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Evolution of a culture protocol for successful blastocyst development and pregnancy

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A cell-free culture system was designed for human embryo development to the blastocyst stage by testing a range of culture conditions in a series of protocols. The culture system that was evolved has a brief 1 h exposure to spermatozoa and then culture of the pronucleate zygote for 2 days in IVF-50 medium. Two or three embryos were cultured together in 20 μl microdrops of medium under oil. Embryos were then regrouped and two or three at a similar stage were cultured together in 50 μl microdrops of Gardner's G2 medium under oil from days 3 to 5. Embryos were transferred to fresh G2 medium on day 5 and cultured for a further 1 or 2 days (day 6 or 7). No serum was used in any of the cultures. The embryo transfer medium and G2 medium were supplemented with human serum albumin. The zonae of all blastocysts to be transferred to patients were completely removed enzymatically. Using this protocol, 52% of zygotes developed to blastocysts and 34 out of 35 patients treated received 82 blastocysts and 11 morulae on day 5 or 6. Twenty-one fetal sacs with positive heartbeats (23% implantation rate) were detected in 13 ongoing pregnancies (38% pregnancy rate/transfer or 37%/patient treated). We anticipate that further improvements in embryo development and the selection of viable embryos can be achieved using this simple and effective culture system.

Key words: blastocyst culture/cell-free culture/serum-free culture

Introduction

In recent years there have been several major advances in assisted reproduction technology. The use of gonadotrophin-releasing hormone (GnRH) agonists allows for greater control and manipulation of ovarian stimulation resulting in higher numbers of oocytes at retrieval. The introduction of intracytoplasmic sperm injection (ICSI) has overcome the last barrier to achieving high numbers of zygotes for patients who previously would have had a poor fertilization outcome. Despite the introduction of these technologies to produce cleavage stage embryos for transfer, pregnancy and implantation rates have remained lower than that routinely achieved for animal embryo production (Gardner, 1994; Gardner *et al*., 1994; Bavister, 1995). Many reasons have been postulated to explain the high rate of embryo wastage, including an increased frequency of genetic abnormalities (Plachot *et al.*, 1987; Bongso *et al.*, 1991; Pellestor *et al*., 1994; Menezo and Ben Khalifa, 1995), inadequacies of present culture systems (Gardner and Lane, 1993a; Bavister, 1995) and an inappropriate synchronization of embryo cell stage and endometrial lining of the uterus as embryos are transferred at least 2 days before they would normally enter the uterus. Whatever the reason, the major difficulty facing the transfer of early cleavage stage embryos is the problem of selecting viable embryos with only gross subjective measurements of morphology available. Extending embryo culture periods beyond 2–3 days *in vitro* would allow for further opportunities for selection because of the elimination of those embryos incapable of converting their development from stored maternal message to an activated embryonic genome (Braude *et al*., 1988).

Early attempts to extend the culture period to the blastocyst stage in the human resulted in limited success (Bolton *et al*., 1991). More recently, the employment of co-culture techniques using various somatic helper cells has been suggested to improve the development of embryos to the blastocyst stage and implantation after uterine transfer (Ménézo *et al*., 1992; Olivennes *et al*., 1994; Schillaci *et al*., 1994). The beneficial effects of using somatic helper cells have been claimed to be both biophysical and biochemical in nature (Bongso *et al*., 1989) and are not specific to cells originating from the human reproductive tract (Wiemer *et al*., 1989; Ménézo *et al*., 1990). However, the claim that the development of human embryos in co-culture is superior to that achieved using conventional culture conditions has been questioned (Bavister, 1992; Van Blerkom, 1993; Sakkas *et al*., 1994). A recent report would seem to confirm that it is technically possible to grow viable human blastocysts in culture medium alone (Scholtes and Zeilmaker, 1996). Over the past few years there has been a resurgence of interest in the culture of the mammalian preimplantation embryo, culminating in the development of several new media. Conventional embryo culture media, such as Earle's (Bolton *et al*., 1989) and human tubal fluid (Quinn *et al*., 1985), do not support high levels of embryo development to the blastocyst stage. Such media lack important regulators of early embryo development such as amino acids (Bavister and McKiernan, 1993; Gardner and Lane, 1993a,b, 1996; Lane and Gardner, 1994, 1997a,b). Furthermore, conventional embryo culture media fail to take into account the changing physiology and requirements of the embryo, which reflect changes in the environment of the female reproductive tract

(Gardner *et al*., 1996). To this end, two sequential media were formulated (Gardner's G1 and G2), the composition of which reflects changes in both carbohydrate and amino acid requirements during embryo development from the zygote to the blastocyst stage (Barnes *et al*., 1995).

The aim of this study was to design and optimize a culture protocol for the development of viable human blastocysts without the need for somatic support cells.

Materials and methods

A total of 53 patients who produced more than eight oocytes at retrieval and who subsequently had more than five fertilized oocytes were included in this study. All patients were aged <40 years and had undergone between zero and 10 previous in-vitro fertilization (IVF) cycles. All patients received human menopausal gonadotrophin (HMG) stimulation (Metrodin HP; Serono, Frenchs Forest, Australia) in doses ranging from 150 to 600 IU/day for up to 14 days. The GnRH agonists nafarelin acetate (Synarel; Searle, Delpharn, France) or leuprorelin acetate (Lucrin; Abbott, Kurnell, Australia) were administered in either the long (down-regulation) or short (flare) protocol. Follicular growth was monitored using ultrasonography and measurement of oestradiol concentrations. Human chorionic gonadotrophin (HCG) (Profasi; Serono) was administered at a 5000 IU dose when there were more than three follicles measuring ⩾16 mm in diameter and the oestradiol concentration was ⩾2000 pmol/l. Oocyte retrieval was scheduled 36 h after HCG injection. Oocytes were recovered transvaginally with ultrasound guidance. The luteal phase was supported by one of three regimens: 16 days of progesterone beginning on the day of oocyte retrieval, administered vaginally at a dose of 400 mg/day or vaginal progesterone at the same dose from the day of oocyte retrieval to day 4 in combination with 1000 IU HCG on days 4, 7, 10 and 13 after oocyte retrieval or only HCG at a dose of 2000 IU on days 3, 6 and 9 after oocyte retrieval.

Oocytes were identified in the laboratory and briefly rinsed free of follicular fluid and blood in handling medium (Gamete-100; Scandinavian IVF Science AB, Gothenburg, Sweden) before being placed in 1 ml pre-equilibrated culture medium (IVF-50; Scandinavian IVF Science AB), one to two oocytes per culture tube (Falcon #2003; Becton-Dickinson Labware, Franklin Lakes, NJ, USA).

Spermatozoa were prepared using a discontinuous Percoll gradient (Pharmacia LKB, Uppsala, Sweden). In all, 30 cycles had oocytes inseminated with 50 000 normal, motile spermatozoa/ml, 4–6 h after collection. Oocytes were exposed to spermatozoa for 1 h, then rinsed briefly in handling medium, before being placed in fresh pre-equilibrated culture medium. A total of 23 cycles had oocytes injected with a single spermatozoon because male factor was the underlying cause of infertility. Oocytes for injection were denuded of cumulus cells following brief exposure to hyaluronidase (HYASE-10X; Scandinavian IVF Science AB) and then assessed for maturity. Metaphase II oocytes were injected using the method described by Barnes *et al.* (1995). All culture was performed in individual sealed chambers at 37°C and a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2. At 16–20 h post-insemination, oocytes were assessed for fertilization. Those oocytes exhibiting two pronuclei and two polar bodies were placed in groups of two or three in 10 or 20 μl microdrops of pre-equilibrated Gardner's G1 medium (Barnes *et al*., 1995) supplemented with 2 mg/ml bovine serum albumin (BSA; Albumax; Life Technologies, Gibco BRL, Grand Island, NY, USA) or 2 mg/ml human serum albumin (HSA; Irvine Scientific, Irvine, CA, USA) under mineral oil (Sigma Chemical Co., St Louis, MO, USA) pre-washed with Gardner's G1 medium, or 20 μl microdrops of pre-equilibrated IVF-50 culture medium under OVOIL-150 (Scandinavian IVF Science AB) in Falcon #3652 culture dishes. The quality of all in-house prepared media and consumables associated with embryo culture was ascertained using a 1-cell mouse embryo bioassay (Gardner and Lane, 1993a). The choice of microdrops under oil, as opposed to open dishes, was based on prior extensive research (Lane and Gardner, 1992; Gardner *et al.*, 1994), as was the culture of embryos in groups in order to maximize any autocrine effects.

Table I. Development to the blastocyst stage following standard insemination by brief exposure to spermatozoa or intracytoplasmic sperm injection (ICSI)

	Standard insemination	ICSI
No. of cycles	30	23
No. of embryo transfers (%)	29 (97)	22 (96)
No. of oocytes fertilized – two-pronucleate zygotes (% oocytes)	323 (59)	271 (72)
No. of zygotes developing to blastocysts by day 7 (% zygotes)	153 (47)[a]	118 (44)[b]

[a]Does not include 15 morulae transferred on day5 or 6.
[b]Does not include nine morulae transferred on day 5 or 6.

At 65–76 h post-insemination embryos were assessed for cell number and degree of fragmentation using Nomarski differential interference contrast optics and a magnification of ×200. Embryos were then transferred to 10 μl microdrops of pre-equilibrated Gardner's G2 medium (Barnes *et al*., 1995) supplemented with 2 mg/ml BSA or 2 mg/ml HSA, or 50 μl microdrops of pre-equilibrated Gardner's G2 medium supplemented with 2 mg/ml HSA. Microdrops of culture medium were set up in Falcon #3652 culture dishes under mineral oil pre-washed with Gardner's G2 medium and equilibrated for at least 4 h before required. Embryos were transferred in their original groupings or into new groups defined by similarity in cell number.

Embryos were assessed for blastocyst development early on the morning of day 5 post-insemination (day 0 = day of insemination, 110–117 h post-insemination). Embryos requiring continued culture were either left in the same culture medium or transferred to fresh pre-equilibrated 50 μl microdrops of Gardner's G2 medium supplemented with 2 mg/ml HSA under mineral oil pre-washed with Gardner's G2 medium.

Morula or blastocyst stage embryos were selected for transfer, the number for transfer being determined by the availability of embryos for transfer and the patient's age and previous clinical history. If the patient was aged >35 years or had failed to achieve a pregnancy after three or more previous IVF cycles then consideration was given to transferring three or four blastocysts, if available, rather than only two blastocysts, which would be the usual recommendation to patients of a younger age or with a limited IVF history. One to four blastocysts were transferred to each patient. All embryo transfers were performed initially on day 5 regardless of morphology. In the latter part of the study, embryos were transferred on either day 5 or 6, the day of transfer being determined by the degree of expansion of the blastocysts. Embryos were transferred either zona intact or zona free after a brief exposure to 0.2% pronase (Sigma Chemical Co.) in HEPES-buffered Gardner's G2 medium. Embryos were transferred in pre-equilibrated Gardner's G2 medium supplemented with 50% patient serum or 8 mg/ml HSA, using a Cook Pivet Laboratory Embryo Transfer Set (#K-PETS-2031-Monash; Cook Australia, Eight Mile Plains, Australia).

Blastocysts in excess of those required for transfer were cryopreserved using a slow cooling protocol with glycerol as the cryoprotectant. Blastocysts were rinsed briefly in Dulbecco's phosphate-buffered

Table II. Summary of culture protocols used to optimize the development of blastocysts and increase the pregnancy rate following transfer

	Protocol							
	1	2	3	4	5	6	7	8
Days 1–3 Embryo culture	10 µl G1 +BSA	10 µl G1 +BSA	10 µl G1 +BSA	10 µl G1 +BSA	10 µl G1 +BSA	10 µl G1 +HSA	20 µl G1 +HSA	20 µl SIS IVF-50
Days 3–5 Embryo culture	10 µl G2 +BSA	10 µl G2 +BSA	10 µl G2 +BSA	10 µl G2 +BSA	10 µl G2 +BSA	10 µl G2 +HSA	50 µl G2 +HSA	50 µl G2 +HSA
Days 5–7 Embryo culture	[a]	[a]	[a]	[a]	[a]	[a]	[a]	50 µl G2 +HSA
Embryos grouped days 1–3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Embryos regrouped days 3–7	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Transfer medium + 50% patient serum	Yes	Yes	No	No	No	No	No	No
Transfer medium + 8 mg/ml HSA	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Transfer zona intact	Yes	Yes	Yes	No	No	No	No	No
Transfer zona free	No	No	No	Yes	Yes	Yes	Yes	Yes
Transfer day 5 only	Yes	Yes	Yes	Yes	No	No	No	No
Transfer day 5/day 6 dictated by morphology	No	No	No	No	Yes	Yes	Yes	Yes

G1, G2 = Gardner's G1 and G2 medium; SIS = Scandinavian IVF Science IVF-50 medium; BSA = bovine serum albumin; HSA = human serum albumin.
[a]Embryos remained in the same culture medium from days 3 to 7.

saline (DPBS; Gibco BRL) supplemented with 2 mg/ml HSA. Blastocysts were then transferred to 10% glycerol (Sigma Chemical Co.) in DPBS supplemented with 2 mg/ml HSA for 15–20 min at room temperature. Blastocysts were loaded into freezing straws (#AA201; IMV, Cassou, France), heat sealed and placed in a cryological freezer (Model CL2000; Cryologic Pty Ltd, Mt Waverley, Australia) at –6.0°C. Straws were manually seeded after 2 min and held at –6.0°C for a further 8 min, then slowly cooled at a rate of –0.5°C/min to –32°C, before rapid cooling to –196°C and storage in liquid nitrogen.

Results

A total of 53 patients were recruited for this study, with 51 patients (96%) having an embryo transfer of morula or blastocyst stage embryos on day 5 or 6 post-insemination. Two patients failed to develop morulae or blastocysts by day 7 and did not have an embryo transfer. In all, 34 patients (64%) had blastocysts in excess of those required for transfer, which were cryopreserved. Oocytes were inseminated using the short sperm exposure protocol in 30 cycles, and in 23 cycles the oocytes were injected using ICSI. There was no significant difference in the numbers of embryos developing to the blastocyst stage with respect to the insemination technique (Table I).

Embryos from the 53 patients recruited for this study were cultured using eight different culture protocols. The culture protocol was adjusted continually in an attempt to optimize the numbers of embryos developing to blastocysts and to increase the pregnancy rate following transfer of these blastocysts. A summary of the eight different culture protocols is shown in Table II.

The first six treatment cycles were cultured using the same culture protocol (Protocol 1). Embryos were cultured from days 1 to 3 post-insemination in 10 µl microdrops of Gardner's G1 medium supplemented with 2 mg/ml BSA and then from day 3 post-insemination onwards in 10 µl microdrops of Gardner's G2 medium supplemented with 2 mg/ml BSA. Embryos were kept in their original groupings when transferred to fresh medium. Blastocysts were selected for transfer on day 5 and transferred, zona intact, in Gardner's G2 medium supplemented with 50% patient serum. When the patient did not have sufficient numbers of blastocysts available on day 5 for transfer, the numbers were made up with morulae. Figure 1 provides an illustration of the embryo developmental stages selected for transfer on day 5 or 6. There did not appear to be any difference in the morphology of the blastocysts across protocols, with the exception that more advanced blastocysts were transferred for protocols 5 and onwards as indicated. A total of 12 blastocysts developed by day 5 (22.6% of fertilized oocytes, mean of 2.0/patient). These blastocysts were transferred to the patients along with five morulae (mean of 2.8 embryos/patient). No pregnancies resulted. An additional four blastocysts developed by day 6 (7.5% of fertilized oocytes or 11.1% of remaining embryos), which were cryopreserved. No further development to blastocysts occurred in the remaining embryos by day 7.

It was decided that for subsequent patients embryos should be regrouped on day 3 post-insemination when transferred to Gardner's G2 medium according to the similarity in cell number. It was proposed that embryos with a low cell number on day 3 (fewer than eight cells, 65 h post-insemination) may have a negative effect on development when cultured with embryos with a high cell number. One patient was treated with the previously described culture conditions with the exception that the embryos were regrouped on day 3 before being transferred to Gardner's G2 medium (Protocol 2). Three blastocysts developed by day 5 post-insemination (25% of fertilized oocytes) and were transferred to the patient. This patient became pregnant with a continuing singleton pregnancy.

A commercial source of HSA was made available to replace human serum at this time. It was decided that embryos should be transferred back to the patient in medium supplemented with 8 mg/ml HSA instead of 50% patient serum because exposure to serum may have a detrimental effect on developmental competence. Two patients had embryos cultured from day 1 to 3 in 10 µl microdrops of Gardner's G1 medium supplemented with 2 mg/ml BSA and, after regrouping, from day 3 onwards in 10 µl microdrops of Gardner's G2 medium supplemented with 2 mg/ml BSA. One blastocyst developed

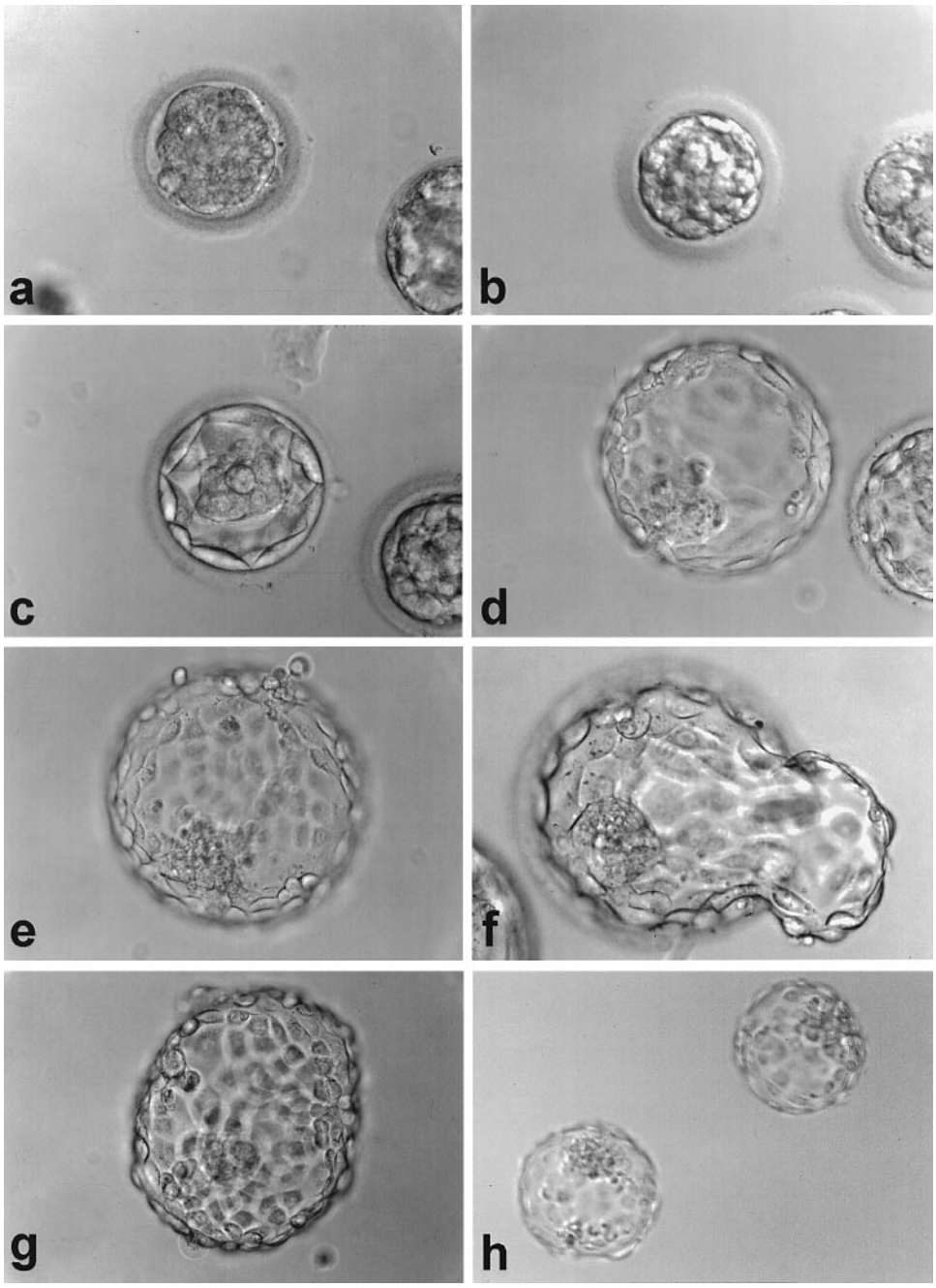

Figure 1. Examples of the embryo developmental stages selected for transfer on day 5 or 6. (**a**) Morula. (**b**) Cavitating morula. (**c**) Early blastocyst showing a clearly defined blastocoele cavity lined by trophectoderm cells and a prominent inner cell mass. (**d**) Expanded blastocyst showing a dilated blastocoele cavity lined by flattened trophectoderm cells and a prominent inner cell mass surrounded by a thinned zona pellucida. (**e**) Hatching blastocyst showing the first few trophectoderm cells emerging from the zona pellucida. (**f**) Hatching blastocyst showing half the trophectoderm cells emerging from the zona pellucida. (**g**) Hatched blastocyst which is completely free of the zona pellucida (not shown). (**a**)–(**g**) Original magnification ×300. (**h**) Two fully expanded blastocysts that were treated with a 0.2% pronase solution to remove the zona pellucida prior to embryo transfer (original magnification ×150).

by day 5 post-insemination (5.0% of fertilized oocytes, mean of 0.5/patient), and this was transferred, along with five morulae (mean of 3.0 embryos/patient), zona intact in Gardner's G2 medium supplemented with 8 mg/ml HSA (Protocol 3). One ectopic pregnancy resulted. A further blastocyst developed by day 6 post-insemination (5.0% of fertilized oocytes or 7.1% of remaining embryos) and was cryopreserved.

From the nine transfers to this date, only two pregnancies had resulted from the transfer of 16 blastocysts and 10 morulae (mean of 2.9 embryos/patient), yielding an implantation rate of 7.7%. It was decided that subsequently all embryos for transfer would be transferred zona-free after exposure to 0.2% pronase to eliminate the possibility that embryos were failing to hatch and implant because of hardening of the zona pellucida from prolonged culture (Figure 1h). One patient's embryos were cultured according to Protocol 3, with the exception that embryos were transferred zona free (Protocol 4). Three blastocysts developed by day 5 post-insemination (30% of

fertilized oocytes), which were transferred on day 5, zona-free. No pregnancy resulted. One additional blastocyst developed by day 6 post-insemination (10% of fertilized oocytes or 14.3% of remaining embryos), which was cryopreserved.

Many of the blastocysts transferred in the previous protocols had only just developed a blastocoele cavity and showed very little sign of expansion (Figure 1c) by day 5 post-insemination. To introduce yet a further element of selection for the most viable blastocysts it was decided that subsequently the day of transfer would be determined by the morphology of the blastocyst, i.e. if the patient did not have blastocysts that were either fully expanded or hatching (Figure 1d–g) on day 5 post-insemination, the embryos were cultured for a further day and transfer of the most advanced blastocysts with the bulkiest inner cell mass (ICM) proceeded on day 6 post-insemination. Six patients' embryos were cultured from day 1 to 3 in Gardner's G1 medium supplemented with 2 mg/ml BSA, regrouped according to cell number and cultured from day 3 onwards in Gardner's G2 medium supplemented with 2 mg/ml BSA. Blastocysts were transferred zona-free on either day 5 or 6 post-insemination in Gardner's G2 medium supplemented with 8 mg/ml HSA (Protocol 5). In all, 11 blastocysts developed by day 5 post-insemination (17.7% of fertilized oocytes). One patient had two fully expanded blastocysts and a morula transferred zona-free on day 5 post-insemination. One patient failed to develop blastocysts by day 5 and continued culture did not yield any further development; therefore the embryo transfer was cancelled. Continued culture of the remaining embryos from the patient having a day 5 transfer and the embryos of the remaining four patients yielded an additional 10 blastocysts (16.1% of fertilized oocytes or 0.2% of remaining embryos) on day 6 post-insemination. Four patients had 10 blastocysts and one morula transferred zona-free on day 6 post-insemination. In total, 12 blastocysts and two morulae were transferred to five patients (mean of 2.8 embryos/patient) on either day 5 or 6 post-insemination. One additional blastocyst developed by day 7 post-insemination (1.6% of fertilized oocytes or 2.6% of remaining embryos). Blastocysts in excess of those required for transfer were cryopreserved on the day of embryo transfer or on the day of subsequent development. A continuing singleton pregnancy resulted from a transfer on day 5 post-insemination and a singleton pregnancy which aborted at 7 weeks resulted from one of the transfers on day 6 post-insemination. The implantation rate for Protocol 5 was 14.3% compared with an implantation rate of 6.9% for Protocols 1–4.

One patient was treated using Protocol 6. Embryos were cultured from days 1 to 3 in Gardner's G1 medium supplemented with 2 mg/ml HSA, regrouped according to cell number and cultured from day 3 onwards in Gardner's G2 medium supplemented with 2 mg/ml HSA. Blastocysts were transferred zona-free in Gardner's G2 medium supplemented with 8 mg/ml HSA on either day 5 or 6 post-insemination depending on morphology. No blastocysts developed by day 5 post-insemination, and continued culture resulted in the development of two blastocysts by day 6 post-insemination (28.6% of fertilized oocytes). Two blastocysts and one morula were transferred zona free on day 6 post-insemination; no pregnancy resulted.

Protocol 7 was the same as Protocol 6 with the exception that embryos were cultured from days 1 to 3 in 20 μl microdrops of Gardner's G1 medium supplemented with 2 mg/ml HSA and from day 3 onwards in 50 μl microdrops of Gardner's G2 medium supplemented with 2 mg/ml HSA. It was felt that the larger drop sizes would more adequately provide for the metabolic needs of the embryos in group culture. One patient was treated using Protocol 7. Two blastocysts developed by day 5 post-insemination (33.3% of fertilized oocytes), with no further development of the remaining embryos on day 6 or 7 post-insemination. Two expanded blastocysts were transferred zona free on day 5 and no pregnancy resulted.

The development of embryos by day 3 post-insemination was thought to be retarded because only 38 (22%) of a possible 170 normal embryos in the preceding seven protocols had developed to the 8-cell stage or beyond by 65 h post-insemination. As oocytes were originally cultured and inseminated in Scandinavian IVF Science AB IVF-50 culture medium, it was decided to incorporate a further protocol change to Protocol 7 whereby embryos would be cultured from days 1 to 3 post-insemination in 20 μl microdrops of IVF-50 culture medium under OVOIL-150. It was also felt that Gardner's G2 medium should be replaced with fresh medium every 48 h to eliminate possible toxic effects of ammonium build up in this complex medium. Embryos were therefore regrouped according to cell number on day 3 post-insemination and cultured from days 3 to 5 in 50 μl microdrops of Gardner's G2 medium supplemented with 2 mg/ml HSA and then transferred to fresh 50 μl microdrops of Gardner's G2 medium supplemented with 2 mg/ml HSA for continued culture (Protocol 8). In all, 35 patients had their embryos cultured using Protocol 8. Of these embryos, 44% developed to the ⩾8-cell stage by 65 h post-insemination compared with 22% in the previous seven protocols. By day 5 post-insemination, 64 blastocysts had developed (15.1% of fertilized oocytes); 132 additional blastocysts developed by day 6 post-insemination (31.1% of fertilized oocytes or 36.8% of remaining embryos) and a further 24 blastocysts by day 7 post-insemination (5.7% of fertilized oocytes or 11.1% of remaining embryos). One patient failed to develop a blastocyst by day 7 post-insemination and the embryo transfer was cancelled. In all, 18 blastocysts and one morula were transferred zona free on day 5 post-insemination to six patients (mean of 3.2 embryos/patient), and 64 blastocysts and 10 morulae were transferred on day 6 post-insemination to 28 patients (mean of 2.6 embryos/patient). Three pregnancies resulted from the transfer of embryos on day 5: two continuing singleton pregnancies and a quadruplet pregnancy. A total of 12 pregnancies resulted from the transfer of embryos on day 6: two blighted ova, six continuing singleton pregnancies, three twin pregnancies and one triplet pregnancy. The overall implantation rate (fetal heart/embryo transferred) for Protocol 8 was 22.6%. Embryo development in the eight different culture protocols is summarized in Table III, and pregnancy outcome is summarized in Table IV.

The continuing pregnancy rate of 38% with blastocyst stage

Table III. Summary of results of embryo culture using Protocols 1–8

	No. of cycles	No. of fertilized oocytes (2PN)	No. embryos with more than eight cells Day 3 (% 2PN)	No. of blastocysts (% 2PN)			
				Day 5	Day 6	Day 7	Total
Protocol 1	6	53	7 (13.2)	12 (22.6)	4 (7.5)	0	16 (30.2)
Protocol 2	1	12	6 (50.5)	3 (25.0)	0	0	3 (25.0)
Protocol 3	2	20	8 (40.0)	1 (5.0)	1 (5.0)	0	2 (10.0)
Protocol 4	1	10	2 (20.0)	3 (30.0)	1 (10.0)	0	4 (40.0)
Protocol 5	6	62	14 (6.5)	11 (17.7)	10 (16.1)	1 (1.6)	22 (35.5)
Protocol 6	1	7	0	0	2 (28.6)	0	2 (28.6)
Protocol 7	1	6	1 (16.7)	2 (33.3)	0	0	2 (33.3)
Protocol 8	35	424	186 (43.9)	64 (15.1)	132 (31.1)	24 (5.7)	220 (51.9)

2PN = two pronuclei.

Table IV. Summary of pregnancy outcome following the transfer of blastocysts produced using eight different culture protocols

	Protocol							
	1	2	3	4	5	6	7	8
No. of embryo transfers	6	1	2	1	5	1	1	34
No. of blastocysts transferred on day 5 (no. of morulae transferred)	12 (5)	3	1 (5)	3	2 (1)		2	18 (1)
No. of blastocysts transferred on day 6 (no. of morulae transferred)					10 (1)	2 (1)		64 (10)
No. of implantations on day 5	0	1	1[a]	0	1		0	6
No. of implantations on day 6					1[b]	0		15[c]
Implantation rate day 5/day 6 (%)	0	33.3	16.7	0	14.3	0	0	22.6
No. of continuing pregnancies day 5	0	1	0	0	1		0	3
No. of continuing pregnancies day 6					0	0		10
Continuing pregnancy rate day 5/day 6 (%)	0	100	0	0	20	0	0	38.2

[a]Includes an ectopic pregnancy.
[b]Includes a singleton pregnancy which subsequently aborted.
[c]Excludes two blighted ova.

transfer can be compared with rates of 19.0 and 18.5% following the transfer of early cleavage stage embryos on day 2 or 3 achieved in this unit.

Discussion

The evolution has been described of a culture protocol using strictly defined culture conditions and serum-free culture media without somatic cell support for the successful development of embryos to the blastocyst stage *in vitro*. In all, 52% of zygotes developed to the blastocyst stage using an 'optimized' culture protocol (Protocol 8) where zygotes were cultured in groups in microdrops of IVF-50 culture medium under oil from days 1 to 3 post-insemination, followed by group culture in microdrops of Gardner's G2 medium under oil from days 3 to 5 and days 5 to 7 post-insemination. This compares favourably with a previous report of 40% of zygotes developing to blastocysts in culture medium alone (Bolton *et al*., 1991).

Many have claimed improved development to the blastocyst stage when embryos are cultured with a variety of somatic cells (for a review see Bongso *et al*., 1995). However, few workers have attempted to culture to the blastocyst stage all embryos rather than those embryos in excess of transfer on day 2 or 3 post-insemination. Ménézo *et al*. (1992) reported that 57% of all zygotes developed to the blastocyst stage when cultured with Vero cells, and Olivennes *et al*. (1994) reported that 40% of all zygotes developed to the blastocyst stage when cultured with Vero cells. Using the same co-culture system, Schillaci *et al*. (1994) observed that 68% of all zygotes developed to the blastocyst stage. In a prospective randomized study, Van Blerkom (1993) cultured embryos in excess of transfer in the presence or absence of a monolayer of Vero cells. He reported on the frequencies of fragmentation, developmental arrest, multinucleation and blastocyst formation over 7 days in culture. With respect to these parameters he could not demonstrate any statistically significant difference in embryo development between cell-free cultures and the co-culture system. The results of our study, in which 52% of all zygotes developed to the blastocyst stage in serum-free culture medium alone, compare very favourably with the results reported using the co-culture systems.

Dokras *et al*. (1991) studied embryos in excess of transfer in continued culture from days 3 to 14 post-insemination. They observed that blastocysts *in vitro* formed between days 5 and 7 and that there was neither a morphological difference nor a significant difference in the secretion of HCG from day 8 onwards by blastocysts formed on days 5 and 6 or 7, suggesting that a slower cleavage rate does not necessarily reflect the growth potential. They also described similar blastocyst development *in vitro* compared with development

reported previously *in vivo*, but that hatching was delayed *in vitro* by at least 1 day. In a further study, Dokras *et al.* (1993) graded blastocysts based on their morphological appearance. Development to the blastocyst stage was poorly associated with the grade of the embryo on day 2 post-insemination, an observation that had been reported previously by Bolton *et al.* (1989), highlighting the difficulty in selecting viable embryos for transfer at early cleavage stages. Blastocyst grade (BG) 1 blastocysts were characterized by early cavitation, resulting in the formation of an eccentric and then expanded cavity lined by a distinct ICM region and trophectoderm layer. BG2 blastocysts exhibited a transitional phase, where single or multiple vacuoles were seen which, over subsequent days, developed into the typical blastocyst appearance of the BG1 blastocysts. A third appearance was defined as BG3, with a vacuolated appearance or degenerative foci in the ICM. These blastocysts collapsed or failed to expand significantly over 24 h. BG3 blastocysts secreted lower levels of HCG and had significantly fewer nuclei than the other two grades. No significant difference in these parameters could be detected for BG1 and BG2 blastocysts. In the present study only 20% of all zygotes had developed to the blastocyst stage by day 5 post-insemination, which meant that for many patients morulae would have to be transferred to meet the numbers required for transfer. It was also often the case that the blastocysts that had developed by day 5 were early blastocysts with a small, poorly defined cavity that made assessment of the ICM difficult if not impossible. The morphology of morulae on day 5 was also impossible to assess. No morphological parameter reflected the ability of the morula to continue development to the expanded blastocyst stage, making selection of the most viable embryos impossible. Culturing blastocysts for a further day allows for a more objective assessment of morphology. For transfer, preference should be given to blastocysts which show signs of hatching or at the very least show complete expansion and a prominent well-defined ICM equivalent to the BG1 of Dokras *et al.* (1993).

To achieve the development of 52% of zygotes through to the blastocyst stage in the present study, the culture protocol was modified several times, although some elements of the culture protocol remained constant. Scandinavian IVF Science AB culture medium was chosen for the culture and processing of gametes because it is a commercially available culture medium that is produced according to the current Good Manufacturing Practices of the pharmaceutical industry, is subjected to very strict quality control assessments and is in routine use in IVF programmes in Scandinavia and Europe. When oocytes were inseminated, exposure to spermatozoa was limited to 1 h (rather than overnight) because it has been reported that a shortened sperm exposure time results in no significant difference in the fertilization rate but a significantly improved pregnancy outcome (Gianaroli *et al*., 1996). A two-step culture system was utilized for embryo growth from days 1 to 3 for early cleavage stages and from day 3 onwards for the development of blastocysts. Recently, Desai *et al.* (1997) reported the use of a two-step culture system (Quinn's human tubal fluid, followed on day 3 by the alpha modification of minimal essential medium, both supplemented with Irvine Scientific Synthetic Serum Substitute) to culture 'spare' embryos to the blastocyst stage. These authors reported a high incidence of 'spare' embryos developing to the blastocyst stage (45.1%), but because the blastocysts were not transferred, viability could not be assessed. Gardner's G2 medium was chosen for embryo development from day 3 onwards because it has been designed to enhance development to the blastocyst stage. Heat-inactivated patient serum was never used as the protein supplement to the culture medium and was included in the transfer medium only until an approved supply of HSA was made available. Serum is not a naturally occurring biological product but rather a pathological fluid formed by blood clotting, a process that may induce chemical alterations with possible detrimental implications for embryo culture (Maurer, 1992; Gardner and Lane, 1993a). Serum albumin is more defined than human serum and has less batch to batch variation. Embryos may be grown up to the blastocyst stage in the absence of serum, and viable pregnancies can result from the transfer of embryos grown in serum-free culture (Ménézo *et al*., 1984; Caro and Trounson, 1986). At the blastocyst stage of development the inclusion of serum in the culture medium has been reported to enhance the development of bovine morulae to blastocysts (Pinyopummintr and Bavister, 1991) and to stimulate cell numbers and hatching of cultured sheep blastocysts (Thompson *et al*., 1992). In-vivo-derived morulae from the rhesus monkey were able to develop to the expanded blastocyst stage in the absence of serum but were unable to hatch from the zona pellucida. Serum may also benefit embryo development because of its anti-oxidant properties (Kan and Yamane, 1983), but this can adequately be replaced by the inclusion of serum albumin in the culture medium. Serum can cause partial opening of epithelial tight junctions in cultured epithelial cells (Mortell *et al*., 1993), an effect that could be detrimental to the culture of blastocysts. More recently, the inclusion of serum in embryo culture medium has been associated with some alarming effects on the embryos of domestic animals. Such effects include abnormal ultrastructure (Dorland *et al*., 1994; Thompson *et al*., 1995), altered metabolism (Gardner *et al*., 1994) and the appearance of abnormally large offspring (Thompson *et al*., 1995). Therefore, it is recommended that serum should not be used in blastocyst culture systems.

Embryos were cultured in groups in microdrops of medium from day 1 post-insemination because studies in mice (Wiley *et al*., 1986; Paria and Dey, 1990; Lane and Gardner, 1992) and sheep (Gardner *et al*., 1994) have demonstrated a beneficial effect of culturing embryos in groups on the development of embryos to the blastocyst stage due possibly to autocrine factors produced by the embryo.

Cohen *et al.* (1990) suggested that, even with the best culture conditions, development was suboptimal compared with that *in vivo*, and that this could lead to hardening of the zona pellucida which would retard or prevent the fully expanded blastocyst from hatching and subsequently implanting in the endometrium. Consequently in Protocols 4–8 in our study all embryos for transfer that remained within the zona pellucida had the zona pellucida removed by enzymatic digestion with a 0.2% solution of pronase prior to embryo

transfer. Pronase digestion of the zona pellucida was chosen as the method for assisted hatching because a normal pregnancy has resulted from the transfer of pronase-exposed day 6 blastocysts (Fong *et al.*, 1997). These authors further reported that blastocysts that have been hatched artificially by exposure to 0.5% pronase attach tightly, spread out and grow on a variety of feeder layers in a manner similar to blastocysts that hatch naturally on their own *in vitro*. The implantation rate per zona-free blastocyst transferred was 22.8% and for zona-free morulae and blastocysts transferred was 20.0%.

In conclusion, this report describes the evolution of a culture protocol utilizing strict quality-controlled serum-free, cell-free culture media for the development of up to 52% of all zygotes to the blastocyst stage. Blastocysts developed *in vitro* from days 5 to 7 post-insemination and varied in morphology from early cavitation to complete expansion and hatching. Transfer of these embryos following enzymatic removal of the zona pellucida resulted in a continuing pregnancy rate of 38% and an implantation rate (fetal heart/embryo transferred) of 23%. Successful culture *in vitro* of zygotes to the blastocyst stage allows for a more objective selection of viable embryos for embryo transfer and may finally afford us the opportunity of decreasing the numbers of embryos transferred per patient without lowering the pregnancy rate, thus reducing the incidence of multiple pregnancy and its attendant obstetric complications.

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