Mitotic activity during preimplantation development of human embryos

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The rate of cell division of human embryos *in vitro* was estimated throughout the preimplantation period. Seventy-five human embryos were assessed by performing nuclei counts on whole mount preparations at 24 h intervals between day 2 and day 6 of development. The results indicate that the cell doubling time increases after the first two division cycles. The mean ($\pm$ SEM) doubling time between day 2 and day 6 was 31 ± 2.51 h. At the end of the designated culture periods, embryos were exposed to a microtubule disrupting agent, which caused the nuclei to arrest in metaphase. Thus, a profile of the mitotic activity at given stages of development was obtained. The numbers of actively dividing cells were frequently lower than anticipated, indicating that nuclei counts cannot be directly correlated with continuing mitotic activity.

Introduction

Standard practice in clinical *in vitro* fertilization (IVF) and embryo transfer treatment is to place embryos in the uterus during their early cleavage stages. The rate of cell division during these stages is positively correlated with embryo viability following IVF and embryo transfer (Trounson *et al.*, 1982; Staessen *et al.*, 1992; Margalioth *et al.*, 1993). Cell division rate is thus used as an important indicator of embryo quality, not only to select suitable embryos for transfer but also to evaluate culture conditions.

Despite the widespread practice of *in vitro* culture of human embryos, little is known about the rate of cell proliferation. Data on the development rate of human embryos *in vitro* are based largely on the number of blastomeres visible during the early cleavage stages (Edwards *et al.*, 1981; Trounson *et al.*, 1982; Cummins *et al.*, 1986). Hardy *et al.* (1989), Winston *et al.* (1991) and Dorkas *et al.* (1993) estimated the number of cells of blastocysts by staining and counting the nuclei. Using this approach, Hardy *et al.* (1989) determined the rate of cell division of blastocysts as fixed between day 5 and day 7. Therefore, the rather sparse information on the rate of cell division in human embryos relates to either the early cleavage divisions or to the blastocyst stage. There are no data available on the rate of cell proliferation of human embryos throughout the preimplantation period.

The assessment of numbers of cells by staining and counting nuclei provides an indication of the number of cell division cycles that have occurred up to the point at which the embryos were stained and is, in this sense, historical information. There is evidence, from studies on the proliferation kinetics of normal human somatic cells, of a loss of growth potential with increasing number of cell replications *in vitro*. This has

been attributed to an increase in the fraction of noncycling cells (Smith and Braunschweiger, 1979; Ponten *et al.*, 1983). Experiments on mouse embryos indicate that, as development proceeds, the rate of cell division slows down (Streffer *et al.*, 1980). In addition, subpopulations of cells become asynchronous and divide at different rates during late preimplantation morphogenesis in a number of species, including humans (Johnson and Ziomek, 1982; MacQueen and Johnson, 1983; Papaioannou and Ebert, 1988; Hardy *et al.*, 1989). Therefore, a more valid profile of the mitotic activity of human embryos needs to take into account the fraction of actively dividing cells at specific times or stages of development. This information is not available for human embryo development.

The relationship between mitotic activity and the onset of compaction and cavitation has not been investigated in humans, although it has been suggested that compaction of human embryos takes place between the fourth and fifth cleavage divisions (Edwards *et al.*, 1981; Cummins *et al.*, 1986; Hardy *et al.*, 1989). Measurement of the mitotic activity in human morulae and blastocysts offers an insight into the relationship between cell proliferation and late preimplantation morphogenesis.

In this study, we set out to estimate the number of cell generation cycles that embryos undergo during each 24 h of culture between day 2 and day 6 of development. By counting the number of nuclei per embryo, the mean rate of cell proliferation between the early and late preimplantation stages was calculated. At the end of the designated culture period, embryos were treated with colcemid which inhibits microtubule assembly and thereby causes cells to arrest in metaphase. The number of metaphases related to the total number of nuclei provides an index of the mitotic activity at specific stages of development.

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Materials and Methods

Source of embryos

The embryos used in this study were donated for research by couples undergoing IVF treatment. Surplus embryos were allocated to the study once the IVF treatment cycle was completed. The study was approved by the Ethics Committee of Newcastle Health Authority and was licensed by the Human Fertilisation and Embryology Authority.

Superovulation was achieved by downregulation of the pituitary with the GnRH agonist, buserelin (Suprefact: Hoechst, Hounslow) and exogenous administration of either human menopausal gonadotrophin (hMG) (Pergonal: Serono, Welwyn Garden City) or FSH (Metrodin: Serono). Buserelin was administered by nasal spray in three doses of 800 µg per day, until the ovarian steroids were in the postmenopausal range and no follicles were seen on the transvaginal ultrasound scan. Thereafter, hMG and FSH stimulation commenced (150–225 iu per day), while the same daily dose (800 µg) of buserelin continued to be taken. Final maturation of follicles was achieved by i.m. injection of either 5000 or 10 000 iu hCG (Profasi: Serono). Oocytes were retrieved by transvaginal ultrasound guided aspiration of follicles 36–38 h after hCG injection.

Culture of gametes and embryos

Oocytes were cultured and inseminated in 200 µl droplets of medium under mineral oil (Aldrich, Dorset). The medium was Earle's balanced salt solution (EBSS; Gibco BRL, Paisley) supplemented with sodium bicarbonate (25 mmol l⁻¹) (Analar grade; BDH, Lutterworth), sodium pyruvate (0.47 mmol l⁻¹) (Analar grade; BDH) and 10% (v/v) human serum albumin (HSA; 5% solution, Albuminar-5, Armour Pharmaceuticals, Eastbourne). The culture environment was 5% CO₂ in air, 37°C and 99% relative humidity. Motile spermatozoa were separated from semen by centrifugation at 200 g for 20 min at 20°C on a discontinuous Percoll gradient (Pharmacia, Uppsala) composed of 1.5 ml each of 90% and 45% Percoll solutions in Hepes-buffered EBSS solutions. Oocytes were inseminated 41–43 h after hCG injection (day 0). On day 1 (18–20 h after insemination) the cumulus cells surrounding the oocyte were mechanically removed either with finely drawn pipettes or needles. Normal fertilization was indicated by the presence of two pronuclei. Fertilized eggs were transferred to a fresh droplet of modified EBSS, supplemented with 15% (v/v) of HSA under mineral oil.

Embryo evaluation and selection

Assessment of the morphology of embryos was performed on an inverted microscope at ×200 magnification. The development of cleavage stage embryos was assessed by counting the number of blastomeres. The degree of cytoplasmic fragmentation and evenness of blastomere size was also noted. The classification of morulae was based on the morphological transition of the embryo from being a cluster of spherical blastomeres to being a rounded mass in which distinct divisions between the blastomeres were not visible. Embryos were

Fig. 1. Micrograph of fixed and stained expanded human blastocyst A: an interphase nucleus; B: a metaphase spread. Scale bar represents 100 µm.

classified as blastocysts when the blastocoelic cavity was surrounded by a distinct layer of trophoblast cells. Blastocyst expansion was subjectively assessed on the basis of volume increase and the degree of zona thinning.

On day 2 of development, 44–50 h after insemination, a maximum of three of the fastest growing and least fragmented embryos were selected for embryo transfer; the mean number of embryos replaced per patient was 2.6. The surplus embryos of consenting couples were then available for research. The embryos were cultured either in their original droplets of medium or they were transferred to 1 ml of the same medium. They were cultured randomly for different periods. Heavily fragmented or degenerating embryos at each stage of culture were not included in further analysis because preparation and staining were not successful. This study therefore reflects the growth of apparently normal embryos.

Staining and counting of nuclei

At the end of the culture period, embryos were treated with 0.5 µg colcemid ml⁻¹ (Gibco BRL) for 16 h before being fixed and stained. Therefore, nuclei counts for each period represent the number of interphase nuclei plus the number of nuclei that entered metaphase during the 16 h incubation with colcemid.

Embryos were fixed in methanol and acetic acid (3:1, v:v) and stained with Giemsa stain. The zonae were removed before fixing by placing the embryos in 0.3 ml solution of trypsin (BactoTrypsin: Gibco BRL), water and 1% (w/v) sodium citrate (1:1:10) and left for 10 min. An equal volume of fixative (3:1 methanol and acetic acid) was added dropwise and left for 5 min. The embryos were then transferred to 0.3 ml fixative and left for 30 min at room temperature. Finally, the embryos were placed in a 3:3:1 solution of methanol, acetic acid and water for 2–3 min, before being placed on an alcohol-cleaned glass slide. The slides were dried under a flow of warm air and placed in a 10% (w/v) solution of Giemsa stain for 2–3 min.

Nuclei were counted at ×400 magnification. Interphase nuclei stained darkly and had a distinct outline (Fig. 1). There was some variation in the size of interphase nuclei but this was

mainly artefactual and due to orientation of the nucleus or its position within the embryo (i.e., the outermost nuclei in the fixed preparation were often more decondensed). Nuclear fragments were seen in some preparations; these were not counted. Of the embryos that were fixed and stained successfully, 38% could not be analysed because of extensive nuclear degeneration. Metaphase nuclei appeared as discrete clusters of chromosomes (Fig. 1); these were counted as one nucleus.

The mitotic index of embryos was calculated as the ratio of metaphases to the total number of nuclei multiplied by 100.

mitotic index =

$$100 \times (\text{number of metaphases} / \text{total number of nuclei})$$

Statistical analysis

The statistical analysis was carried out using generalized linear models from the GLIM4 statistical analysis package (The GLIM System, Release 4 manual, 1993). This allowed the correct analysis of the mitotic activity, considering a binomial model with successes being the number of nuclei in mitosis out of the total number of cells in the embryo. The analysis of development rates considered normal analysis of variance techniques.

Results

A total of 216 embryos from 50 patients were allocated to this study. Fifty-five embryos were lost during the fixing process. Most of the embryos lost in the fixative were morphologically abnormal and many were undergoing degeneration. Forty-six embryos which were fixed and stained successfully could not be analysed because of nuclear fragmentation. Forty day 6 embryos were not fixed because they were in an advanced state of degeneration and it was expected that they would be lost during processing. Thus, the analysis of nuclear counts and mitotic indices were based on data from 75 embryos donated by 29 couples (range 1–5 embryos per couple). Colcemid (1.25 mol l^{-1}) was added to embryos at the following times after insemination: 48–50 h (day 2; $n = 12$); 72–74 h (day 3; $n = 11$); 96–98 h (day 4; $n = 12$); 108–110 h (day 5; $n = 20$); 132–134 h (day 6; $n = 20$). Embryos were fixed following 16 h exposure to colcemid.

The nuclei counts and mitotic indices of individual embryos for each culture period are illustrated in Fig. 2. The total nuclei count for each embryo represents the number of interphase nuclei plus the number of metaphase nuclei. The patient origin of embryos is also indicated.

At the time of addition of colcemid, all of the day 2 and day 3 embryos were classified as being in the cleavage stages; the number of blastomeres ranged from 2 to 12. The day 4 and day 5 embryos consisted of a mix of cleavage stage morula and blastocyst stage embryos. All of the day 6 embryos were blastocysts; five were unexpanded and 15 were expanded. The nuclei counts of embryos that were identified as morulae ($n = 6$), blastocysts ($n = 12$) and expanded blastocysts ($n = 16$) on days 4, 5 and 6 are shown in Fig. 3.

The percentage of mitotic nuclei on each day was 44% on day 2, 15% on day 3, 18% on day 4, 25% on day 5 and 25.6%

on day 6. From these data, the probability of nuclei being in mitosis at the end of each culture period is illustrated in Fig. 4. The difference in the proportions of actively dividing cells on each day was not significant ($P = 0.53$), although the wide confidence limits indicate that a larger study is required to confirm this apparent effect.

The number of nuclei per embryo was plotted on a $\log_2$ scale against time (Fig. 5). $\log_2$ of the number of nuclei per embryo was taken, as it represents the number of nuclear replications undergone during the culture period. The overall rate of increase between day 2 and day 6 of 75 embryos was linear on a log scale and is expressed by the following equation:

$$\log_2 n = a + bt$$

where n is the number of nuclei, t is the incubation time (days) and a and b are the regression coefficients. A regression gave values $\pm \text{SEM}$ of $a = 0.735 \pm 0.26$ and $b = 0.76 \pm 0.06$. The overall doubling time of this population of embryos was 31.6 ± 2.51 h. This corresponds to a theoretical mean of 50% of all nuclei entering metaphase during the 16 h exposure to colcemid.

Again, taking $\log_2$ of the absolute number of nuclei per embryo as a rate of cell division over the whole culture period, there was wide variation in the numbers of nuclei per embryo within each day. To test the hypothesis that this was due to variation between donating couples, analysis of variance was performed. The analysis showed a significant person effect ($P = 0.021$) after adjustment for day effect.

The relationship between mitotic indices and late preimplantation development was investigated. The mitotic indices after 16 h exposure to colcemid of morulae ($n = 6$), unexpanded blastocysts ($n = 12$) and expanded blastocysts ($n = 16$) were compared. A trend in the mitotic activity was observed, with the morulae tending to have the lowest mitotic indices; this was not statistically significant ($P = 0.26$).

Discussion

This study of nuclei counts and mitotic indices in 75 human embryos after 2–6 days in culture has provided an estimate of the rate of cell proliferation *in vitro* of human embryos throughout the preimplantation period. We conclude that, while the mean doubling time of cells during the first two division cycles is approximately 20 h, the mean duration of the cell cycle of subsequent divisions is 31 h. The rate of nuclear replication is linear on a log scale between day 2 and day 6.

In the human preimplantation embryo, the rate of nuclear replication can only be taken as an estimate of the rate of cell division, since there is a high incidence of multinucleated blastomeres at all stages of development (Lopata *et al.*, 1983; Plachot *et al.*, 1986; Tesarik *et al.*, 1987; Winston *et al.*, 1991). Murray *et al.* (1986) and Winston *et al.* (1991) suggest that the presence of multinucleated cells in blastocysts represents giant cell formation in the trophoblast.

Previous estimates of the mean cell doubling time of *in vitro* cultured human embryos have ranged from 11.9 to 24 h

Fig. 2. Number of interphase (□) and mitotic (▨) nuclei per human embryo on (a) day 2 (44–50 h after insemination); (b) day 3 (68–70 h after insemination); (c) day 4 (96–98 h after insemination); (d) day 5 (120–122 h after insemination) and (e) day 6 (144–146 h after insemination). Embryos from individual couples are denoted by the letters a–k.

(Edwards *et al.*, 1981; Trounson *et al.*, 1982; Cummins *et al.*, 1986; Hardy *et al.*, 1989). In an analysis of the growth rate of 208 embryos from 22 to 65 h after insemination, Cummins *et al.* (1986) found a mean doubling time of 11.9 h. However, it is likely that this value is artificially low because the analysis was based on the assumption that embryos at the morula stage had 64 cells; the evidence from the present study and that of Hardy *et al.* (1989) suggests that embryos containing this number of cells would already have become blastocysts. From a study of nuclei counts of human blastocysts on days 5, 6 and 7, Hardy *et al.* (1989) conclude that the mean doubling time of

human embryos between the one cell and the blastocyst stage is 24 h.

The results of the present study show that the mean number of cell replication cycles completed by day 2 (48–50 h after insemination) was 2.23. It is not possible to determine accurately the mean doubling time for the first two division cycles because the exact time at which fertilization occurred is not known. However, Trounson *et al.* (1981) reported that the first cleavage division is not observed until at least 27 h after insemination. On this basis, the time taken to complete the second cell division cycle in our study is 17–19 h, which is in

Fig. 3. Correlation between the number of nuclei and different stages of development in humans of later preimplantation development on (□) day 4; (○) day 5 and (△) day 6. M: morula ($n = 6$); B: blastocyst ($n = 12$); XB: expanded blastocyst ($n = 16$).

Fig. 5. Total number of nuclei of 75 embryos after 2–6 days of culture *in vitro*.

Fig. 4. Probability of human nuclei being mitotic after exposure for 16 h to colcemid at the end of 2, 3, 4, 5 and 6 days of culture *in vitro*. Vertical bars represent 95% confidence intervals.

close agreement with other reports of the mean doubling times during the early division cycles of 18 h (Trounson *et al.*, 1982) and 15.8 h (Edwards *et al.*, 1981). In the present study, the overall doubling time between day 2 and day 6 was 31.6 h. It would therefore appear that the rate of human embryonic cell proliferation *in vitro* slows down between the second and third replication cycle, with the mean time taken to complete a division cycle increasing by almost 50% after the first two cycles.

The timing of a reduction in the rate of cell proliferation coincides with the reported timing of activation of the human embryonic genome (Braude *et al.*, 1988). Artley *et al.* (1992) were unable to demonstrate a direct causal relationship between cleavage arrest and the onset of gene expression in

human embryos. However, a number of models for the regulation of mitogenic activity during embryogenesis in nonmammalian species have proposed that the transition from regulation of cell function by maternal gene products to embryonic genome expression is related to the observed reduction in the rate of cell proliferation between the early and late division cycles (O'Farrell *et al.*, 1989; Hartwell and Weinert, 1989; Obeyesekere *et al.*, 1994). It is conceivable that, once the embryonic genome assumes control, human embryos require an extracellular stimulus to promote mitosis and that the absence of such a factor in the culture medium may be responsible for the generally low mitotic activity reported here.

With the exception of day 2, the mitotic indices were lower than would be expected from the calculations based on numbers of cells on each day. This may reflect the progressive inability of the culture medium to support development owing to increased metabolic requirements of the embryos or build up of inhibitory factors in the culture environment. Studies on a number of species have shown that embryos cultured *in vitro* grow at a slower rate than do embryos *in vivo* (Steffier *et al.*, 1980; Papaioannou and Ebert, 1988). Such a comparison is not possible in humans because of the paucity of data on development *in vivo*. Steffer *et al.* (1980) suggest that there may be a physiological basis for the observed reduction of mitotic activity *in vitro*, because mouse embryos developed *in vivo* show a reduction in the rate of replication after the third cell cycle; this is attributed to an extended G2 phase.

Despite the fact that degenerating and morphologically abnormal embryos were excluded from the present analysis either because they were lost in the fixative or because the extent of nuclear degeneration did not permit an accurate estimate of the number of nuclei, it is possible that some arrested embryos were included in the analysis and therefore contributed to the overall low rate of mitosis. As the specific stage of the cycle of those cells with interphase nuclei was not determined, we cannot state confidently whether these cells had stopped cycling. Furthermore, the finding that a number of embryos, particularly at the later stages of development, had low total nuclei counts but high mitotic indices cautions against the use of arbitrary definitions of developmental arrest.

Hardy *et al.* (1989) report a high rate of cell death, as evidenced by nuclear degeneration. While a substantial proportion of fixed embryos were excluded from our analysis because of extensive nuclear degeneration, this was not a feature of the embryos that were analysed.

It could be argued that either the concentration of colcemid used (1.25 mol l^{-1}) was not sufficiently potent to arrest the cells in metaphase or a nonspecific toxic effect inhibited progression through the cell cycle to the metaphase stage. Siracusa *et al.* (1980) found that a concentration of 3 mol l^{-1} was effective in inhibiting cleavage of mouse embryos and that this effect was reversible, indicating low toxicity at this concentration. Copp (1978) injected mice with $60 \mu\text{g}$ colcemid and found no difference between the dead cell indices of colcemid-treated and of control embryos.

The wide range in the number of nuclei of blastocysts of equivalent chronological age is in agreement with the findings of Hardy *et al.* (1989), Winston *et al.* (1991) and Dorkas *et al.* (1993). In the present study, we found that there was a degree of overlap in the nuclei counts of cleavage stage, morula and blastocyst stage embryos; for example a day 5 embryo was classified as an uncompact 12–16 nuclei cell but was found to contain 19 nuclei, while a newly formed blastocyst was found to contain only 12 nuclei with no evidence of nuclear degeneration. This suggests that control of compaction and cavitation in human embryos is independent of the number of nuclear replications. The work of Dean and Rossant (1984) shows that inhibition of DNA replication in mouse embryos does not prevent the onset of cavitation. In the light of the proposal by Smith and McLaren (1977) that the nucleocytoplasmic volume ratio may be responsible for the control of blastocoel formation, it is worth noting that the uncompact embryo with 19 nuclei had a larger cytoplasmic volume (approximately 33% more) than did other embryos from the same cohort.

Finally, there is wide variation in the number of cells of embryos of equivalent chronological ages and stages of development; our results demonstrate that this was due in part to a significant difference ($P=0.02$) in the replication rate of embryos from different subjects. It should also be remembered that up to three of the fastest growing embryos from each cohort had been selected for embryo transfer. These points illustrate the difficulties encountered in the study of human embryogenesis. Much larger studies are needed to overcome such difficulties if significant results are to be produced.

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