Increased number of cells and metabolic activity in male human preimplantation embryos following *in vitro* fertilization

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The number of cells and metabolic activity of male and female human preimplantation embryos were examined to determine whether male embryos are more advanced than female embryos following *in vitro* fertilization (IVF). The metabolic activity of embryos fertilized normally was assessed daily by non-invasive measurement of pyruvate and glucose uptake and lactate production between days 2 and 6 after insemination. On day 6, the numbers of nuclei from the trophectoderm and inner cell mass of blastocysts were counted by differential labelling and fluorescence microscopy. Nuclei were then recovered and the sex of the embryos identified using nested primers to amplify the amelogenin gene and pseudogene sequences on the X and Y chromosomes, respectively. Development of male and female embryos were then compared retrospectively. From 69 of 178 (39%) embryos that developed to the blastocyst stage, the sex of 57 was determined; 21 (37%) were male and 36 (63%) female. The number of cells in male embryos was significantly greater on day 2 ($P < 0.005$), and this difference was maintained up to the blastocyst stage (in both the trophectoderm and the inner cell mass), although differences were not always significant. Pyruvate uptake was significantly higher by male embryos between days 2 and 5 ($P < 0.05$). Glucose uptake and lactate production were significantly higher in male embryos on days 4–5 ($P < 0.05$); this difference was not significant on days 5–6. Extrapolation from differences in the number of cells indicates that female embryos are approximately 4.5 h delayed in their development from day 2 onwards compared with male embryos. As there was no evidence for a differential cleavage rate beyond this stage, it is suggested that differences between males and females occur early, either at fertilization, or during the first or second cleavage.

Introduction

Sexual dimorphism at pre- and postnatal stages of development has been recognized in several mammals. For example, Karn and Penrose (1951) examined 13 000 new-born human babies and found that males were on average 100 g heavier than females. This early difference is thought to be caused by the effects of androgens and other male-specific hormones which are later responsible for the development of the secondary sexual characteristics. Similarly, Pedersen (1980), who measured body length and skull diameter of fetuses, reported that between weeks 8–12 female fetuses are delayed by one day compared with male fetuses and that the difference increased to 6–7 days at term. Furthermore, extrapolation suggests that this divergence begins a few weeks after conception, that is, before gonadal differentiation and the production of hormones.

Differences between male and female embryos have been demonstrated as early as the preimplantation stage. Tsunoda *et al.* (1985) classified mouse embryos into slow, medium and fast developing groups at the time of blastocoel formation.

Embryos from each group were transferred to separate pseudopregnant recipients. At birth, there were more females (80%) in the slow developing group and more males (71%) in the fast developing group. More recently, Valdivia *et al.* (1993) confirmed this difference by identifying the sex of preimplantation embryos directly by DNA amplification of Y-specific sequences using the polymerase chain reaction (PCR). Increased numbers of cells in male blastocysts of the CD1 mouse strain were reported by Burgoyne (1993) and by Peippo and Bredbacka (1995) in mouse embryos cultured *in vitro*. Similar observations have also been reported for bovine embryos (Avery, 1989; Avery *et al.*, 1989, 1992; Xu *et al.*, 1992; Yadav *et al.*, 1993) and for pig embryos (Cassar *et al.*, 1994).

The development of *in vitro* fertilization (IVF) for the treatment of infertility provides an opportunity to examine whether there are similar differences in the rate of development of human male and female embryos. Recently, Pergament *et al.* (1994) examined this indirectly by correlating the average number of cells of embryos transferred on day 2 after insemination with sex at birth in a small series of 36 singleton pregnancies. The mean numbers of cells on day 2 after insemination was 3.45 for females, and 3.76 for males, although

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this difference was not significant. However, when the distribution of male and female embryos was correlated with the number of cells, a significant trend was demonstrated. For example, when the average number of cells of the transferred embryos was greater than 4, male births exceeded those of females by a ratio of 6:1.

In the last few years, methods have been developed for identifying the sex of human preimplantation embryos for selective transfer of females in couples at risk of X-linked disease (Handyside and Delhanty, 1993). Two approaches for analysis of single cells biopsied from cleavage stage embryos have been used reliably: fluorescent *in situ* hybridization with X- and Y-specific probes, and PCR for the amplification of X- and Y-specific sequences (Harper and Handyside, 1994). Using both methods, Munné *et al.* (1994) failed to demonstrate a significant difference in the numbers of nuclei of arrested male and female embryos on day 4 after insemination, although male embryos appeared to be more fragmented.

This study examined differences in male and female embryos developing normally to the blastocyst stage, using morphological data, number of cells and metabolic criteria. The sex of each embryo was identified by PCR. Embryos were cultured individually from days 2 to 6 and the number of cells counted on days 2, 3 and 6. In addition, the energy substrate metabolism of individual embryos was measured non-invasively at daily intervals. All embryos developing to the blastocyst stage were differentially labelled to count trophoctoderm and inner cell mass nuclei and subsequently sexed by PCR from the labelled nuclei. Results indicate that normally developing male preimplantation embryos have higher number of cells and metabolic activity than do those of females at preimplantation stages from day 2 onwards.

Materials and Methods

Source of human embryos

The embryos used in this study were surplus embryos following embryo transfer, and were donated with informed consent by patients undergoing IVF treatment at the Wolfson Family Clinic, Hammersmith Hospital. The work was carried out with approval from the Research Ethics Committee of the Royal Postgraduate Medical School, and under licence from the Human Fertilization and Embryology Authority. The super-ovulation, IVF and embryo transfer procedures were as described by Hillier *et al.* (1984, 1985) and Rutherford *et al.* (1988).

Analysis of number of cells

The number of cells of each embryo was counted by visual inspection on the morning of day 2 (40 to 42 h after insemination) and again on day 3. After compaction on days 4 and 5, accurate cell counts were not possible and further analysis was delayed until the blastocyst stage at the end of the culture period on day 6 (see below).

Differential labelling of blastocyst nuclei

On day 6, numbers of trophoctoderm and inner cell mass cells of the blastocysts were obtained by differential labelling of

the nuclei with polynucleotide-specific fluorochromes as described in Hardy *et al.* (1989a). The trophoctoderm nuclei were labelled with propidium iodide during immunosurgical lysis, before fixing the embryo and labelling both sets of nuclei with bisbenzamide. Differentially labelled nuclei were mounted in glycerol, partially disaggregated and counted under fluorescence microscopy.

Non-invasive assessment of energy substrate metabolism

Earle's balanced salt solution, modified to contain 1 mmol glucose l^{-1} (Conaghan *et al.*, 1993), and supplemented with 25 mmol sodium bicarbonate l^{-1} (BDH Chemicals Ltd, Poole, Dorset), 0.47 mmol pyruvate l^{-1} (Sigma Chemical Co., Poole, Dorset), antibiotics (Sigma) and 10% v/v human serum albumin (HSA) (Zenalb 20; Bio Products Laboratory, Elstree, Herts) was used for embryo culture. Normally fertilized (two pronucleate) embryos were placed individually in a 5 μl drop of medium under filter-sterilized silicone fluid (Dow Corning 200/50 cs; BDH) in a gas phase of 5% CO_2 in air at 37°C. Identical microdroplets without embryos served as control for substrate degradation.

At intervals of 24 h, embryos were washed through three changes of media and transferred to fresh microdrops. Duplicate 2 μl samples of the incubation and control droplets were then recovered. The first 2 μl was diluted with 398 μl double-distilled water and assayed for pyruvate and glucose content, and the second 2 μl was diluted in 118 μl of 5 μmol lactate l^{-1} standard solution and assayed for lactate content. If not assayed immediately, samples were stored frozen at $-20^\circ C$.

The concentrations of pyruvate, glucose and lactate in the control and incubation drops were determined by modification of methods described in Hardy *et al.* (1989b) and Leese and Barton (1984) using a Cobas Bio autoanalyser (Roche products, Welwyn Garden City, Herts) as described in Conaghan *et al.* (1993). All the assays are based on the fluorescence of the coenzymes NADH or NADPH when oxidized and are based on those described in Stappenbeck *et al.* (1990). The uptake and production of substrates was determined by subtracting the values obtained for the incubation droplets from those of the controls.

Identification of sex by PCR

Embryos were sexed using a nested PCR amplification protocol with primers specific for the amelogenin gene on the X chromosome and an amelogenin-like sequence on the Y chromosome giving rise to 290 bp fragment for the X and 105 bp for the Y. The outer primers were as described in Nakahori *et al.* (1991), and the inner primers (kindly donated by R. Mountford, St Mary's Hospital, Manchester) were 5' TGA CCA GCT TGG TTC TA(A/T) CCC and 5' CA(A/G) ATG AG(A/G) AAA CCA GGG TTC CA, and were synthesized by Pharmacia Biotech Ltd, Herts. Under a binocular microscope, arrested cleavage-stage embryos on day 5, or differentially labelled day 6 blastocyst nuclei, were recovered from the microscope slide (split into two or three separate samples when possible), and transferred into an Eppendorf tube containing 5 μl of lysis buffer using a mouth-operated hand-pulled glass

pipette. For each sample, a control blank was also prepared. For the arrested embryos, this was a sample of the culture medium, and for the blastocysts, a sample of the glycerol mountant was used. The lysis procedure was as described by Cui *et al.* (1989). PCR mix was added to obtain $2.5 \text{ mmol MgCl}_2 \text{ l}^{-1}$ (Sigma), $0.0001 \text{ mg gelatin l}^{-1}$ (Sigma), $10 \text{ mmol Tris-HCl l}^{-1}$ (Sigma), pH 8.3, $0.2 \text{ mmol dNTP mix l}^{-1}$ (Pharmacia Biotech Ltd, St Albans, Herts), $0.8 \text{ }\mu\text{mol primers l}^{-1}$; 1 U Perkin Elmer Taq polymerase (Applied Biosystems Ltd, Warrington, Cheshire). Two microlitres of the outer reaction product was transferred to a fresh tube containing $50 \text{ mmol KCl l}^{-1}$, $10 \text{ mmol Tris-HCl l}^{-1}$, pH 8.3, $1 \text{ mmol MgCl}_2 \text{ l}^{-1}$, $0.2 \text{ mmol dNTP mix l}^{-1}$, $0.8 \text{ }\mu\text{mol primers l}^{-1}$, 1 U Perkin Elmer Taq polymerase. Cycling was performed on a Hybaid Omnigene Thermal Cycler (Hybaid Ltd, Hampton Hill, Middx) and was preceded by 3 min at 94°C and ended by 5 min at 72°C . It consisted of 28 cycles at 94°C for 30 s, 58°C for 1 min, 72°C for 1 min for the outer primers and of 94°C for 30 s, 68°C for 90 s, 72°C for 30 s for the inner amplification. Ten microlitres of the amplification product was run on 10% (w/v) polyacrylamide gel (Maniatis *et al.*, 1989 6.39–6.44) using a Protean II minigel (Bio-Rad Laboratories Inc., Hemel Hempstead, Herts) in TBE buffer ($89 \text{ mm Tris-borate l}^{-1}$, $2 \text{ mmol EDTA l}^{-1}$, pH 8.0) at 200 V constant voltage (Powerpac 3000; Bio-Rad) for 30 min. Ethidium bromide stained gels were visualized under UV light.

Statistical analyses

Mean substrate uptake or lactate production was expressed as $\text{pmol per embryo h}^{-1} \pm \text{SE}$. Differences in number of cells and substrate uptake between groups were compared using the Wilcoxon Rank-Sum (Mann-Whitney) test. Statistics on the sex ratio were carried out using the hypothesis test for a single proportion. All statistical analyses were performed using STAT VIEW II (Abacus Concepts Inc., Berkeley, CA).

Results

Sex ratio

In this study, 69 of 178 (39%) embryos from 58 patients developed to the blastocyst stage by day 6. Fifty-seven of 60 blastocysts were successfully double-labelled and sexed by PCR (Fig. 1). Of these, 21 (37%) were male and 36 (63%) female. In nine cases, three separate samples from the same blastocyst were analysed. In six of these, there were three concordant results, in two, two concordant results and one amplification failure, and one set of samples failed to amplify. Two samples were analysed from 44 blastocysts: concordant results were obtained from 35; two were discordant due to contamination or allele-specific failure of amplification; and only one result was obtained from the remaining seven embryos. In addition, one sample was analysed from seven blastocysts and were all successful. For each embryo, a blank was included as a control for contamination. Five of 60 (8.3%) control blanks were positive (4 female and 1 male).

The remaining 109 (61%) embryos arrested at various cleavage stages. Fifty-eight of these, which showed no sign of cavitation and which had not degenerated on day 5, were also

Fig. 1. Polyacrylamide gel electrophoresis of PCR products obtained after amplification of double-labelled embryos or half-embryos using the amelogenin primers for sex determination. M: molecular mass marker: pBR322 digested with *Hae* III. E1 to E5: embryos or half-embryos retrieved from the microscope slides after double labelling; B: their corresponding blank control.

sexed. Results were obtained for 49 (84%), of which 18 were male (37%) and 31 female (63%). Five out of 58 blanks (8.6%) gave a false positive signal.

Number of cells on days 2 and 3

The distribution of male and female embryos on days 2 and 3 after insemination and the average number of cells are given (Table 1). On days 2 and 3, division of embryos into late and advanced groups indicated that the majority of advanced embryos were male (82% on day 2 and 81% on day 3), whereas females were more equally distributed between the two groups. The average number of cells in male embryos on day 2 and day 3 was higher than in females but the difference was only significant on day 2 ($P < 0.005$).

Morphology on days 4, 5 and 6 after insemination

The large variation in quality and development of the human embryos made morphological observations between days 4 and 6 subjective. The number of male and female embryos that had reached the morula stage on day 4 was comparable (38 and 36%, respectively). Similarly, 71% of male embryos and 63% of female embryos showed some signs of cavitation or had reached the full blastocyst stage by day 5. On day 6, 76% of male embryos had reached or passed (had hatched or collapsed) the expanded blastocyst stage compared with 69% of female embryos.

Number of cells in blastocyst on day 6

Mean number of cells in the trophectoderm and inner cell mass was greater in male than in female embryos, although the differences were not significant (Fig. 2). Similarly, the ratio of trophectoderm to inner cell mass cells between male and female embryos was not significantly different (1.74:1 and 1.96:1, respectively). The percentage difference in the number of cells

Table 1. Number of fast and slow developing human embryos and average number of cells on day 2 and day 3 after insemination in the male and female populations

Day	Sex	≤3 cells	4–7 cells	8–16 cells	Mean number of cells
2	Male	7 (18%)	32 (82%)	—	3.97 ± 0.19*
	Female	33 (50%)	34 (50%)	—	3.28 ± 0.16*
3	Male	—	4 (19%)	17 (81%)	7.62 ± 0.29
	Female	—	15 (42%)	21 (58%)	7.36 ± 0.29

*Values are significantly different from each other, *P* < 0.005.

Fig. 3. Decimal logarithm of number of cells in male (—●—) and female (---□---) embryos from day 2 to day 6 after insemination. Equations for the simple curve fit for male and female values are: $y = 6.2168e - 3 + 0.29397x$ and $y = - 5.2058e - 2 + 0.29580x$, respectively.

and lactate production were significantly higher in male embryos (*P* < 0.05, Fig. 4b, c). Although male embryos maintained this higher glucose uptake and lactate production to days 5–6, these differences were not significant.

Discussion

It was possible, using PCR, to amplify an X- and Y-specific sequence, to examine directly the development and metabolic activity of male and female human preimplantation embryos. The number of cells on day 2 was significantly higher in male embryos and this difference was maintained, although reduced, from day 3 up to the blastocyst stage; however, the difference was not significant at these later stages. Metabolic activity was also increased throughout this period in male embryos. Pyruvate uptake was significantly higher in males from days 2 to 5, and the increase in glucose uptake and lactate production was significantly higher from day 4 to day 6.

The sex ratio in both groups of embryos sexed (developing and arrested) was skewed toward the females (1.7:1). Although this ratio is significantly different from 1:1 (*P* < 0.01), we believe it represents the true proportion of each sex in the studied group. For a male embryo to be mistakenly identified as

Fig. 2. Number of cells in the trophectoderm and inner cell mass lineages for male (■) and female (□) human embryos that developed to the blastocyst stage by day 6 after insemination. Values are means ± SEM.

between male and female embryos was similar on day 2 (17.4%) and at the blastocyst stage on day 6 (12.3%). The difference was less marked on day 3 (3.4%). Analysis of number of cells on days 2, 3 and 6 (Fig. 3) indicates an average cell cycle of about 24 h for both male and female embryos. The time difference between male and female embryos calculated from a simple curve fit (Fig. 3) is about 4.5 h throughout this period.

Metabolic measurements from day 2 to day 6

Pyruvate and glucose uptake, and lactate production by embryos that developed to the blastocyst stage between days 2 and 6 are given (Fig. 4). Male embryos had a significantly higher pyruvate uptake compared with female embryos from day 2 to day 5 (*P* < 0.001 on days 2–3, and *P* < 0.05 on days 3–4 and 4–5, Fig. 4a). This difference was not present on day 6. On days 2–3 and 3–4, glucose uptake was similar in male and female embryos, but on days 4–5, glucose consumption

Fig. 4. Uptake of (a) pyruvate, (b) glucose and (c) production of lactate by male (□) and female (○) human embryos that reached the blastocyst stage by day 6 after insemination. Values are means $\pm$ SEM. *Significantly higher than female value ($P < 0.05$).

female, either a failure to amplify the Y allele, or a total failure to amplify from the embryo nuclei combined with contamination with female DNA, would be required. The probability of this occurring in multiple samples from the same embryo is very low. Only two of 43 embryos (4.6%) that gave more than one result had to be eliminated because of discordant results. In addition, the embryos used in the present study were spare

embryos (that is, ones remaining after selection of usually the best two embryos for transfer). Since embryos are selected for transfer on the basis of morphology and advanced developmental stage, our results suggest that more male embryos are selected for transfer, explaining the observed predominance of females among the spare embryos. However the sex ratio at birth after IVF at the Hammersmith Hospital does not appear to be skewed towards males (K. Dawson, personal communication). Pergament *et al.* (1994) reported that the average number of cells of embryos transferred on day 2 is correlated with sex at birth. Eighty-five per cent of births that resulted following transfer of multiple embryos, with an average number of cells greater than four, were male. Compensating for sex ratio, we also observed that the majority of embryos (61.5%) in this category are male, but the effect is less marked. Similarly, the majority of embryos (58.1%) with 8–16 cells on day 3 were male (data not shown).

The fact that the percentage difference in the number of cells between male and female embryos was similar on day 2 and at the blastocyst stage suggests that the difference arises at an earlier stage of development, either at fertilization or during the first or second cleavage divisions. The less marked difference observed on day 3 indicates that there may be some remaining synchrony among blastomeres in early divisions that causes periodic fluctuation in the ratio. The estimate for average cell cycle of about 24 h for both male and female embryos is similar to that given by Hardy *et al.* (1989a). The time difference of about 4.5 h observed between male and female embryos is unlikely to be accounted for by a difference in the timing of fertilization by X- and Y-bearing spermatozoa *in vitro*, although it is not known how rapidly fertilization takes place following insemination in humans. Oocytes are usually incubated with the spermatozoa for a period of about 16 h to ensure maximum rates of fertilization. However, Valdivia *et al.* (1993) demonstrated that, in mice, 98.3% of eggs are fertilized within 30 min, and 100% within 1 h of insemination.

A difference in the timing of the first or second cleavage division between male and female embryos is surprising, since the embryonic genome is not thought to be activated until the 4–8 cell stage on day 3 (Braude *et al.*, 1988; Tesarik *et al.*, 1986). However, there is now evidence using reverse transcription PCR, that Y-linked transcripts are present in zygotes in several species. *SRY* and *ZFY* have been shown to be expressed as early as the two-cell stage in mice (Zwingman *et al.*, 1993), and as early as the zygote stage and through to the blastocyst stage in humans (Ao *et al.*, 1994). *SRY* is a critical determinant of testis formation (Koopman *et al.*, 1991; Ferguson-Smith, 1992) and has been shown to be a transcription regulator (Sinclair *et al.*, 1990; Lovell-Badge, 1992), and *ZFY* codes for a zinc finger protein. It is not clear how such genes could affect cell division. Also, it is not known whether transcripts are present in a physiologically significant copy number or if the corresponding proteins are expressed at these early stages. If one (or both) of these Y-linked products accelerate the cleavage rate of expressing cells, the difference between the male and female population should increase with each cell division since both transcripts appear to be expressed throughout preimplantation stages. Our data shows that the difference is present as early as day 2 and is maintained but not increased during preimplantation development.

Yadav *et al.* (1993) also reported early differences in the number of cells in cattle embryos which are maintained, but not increased, at later stages. Analysis of their data shows that 75% of male embryos completed first cleavage by 30 h compared with only 46% of female embryos. These percentages are very similar to those obtained in the present study for the second and third cleavage divisions and suggest that the advance of male embryos in humans may also occur during the first cleavage division. It may be caused by another Y-linked gene, which is expressed only at the zygote stage, or by gene products or proteins carried by the Y-bearing spermatozoa and released at fertilization. Such gene products may induce the transcription of other genes exclusively in male embryos giving them a developmental advantage.

Comparison of the timing of different morphological criteria, such as time of compaction or time of blastocoel formation, were inconclusive. Precise timing was not possible and perhaps a difference between the two populations studied could have been observed if a more frequent scoring had been performed. However, the trend that male embryos are more advanced developmentally was preserved when looking at morphological factors between days 4 and 5 after insemination, for example, 71% of male embryos had reached the blastocyst stage on day 5, compared with 63% of females.

The metabolic activity of male embryos increased throughout development. The greatest difference between the two groups was observed for each substrate at the peak of its uptake or production. For instance, embryos rely on pyruvate as their main energy substrate from fertilization to about days 3–4, from which time glucose uptake increases steadily to the blastocyst stage; lactate production follows this latter pattern closely (Conaghan *et al.*, 1993). The greatest difference in pyruvate uptake was found on day 2, when female embryos consumed 21% less pyruvate than did male embryos. A difference of 23% on days 4–5 and 18% on days 5–6 was observed in glucose uptake between male and female embryos. The corresponding figures for lactate production were 15 and 12%. Tiffin *et al.* (1991) demonstrated that in male cattle embryos glucose metabolism was twice that of females at the morula–blastocyst stage. Paradoxically, Gardner and Leese (1987) reported that, in the small series of mouse blastocysts they studied, female embryos took up slightly more glucose than did male embryos. The differences between male and female embryos observed in the present study in metabolic profiles may be accounted for by the differences in the number of cells as the percentage differences are similar. For example, the difference in the number of cells on day 2 is 17.4% and in pyruvate uptake is 21%. In any case, the combination of the two reinforces our conclusion that male embryos are more advanced than are female embryos from day 2 onwards.

Precise timing of fertilization, either by direct observation, or with the use of intracytoplasmic sperm microinjection, and timing of the first two cleavage stages will be necessary to find out when the shift in the rate of development occurs. If it is shown to occur during the first cleavage division, this would strengthen the hypothesis that a Y-linked sperm factor brought in at fertilization is responsible for this effect. It is possible that the isolation and characterization of such a factor, perhaps from a Y-enriched sperm sample, would then be the next step.

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