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Blastocyst Culture – Clinical and Future Applications

K. L. Martin

In the last few years, methods for culturing human embryos in vitro have improved. This includes the development of sequential, serum-free culture media, which support the development of approximately 50 % of zygotes to the blastocyst stage in vitro. As a result, the application of blastocyst culture to clinical practice has become more common. Blastocyst culture benefits several areas of medicine including in vitro fertilisation, embryo cryopreservation, preimplantation genetic diagnosis and human embryonic stem cell derivation. However, there remains concern regarding the use of blastocyst culture, in particular, that the culture conditions for human embryo development are still sub-optimal and may negatively affect development. The long-term effects of blastocyst culture and transfer in IVF on offspring are also unknown. Areas of research aimed at improving human blastocyst culture in vitro are briefly discussed.

In den letzten Jahren wurden die Methoden der in vitro-Kultivierung von menschlichen Embryonen verbessert. Dies beinhaltet die Entwicklung sequentieller, serumfreier Kulturmedien, die die in vitro-Entwicklung von ca. 50 % der Zygoten zum Blastozystenstadium unterstützen. Daraus resultiert, daß die Blastozystenkultur in der klinischen Praxis vermehrt angewendet wird. Die Blastozystenkultur unterstützt verschiedene medizinische Anwendungsbereiche, wie die in vitro-Fertilisierung (IVF), die Kryokonservierung von Embryonen, die Präimplantationsdiagnose (PGD) und die Gewinnung embryonaler Stammzellen. Dennoch bestehen Bedenken hinsichtlich der Anwendung der Blastozystenkulturen, insbesondere, da die Kulturbedingungen für menschliche Embryonen noch immer suboptimal sind und negative Auswirkungen auf deren Entwicklung haben können. Ebenso sind die Langzeitauswirkungen von Blastozystenkultur und -transfer bei IVF auf die Kinder unbekannt. Darüber hinaus werden die Forschungsschwerpunkte zur Verbesserung der Blastozystenkultur in vitro andiskutiert. **J Fertil Reprod 2004; 14 (1): 13–18.**

Preimplantation human embryo development begins with fertilisation of the oocyte and culminates in the formation of a differentiated structure, the hatched blastocyst, approximately 5–6 days later (Figure 1). During this time the embryo undergoes various processes including cell proliferation, compaction, cavitation and finally differentiation into two distinct cell populations, (a) an eccentrically placed group of cells, the inner cell mass (ICM), and (b) an outer single layer of epithelial cells, the trophectoderm (TE). Over the past few years there has been considerable interest in improving the culture conditions for growing human embryos to the blastocyst stage *in vitro*. The aim of this article is to (1) briefly review recent developments in blastocyst culture methods, (2) consider some of the clinical benefits of blastocyst culture, (3) discuss some of the disadvantages of this technique, and (4) briefly discuss the future of blastocyst culture.

Blastocyst Culture

Blastocyst development *in vitro* is dependant on the culture conditions used [1]. Sub-optimal culture conditions can compromise many aspects of embryo development, even within a few hours [2]. Therefore, the challenge for many scientists has been to optimise culture conditions so that genetically competent human embryos can realise their developmental potential and develop into viable blastocysts *in vitro*.

As the human preimplantation embryo is dependent on exogenous nutrients and metabolites for development to the blastocyst stage, one of the most crucial factors of culture *in vitro* is the composition of the incubation medium. This is particularly important as evidence shows that the human embryo has both specific and dynamic nutritional requirements which alter during preimplantation development from the zygote to the blastocyst stage [3, 4]. Until relatively recently, the composition of media used in human IVF was based on those designed for culturing adult somatic cells (eg, Hams F-10) and other species of mammalian embryo, particularly the rodent (eg, Tyrodes-6 medium). Although these media can support reasonable numbers of human embryos to the blastocyst stage, the implantation rates following embryo transfer on day 5 post-insemination are low. In the study by Bolton *et al.* [5], 40 % of embryos reached the blastocyst stage after culture in Earle's balanced salt solution plus 10 % maternal serum, but transfer resulted in only a 10 % viable pregnancy rate. This would suggest that such media are sub-optimal and compromise the implantation potential of the embryos.

In recent years, two approaches have been developed with the aim of improving the conditions for blastocyst culture. The first has been to co-culture embryos with autologous or heterologous adult somatic cells, or established cell lines [6, 7]. This has been utilised in human IVF with some degree of success by several units [8, 9]. However, co-culture is time-consuming and technically difficult. The second area that has been developed is the use of defined, serum-free, sequential culture media. One principle in the design of such media has been to reflect the changing nutritional needs of the preimplantation embryo during development [10] and the changing environment of the maternal reproductive tract [11]. Hence, two or more sequential media that differ in their composition are used for culture to the blastocyst stage [12, 13]. On average ~50 % of embryos develop to the blastocyst stage when cultured in the sequential media of which up to 50 % are viable

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[14–16]. However, this strategy of a two-step protocol has recently been challenged following the publication of similar results using a one-step protocol [17, 18]. Whether it is the use of co-culture, or a one or two-step protocol using a defined culture medium, the application of blastocyst culture in medicine is now more readily used.

Advantages of Blastocyst Culture

Blastocyst transfer

A much reported clinical benefit of blastocyst culture since the development of improved culture systems, is its application to human *in vitro* fertilisation (IVF). Several studies suggest that embryo transfer at the blastocyst stage results in higher implantation rates compared to early cleavage stage replacement on day 3 [14, 19, 20]. In a prospective, randomised trial of patients that responded well to ovulation induction, the implantation rate following blastocyst transfer was significantly higher (50.5 %) than embryo replacement on day 3 (30.1 %), although it should be noted that different culture systems were used for the two treatment groups [15].

Two explanations may account for the reported increase in implantation rates associated with blastocyst transfer. Firstly, transfer on day 5 is more physiological, as it synchronises embryo stage with the female reproductive tract and minimises embryo exposure to the hyperstimulated maternal environment [12]. Secondly, extending the culture period to day 5 or 6 post-insemination, after activation of the embryonic genome (at the 4–8-cell stage) [21], helps to select embryos with a greater chance of implanting.

The increase in implantation rates associated with blastocyst transfer is reported to benefit IVF in two ways. Firstly, higher implantation rates help to improve the low pregnancy rates of IVF [14, 20, 22]. In a retrospective analysis, Marek *et al*. [19] reported a significant increase in the pregnancy rate with day 5 (61.6 %) compared to day 3 transfers (47.5 %), even though fewer embryos were replaced at the blastocyst stage (mean = 2.5) in contrast to early cleavage stage transfer (mean = 3.0). Secondly, higher implantation rates associated with blastocyst transfer mean that fewer embryos need to be replaced to achieve a pregnancy, which in turn helps to reduce the high incidence of multiple gestations associated with IVF [16]. In the future prolonging the period of embryo culture to day 5 post-insemination will also benefit IVF by increasing the window of opportunity of finding quantitative criteria for selecting viable embryos for transfer, making single blastocyst transfer more of an achievable goal.

Whilst blastocyst culture and transfer in human IVF appears promising and is increasingly employed by IVF units, the efficacy of this technique awaits further critical evaluation in a well-designed, prospective, randomised trial [23]. All of the randomised studies to date that have compared the success rates of day 5 versus day 3 embryo transfer have either used different culture conditions for the two treatment groups [15, 24] or have analysed the data retrospectively [19, 25]. Moreover, meta-analysis of ten trials comparing day 2/3 and day 5 embryo transfer concluded, that there is little difference in the major outcome parameters and that routine practice of blastocyst culture should be offered with caution to patients [26]. Finally, and perhaps of greater concern, is that the effects of blastocyst culture and transfer on the long-term health of resulting offspring are yet unknown (see 'Disadvantages of blastocyst culture').

Blastocyst cryopreservation

Blastocyst cryopreservation offers several advantages over freezing at the early cleavage stage. Based on the assumption that blastocyst culture helps to select embryos with a greater chance of implanting, fewer but more viable embryos are cryopreserved on day 5 [27]. Consequently, blastocyst cryopreservation is more efficient and as the embryos have a higher developmental potential, the implantation and pregnancy rates following frozen embryo replacement (FER) of blastocysts should be higher than those with early cleavage FER. However, the efficiency and efficacy of blastocyst cryopreservation also awaits further critical evaluation [26]. Moreover, further technical developments are required to improve blastocyst cryopreservation methods. Vitrification, using cryoloops, in high concentrations of cryoprotectants and rapid cooling to –196°C in liquid nitrogen, appear to offer some advantages over conventional slow-freezing techniques [28, 29].

Preimplantation genetic diagnosis

Blastocyst culture also facilitates preimplantation genetic diagnosis (PGD). Following cleavage stage biopsy on day 2–3 post-insemination, continued culture up to day 5/6 increases the time available for genetic analysis and allows further assessment of the embryo [30]. In addition, culture to the blastocyst stage permits biopsy of the mural trophectoderm for PGD [31]. TE biopsy has three main advantages over removal of 1 or 2 cells at the 8-cell stage. Firstly, only non-embryonic tissue is removed (the placenta and other extra-embryonic tissues are derived from the TE, whilst the embryo proper is derived only from the ICM). Secondly, several cells can be removed with TE biopsy compared to a maximum of only two at the 8-cell stage, thus improving the accuracy of the diagnosis [32]. Finally, the incidence of mosaicism is reduced in the blastocyst (~10.5 %) compared to early cleavage embryos (43 %) [33]. Muggleton-Harris *et al*. [34] have also suggested that dual-stage biopsy is possible with blastocyst culture, ie, at the ~8-cell and then at the blastocyst stage. With this approach PGD at the blastocyst stage may be conducted to confirm the results from biopsy at the early cleavage stage or to carry out additional tests. Whilst TE biopsy is perhaps technically more difficult than at earlier stages, the use of laser technology may enhance this methodology in the future [35].

Human embryonic stem cells

Human embryonic stem cells (hES) are pluripotent cells derived from the ICM of blastocysts [36]. *In vitro* these cells can be induced to differentiate into many cell types of the body, including heart, muscle and nerve cells. The potential benefits of hES in therapeutic medicine and research are enormous [37]. For example, the ability to direct the differentiation of hES into specific cell lineages followed by their transplantation into patients could revolutionise the treatment of debilitating degenerative diseases, including Parkinson's, diabetes and Alzheimer's. To date, however, few hES cell lines have been derived. The efficient procurement of human blastocysts with a viable ICM, from embryos donated with informed patient consent research (as permitted by UK law), is fundamental if the potential of hES in medicine is to be realised.

Disadvantages of Blastocyst Culture

Although there are a number of advantages of blastocyst culture, there are also justified concerns regarding its application in clinical treatment.

Poor rate of blastocyst development in vitro

The first disadvantage of blastocyst culture is the high rate of embryo loss *in vitro* (Figure 2). Only ~50 % of embryos develop into blastocysts, even with the improved culture systems [1]. Also, due to the considerable heterogeneity between patients, the number of blastocysts obtained varies dramatically. As such, some IVF patients have no blastocysts for transfer on day 5 whilst others have 100 % blastocyst formation [15]. Consequently, there is an increased risk of cancellation of the cycle at the embryo transfer stage.

Long-term effects of blastocyst culture

Despite recent improvements, human embryo culture conditions are still sub-optimal for blastocyst formation, having been developed from those for culturing adult somatic cells and other mammalian embryos [1]. Embryo exposure to sub-optimal culture environments, particularly with extended culture to the blastocyst stage, can compromise many aspects of development including metabolism [10], differentiation [38], gene expression [39], imprinting [40] and subsequent fetal development after embryo transfer [41] in several mammalian species. Consequently, concerns have been expressed regarding the use of human blastocysts cultured in sub-optimal media for IVF treatment [42] and more recently for the derivation and therapeutic use of hES [43]. Whilst apparently healthy children have been born following blastocyst culture and transfer [44], this does not preclude any negative effects on the long-term health of these children [42]. This highlights the need for continued rigorous research into the optimisation of human embryo culture conditions (using donated human embryos) and the close observation and follow-up of all children conceived after blastocyst culture and IVF.

Monozygotic twinning

Several studies have reported an increase in the incidence of monozygotic twinning with blastocyst transfer compared to early cleavage stage transfer [45, 46]. For example, the study by Da Costa *et al.* [46], reported an increase in monozygotic twinning of from 3.9 % (day 2/3 ET) to 10.5 % (day 5 ET). Whilst this may not be considered a disadvantage of blastocyst culture, it is important that this phenomenon is taken into account when deciding on the number of blastocysts to transfer and patients should be appropriately counselled.

Perturbed sex ratio

Reports in the literature have also suggested that there is a bias towards the birth of male offspring following blastocyst culture and transfer in IVF compared to early cleavage stage replacement [44, 47]. Menezo *et al.* [44] reported that more male infants were born than females following selection of embryos on day 5 (sex ratio: 1.3). However, Kauche *et al.* [47] found that whilst there was a trend towards more males with blastocyst transfer this was not significant when embryos were selected on day 6 (sex ratio: 1.29 day 5 ET vs. 1.00 day 2/3 ET).

Blastocyst culture – is it the future?

Despite the advantages of blastocyst culture there are still concerns about both the usefulness and the safety of this technique (Table 1). It is clear, that further research into the optimisation of culture conditions is required to overcome concerns about the potential negative effects of blastocyst culture on the long-term health of any resulting offspring.

Table 1: Comparison of the advantages and disadvantages of embryo transfer at the blastocyst stage (day 5/6) versus early cleavage stage (day 2/3).

	Day 2/3 embryo transfer (Early cleavage)	**Day 5/6 embryo transfer (Blastocyst)**
Advantages	~ 80 % reach 2–8-cell stage Minimum time exposed to sub-optimal culture conditions	Higher implantation rates (> 50 %) Blastocyst cryopreservation Preimplantation Genetic Diagnosis Improves embryo selection Embryo & uterine synchrony
Disadvantages	Only ~ 20 % implantation rate No synchrony between embryo & uterus	< 50 % reach blastocyst stage Concerns regarding long-term exposure to suboptimal culture conditions Increased incidence of monozygotic twinning Perturbed sex ratio

Whilst significant improvements in culture conditions have been seen over the last five years, the rate of embryo development *in vitro* is ~12–14 h slower than that *in vivo* [48, 49]. Furthermore, the culture media used today in IVF clinics have been developed from studies using other mammalian species. Since there are significant differences in the metabolism of human and other mammalian embryos, further research into the nutritional requirements of human embryos is required to optimise media specifically for use in IVF, including studies of the maternal environment. Furthermore, the composition of human embryo culture media are still relatively simple compared to the luminal secretions of the reproductive tract. For example, the human embryo is exposed to a variety of growth factors and cytokines *in vivo* [50]. Whilst these are not routinely added to embryo culture media, *in vitro* studies suggest that growth factors have many pleiotropic effects on embryo development. This includes blastocyst formation and hatching (Figure 3) [51], rate of cleavage [52], cell proliferation [53] and apoptosis [54]. The inclusion of a physiological mixture of growth factors may therefore be one way of optimising human embryo culture conditions in the future [55].

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