maternal factors in the form of hormonal imbalances or endometrial lesions. Li *et al.* (1991) found that women with endometriosis and unexplained infertility had a significantly higher prevalence of retarded endometrium than women with normal fertility or women with tubal infertility. In such women, the endometrium may be insufficiently prepared to receive an implanting embryo such that its quality will always be irrelevant. Subjects with unexplained infertility who have good quality embryos falling in the intermediate range of pyruvate consumption that do not achieve a pregnancy may possibly be those individuals with endometrial lesions.

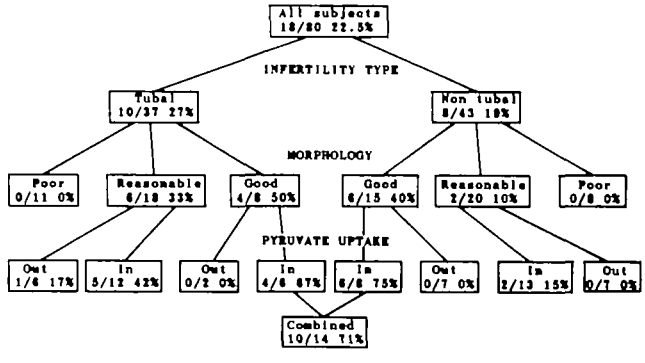

Fig. 2. Flow chart to demonstrate how the pregnancy rate is increased after applying selection by (i) infertility type, (ii) embryo morphology, followed by (iii) pyruvate uptake, where 'In' denotes values within the range 10–30 pmol/embryo/h and 'Out' denotes values outside this range. The within-group pregnancy rates are shown following the numbers of subjects for each of the sub-selections.

The ability of an embryo to implant, therefore, appears to be multifactorial. This is not perhaps surprising for such a biologically complex process, and it may well be that no factor on its own will ever be able to predict accurately the likelihood of implantation. The results of this study suggest that pregnancy rates could be improved in instances where there is a choice of embryos, for example in stimulated IVF, by taking into account both embryo morphology and pyruvate consumption in the first 24 h after fertilization. Whilst the maternal influence on the outcome of embryo transfer will always be a limiting factor in such studies, identification of other embryo factors may improve success rates still further.

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