Behaviour of human embryos *in vitro in the first 14 days: blastocyst transfer and embryonic stem cell production*

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**Introduction**

In women, fertilization occurs in the ampullary–isthmic region of the fallopian tube, and the developing embryo is then transported along the tube into the uterus. The first 4–5 days of early embryonic development, from syngamy to early cavitating blastocyst, occurs in the fallopian tube. The young blastocyst then descends into the uterus, cleaving for approximately another 48 h until it undergoes expansion and early hatching. Finally, the blastocyst implants in the endometrium of the uterus.

The advent of *in vitro* fertilization (IVF) has promoted the study of these early embryonic events *in vitro*. However, in almost all IVF centres worldwide, the replacement of oocytes fertilized *in vitro* into the subfertile patient’s uterus is carried out on day 2 at the 4-cell stage, for fear of degenerative changes occurring in the embryo if it is kept longer *in vitro*. As a result, the rate of successful pregnancy and delivery (‘take-home babies’) has been low, not exceeding 10–15% [1]. Discordance between the stage of embryo replaced and the receptivity of the uterus significantly limits the ‘take-home’ success rate. Physiologically, the embryo enters the uterus on day 4 or 5, at the blastocyst stage. However, the replacement of blastocysts was previously impossible, because only 15–20% of human embryos actually reach the blastocyst stage under conventional culture conditions in IVF laboratories [2]. Conventional culture was therefore suboptimal for later embryonic growth.

To increase blastocyst production rates, tubal conditions may be simulated *in vitro* by growing human embryos on a feeder layer of epithelial cells harvested from the epithelial lining of the fallopian tubes from fertile women undergoing hysterectomy [3]. This co-culture technique improves the quality of 4-cell stage embryos and increases blastocyst production rates to as high as 68% [3–5]. The replacement of such co-cultured embryos in the uterus produces pregnancy in up to 42% of patients aged 35 years or older who had experienced two failures at IVF [6]. Recently, several other cell types have been successfully used in co-culture systems to generate more blastocysts and higher pregnancy rates. These cells include bovine oviductal epithelial cells [7], monkey kidney cells [8], human granulosa cells [9] and human cumulus cells [10]. Embryos grown on such monolayers showed less fragmentation, had expanded blastomeres, and cleaved faster, at rates close to those occurring *in vivo*. Expanded blastocysts of improved viability could be produced on day 5, and hatching commenced on day 6. A grading system for blastocyst quality is now available [11].

It is possible to observe the behaviour of such human embryos in a laboratory dish during co-culture for up to 6 or 7 days. In one study, endometrial epithelial cells were obtained from the same subfertile patient and used as a feeder layer to grow her own embryos, to decrease the risk of transmission of microbes from donor cells [12]. An endometrial biopsy is taken in the cycle just before the IVF treatment cycle, and the epithelial cells from this biopsy are cultured and later frozen in liquid nitrogen. When the patient is ready in the next treatment cycle, her embryos are grown on her frozen–thawed endometrial epithelial monolayers to the blastocyst stage. A pregnancy rate of 45% was reported using this procedure [12].

The exact mechanisms of action of the co-culture system are unknown. A combination of positive and negative conditioning of the culture medium in which the embryos grow may produce the higher rate of pregnancy [13]. Positive conditioning depends on the release by the cells of putative embryotrophic factors which enhance embryo growth. Negative conditioning depends on the removal by the cells of toxic or unwanted metabolites from the medium. The possible embryotrophic factors include growth factors such as insulin-like growth factor-1, platelet-derived growth factor, transforming growth factor, and also glycoproteins. Negative conditioning may consist of alterations in the energy substrates of the medium, mainly glucose, pyruvate and lactate, or stabilization of the oxygen and carbon dioxide tensions in the medium [13].

There have been few prospective randomized controlled studies evaluating the ‘take home baby’ rates after blastocyst transfers in IVF programmes. A recent study in 85 patients reported a significant difference in pregnancy rate between co-cultured blastocyst-stage transfers on day 5 (52.8%) and co-cultured 4-cell stage transfers on day 2 (40.4%). Vero (monkey kidney) cells were used in the co-culture system, and the patients had had several repeated failures at IVF [14]. A major concern in carrying out blastocyst-stage transfers is consistency in producing a blastocyst for every patient going through IVF. Success in this regard is usually difficult to predict, because after day 2 (the 4-cell stage), some embryos may not grow further due to known *in-vitro* embryonic blocks, and others may undergo severe fragmentation due to unknown problems intrinsic to the embryo. An approach our group has adopted, with encouraging results, is a sequential two-stage transfer of the embryo on days 2 and 5. This involves replacing two 4-cell stage embryos in the uterus on day 2, and growing the
remaining embryos in co-culture to blastocyst stage in the hope of replacing one more blastocyst embryo on day 5. This approach needs very careful and gentle transfers by an experienced clinician, who should ideally perform both transfers. Of 20 patients undergoing sequential transfer, 11 became pregnant in our programme.

The concept of co-culture has led to the growth of human embryos beyond day 7. Cell monolayers based on SIM embryo-derived thioguanine- and ouabain-resistant fibroblasts [15], human amniotic cells [16], bovine oviductal epithelial cells [17] and buffalo rat liver cells [18] are used as feeder layers. The feeder cells encourage murine, bovine, ovine and human embryos to hatch completely out of their shells, and to continue their growth as inner cell mass (ICM) and trophectoderm cells in culture, finally producing embryonic stem (ES) cells. Our group recently reported the first isolation and culture of human ES-like cells from 5-day-old embryos [16]. To encourage hatching and continued non-differentiated growth, we renewed, every 48 h, an enriched complex culture medium such as Chang's medium, supplemented with a differentiation inhibitor such as human recombinant leukaemia inhibitory factor (hLIF). After day 7 the medium was changed to Chang's medium containing hLIF, and the dishes left undisturbed for 72 h (to day 10), to allow the hatched ICM and trophectoderm to attach to the feeder monolayer. The monolayer was made inactive and amitotic by irradiation. The ICM formed lumps which piled up and grew vertically, while the trophectoderm formed large, flat surrounding cells which grew horizontally. The ICM lumps were then mechanically separated, disaggregated into clusters of cells using trypsin–EDTA, and recultured on fresh inactive feeder layers bathed in Chang's medium containing hLIF. It is possible to grow ICM cells up to only two cell generations without differentiation (i.e. 14 days). The ICM cells had the typical characteristics of ES-like cells. They were small and round, with high nuclear–cytoplasmic ratios, stained positively for alkaline phosphatase, and maintained a normal diploid karyotype.

The ICM cells of the mammalian blastocyst contain non-committed cells which have the potential to enter a full range of developmental pathways. If, however, ICM cells differentiate they lose the capacity to enter these pathways. Human ES cells developed from the ICM have potential uses in the treatment of neurodegenerative and genetic disorders, and the development of monoclonal antibodies to human proteins for diagnostic and therapeutic use. The cells may also model the events involved in embryogenesis and genomic manipulation. As research using whole human embryos is restricted, such ICM cells may be studied instead.

Ethical issues do not pose a problem so long as ES cells are used for these specified purposes. The utmost caution should be taken to minimize the risks of unintentionally producing clones by transplanting ES cells into enucleated oocytes and then activating them to produce more embryos in vitro. During in vitro culture, the organization of the embryo is lost after hatching has finished, and the ICM cells are merely a cell monolayer with no possibility of becoming a human being unless cloned.

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References