A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization

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The effectiveness of blastocyst culture and transfer in human in-vitro fertilization (IVF) was evaluated in a prospective randomized trial in patients having a moderate to good response to gonadotrophin stimulation. Embryos were transferred either on day 3 after culture to around the 8-cell stage in Ham's F-10 medium supplemented with fetal cord serum, or on day 5 after culture to the blastocyst stage in the sequential serum-free media G 1.2 and G 2.2. The pregnancy rates after transfer on day 3 or day 5 were equivalent, 66 and 71% respectively; however, significantly more embryos were transferred on day 3 (3.7) than on day 5 (2.2). The number of blastocysts transferred did not affect the implantation rate, and pregnancy rates when either two or three blastocysts were transferred were 68 and 87% respectively. The implantation rate of the blastocysts (50.5% fetal heart beat) was significantly higher compared to the cleavage stage embryos transferred on day 3 (30.1%). The percentage of blastocyst development was not affected by the number of 2-pronuclear embryos, or by maternal age. Irrespective of the number of blastocysts formed, pregnancy rates were similar. Furthermore, the pregnancy rate following blastocyst transfer in patients with 10 or more follicles at the time of human chorionic gonadotrophin administration was not affected by patient age. More than 60% of patients having blastocyst culture and transfer had supernumerary embryos for cryopreservation. The establishment of a pregnancy following thaw and transfer confirmed the viability of cryopreserved blastocysts cultured in the absence of serum or co-culture. The ability to transfer just two blastocysts while maintaining high pregnancy rates will therefore help to eliminate high order multiple gestations and improve the overall efficiency of human IVF.
Key words: blastocyst/embryo transfer/implantation rate/pregnancy/sequential media

Introduction

In human in-vitro fertilization (IVF), the embryo is routinely transferred to the uterus on day 2 or 3 of development, when it has between 4 and 8 cells. This procedure results in implantation rates between 5 and 30%. Such rates have remained relatively constant for the past two decades. As a direct result of the low implantation rate of the cleavage stage human embryo, clinics transfer more than one embryo in order to obtain acceptable pregnancy rates. In 1995 in the USA, the average number of embryos replaced in patients of all ages was four (Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine, 1998). Pregnancy rates do increase with the number of embryos replaced, but so does the incidence of multiple gestations.

In contrast to the cleavage stage embryo, the implantation rate of the human blastocyst, developed *in vivo* and transferred to recipients, is >50% (Buster *et al.*, 1985). Should it be possible to obtain such viable blastocysts *in vitro* after culture from metaphase II oocytes, then the resultant high implantation rate would reduce the need for multiple embryo transfers and thereby eliminate high order multiple gestations (Lopata, 1992). Furthermore, the routine ability to culture and transfer viable blastocysts should improve the overall efficiency of assisted conception in humans.

Recent studies have indicated that it is possible to obtain viable human blastocysts in defined culture systems, in the absence of serum and somatic helper cells (Scholtes and Zeilmaker, 1996). Specifically the development and use of sequential culture media, designed to cater for the changing requirements of the embryo as it develops and differentiates, has been shown to produce blastocysts of high viability (Gardner *et al.*, 1997, 1998; Jones *et al.*, 1998). In all studies, the implantation rate of the blastocysts was higher than that of cleavage stage embryos. We have therefore undertaken a prospective randomized study to determine the efficacy of sequential culture media for human blastocyst development and transfer on day 5.

In January 1998, the Colorado Center for Reproductive Medicine formed a consultant agreement with IVF Science Scandinavia, Gothenburg, Sweden.

Materials and methods

Patient selection

The study was open to all patients who consented to enter the randomized trial between October 1997 and March 1998. The trial was approved by the Institutional Review Board. Requirements for IVF included: (i) basal follicle stimulating hormone (FSH) <15 mIU/ml, (ii) female age <45 years, (iii) presence of normal uterine cavity, (iv) adequate semen parameters for IVF or intracytoplasmic sperm injection (ICSI), (v) absence of any contraindications to pregnancy. In addition, to be included in the study, at least 10 follicles ≥12 mm in diameter, visible by transvaginal ultrasound, were required on the

Table I. Effect of day of transfer on implantation and pregnancy outcome

	Day 3	Day 5	P
No. of patients	47	45	
Age (years) (mean ± SEM)	34.5 ± 0.6	33.6 ± 0.7	NS
Age range (years)	26–43	26–43	
Previous no. of cycles (mean ± SEM)	0.21 ± 0.07	0.62 ± 0.14	<0.05
FSH (mIU/ml) (mean ± SEM)	7.7 ± 0.3	7.3 ± 0.4	NS
Patients with ICSI (%)	34	33	NS
No. of pronuclear embryos (mean ± SEM)	10.9 ± 0.7	11.6 ± 0.8	NS
No. of embryos transferred (mean ± SEM)	3.7 ± 0.1	2.2 ± 0.1	<0.01
Patients with embryo freezing (%)	30	64	<0.01
No. of frozen embryos (mean ± SEM)	3.0 ± 0.7	3.2 ± 0.5	NS
Implantation rate[a] (fetal sac) (%)	37.0	55.4	<0.01
Implantation rate[a] (fetal heart) (%)	30.1	50.5	<0.01
Clinical pregnancy rate (%)[a]	66	71	NS

[a]Includes two patients in the blastocyst culture group who did not have an embryo transferred on day 5 due to embryonic arrest at the cleavage stages.
[b]Per embryo transferred.

day of human chorionic gonadotrophin (HCG) administration. In our experience, this group of patients is most at risk of a high order multiple gestation. Patients entering the trial were allocated to either day 3 transfer or day 5 transfer using a computer-generated randomization table. There were no differences in mean age and mean FSH concentrations on cycle day 3 (~7.5 mIU/ml by in-house assay) between patients having embryo transfer on day 3 or day 5 (Table I), or in their primary cause of infertility. The primary cause of infertility for patients having transfer on day 3 was: tubal ($n = 12$), endometriosis ($n = 8$), ovulatory disorders ($n = 3$), idiopathic/unexplained ($n = 15$) and male factor ($n = 9$). The primary cause of infertility for patients having transfer on day 5 was: tubal ($n = 10$), endometriosis ($n = 11$), ovulatory disorders ($n = 3$), idiopathic/unexplained ($n = 9$) and male factor ($n = 12$).

Ovarian hyperstimulation was initiated with leuprolide acetate (Lupron; TAP Pharmaceuticals, North Chicago, IL, USA) administered from the midluteal phase at 1.0 mg s.c. daily for 10 days and subsequently reduced to 0.5 mg daily until the morning of HCG injection. Human menopausal gonadotrophin was begun after pituitary down-regulation (serum oestradiol <35 pg/ml; 128 pmol/l) and continued until at least 10 follicles reached a mean diameter of 12 mm. HCG was administered when at least two follicles had a mean diameter of 18 mm. Oocyte retrieval was scheduled for 35 h after HCG injection.

Embryo culture

Media were prepared weekly in the laboratory. Ham's F-10 (Flow Laboratories, McLean, VA, USA) was reconstituted with HPLC grade water (Mallinckrodt, Paris, KY, USA) and supplemented with 15% fetal cord serum (FCS) (Meldrum *et al.*, 1987). Media G 1.2 and G 2.2 (modifications of the original G 1 and G 2; Gardner, 1994; Barnes *et al.*, 1995) were supplemented with 5 mg/ml human serum albumin (IVF Science Scandinavia, Gothenburg, Sweden). Modifications to the original culture media formulations included reduced glutamine, EDTA and phosphate concentrations, the inclusion of specific vitamins in medium G2.2, and the use of human serum albumin rather than bovine serum albumin. The exact formulation of several of the more recent culture media used in human IVF have not been disclosed for commercial reasons. All media were screened prior to use with a mouse embryo bioassay (Gardner and Lane, 1993). Semen preparation

was carried out with a 50–70–95 discontinuous gradient or a mini-gradient method using Pure Sperm (Nidacon, Gothenburg, Sweden) depending on the initial semen parameters. The resulting pellet was washed in Ham's F-10 and stored in the incubator until insemination. Each oocyte was inseminated with 100 000 sperm/ml in Ham's F-10 + FCS. Fertilization was assessed 15–18 h post insemination. Cumulus and corona cells were removed by dissection with 27-gauge disposable needles in an organ culture dish. All gamete and embryo manipulations occurred in a paediatric isolette designed to control humidity, temperature and pH fluctuations.

If ICSI was performed, the oocytes were denuded using hyaluronid-ase and drawn pipettes. Each mature oocyte was placed in a 6 µl droplet of phosphate-buffered saline supplemented with 15% FCS. The partner's spermatozoa were placed in a 6 µl droplet of polyvinyl-pyrrolidone (PVP; IVF Science Scandinavia). All droplets were overlaid with paraffin oil (BDH, Poole, Dorset, UK). ICSI was performed on a Nikon inverted microscope with Narishige micromani-pulators. The injected oocytes were then rinsed and placed in tubes of Ham's F-10 with 15% FCS until fertilization was assessed.

Patients having embryo transfer on day 3 had embryos with two pronuclei cultured in groups of 3–4 in 1 ml of Ham's F-10 + FCS in pre-rinsed 5 ml Falcon culture tubes, and left for 48 h (to day 3 of development). On day 3, the majority of embryos for transfer underwent assisted hatching. The criteria for assisted hatching were: patients aged ≥39 years, FSH >10 mIU/ml, a previous failed cycle, for specific embryos where there was ≥20% fragmentation or where there was increased zona thickness. The procedure of selective assisted hatching for embryos with thicker zonae pellucidae has been associated with an increase in implantation and pregnancy rates after the transfer of cleavage stage embryos (Cohen *et al.*, 1992). Embryos were then transferred to the patient in medium Ham's F-10 + FCS. The mean cell number of embryos transferred on day 3 was 7.9 ± 0.1 (± SEM). For those patients having transfer on day 5, embryos with two pronuclei were cultured in groups of three or four in 1 ml of medium G 1.2 in pre-rinsed 5 ml Falcon tubes. Around noon on day 3 all embryos were transferred to 1 ml of medium G 2.2 for a further 48 h of culture (to day 5 of development). None of these embryos underwent assisted hatching. On day 5 the percentage blastocyst formation was determined and up to three blastocysts were selected for transfer. Blastocysts were selected based on the presence of an inner cell mass and a trophectoderm comprised of many cells which formed a distinct epithelium (Figure 1). The most expanded blastocysts within a given cohort were selected for transfer. Blastocysts were transferred in medium G 2.2 + 5 mg/ml HSA. No embryos in the blastocyst group underwent assisted hatching or complete zona removal. The gas phase for all embryo cultures was 5% CO_2 in air.

Embryo transfer and luteal support

All embryo transfers were performed using a Wallace catheter (Edwards–Wallace catheter; Marlow Technologies, Inc., Willoughby, OH, USA) and ultrasound guidance. Methylprednisolone (16 mg once per day) and tetracycline (250 mg four times per day) were administered to all patients for 4 days beginning on the day of oocyte retrieval. Luteal support for all embryo transfers comprised 50 mg i.m. progesterone in oil beginning 2 days following oocyte retrieval.

Embryo freezing

Blastocysts were cryopreserved using the method of Ménézo *et al.* (1992a). On day 3 an embryo had to have at least 6 cells and <10% fragmentation for it to be frozen. The method used was based on Cohen *et al.* (1988). For an embryo to be frozen on day 5 or 6, it had to have an intact trophectoderm, a blastocoel and a visible inner cell mass.
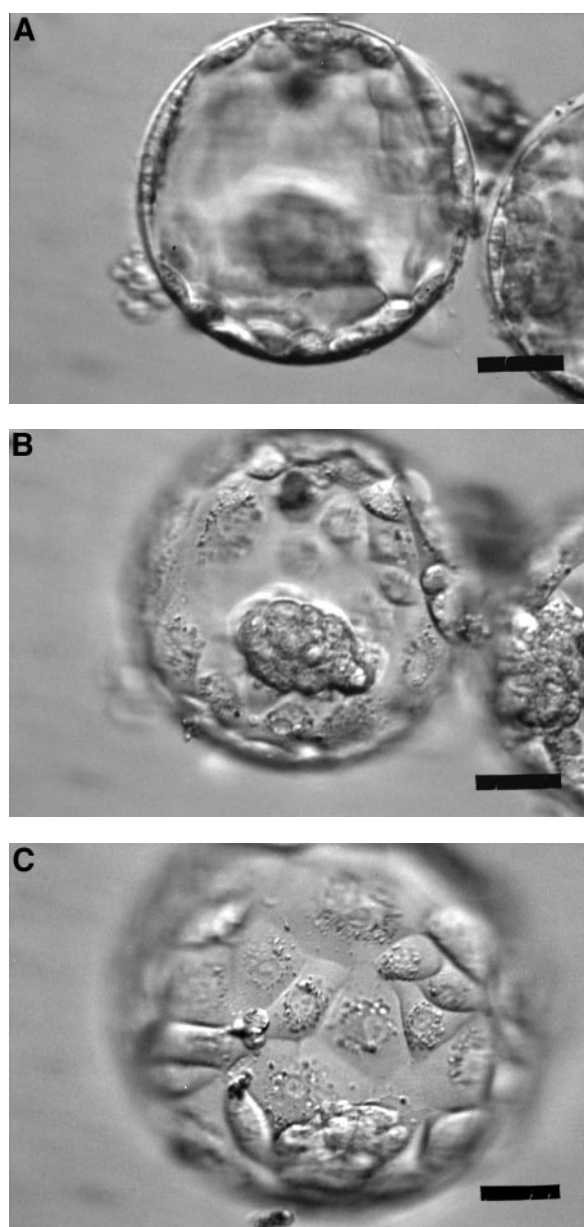
Figure 1. Photomicrographs of human blastocysts. (**a**) Human blastocyst on the morning of day 5 after culture in medium G 1.2 for 48 h from the pronucleate stage, followed by culture in medium G 2.2 for 48 h from the 8-cell stage. Note the thinning of the zona pellucida. A dense group of cells, the inner cell mass (ICM), can be seen around the 6 o'clock position. (**b**) The same blastocyst, at a different focal plane. The ICM is clearly visible. (**c**) The trophecto-derm of the blastocyst can be seen to be made up of many cells. Bar = 35 μm.

Statistical analysis

Statistical analysis of results was performed using unpaired *t*-tests. Percentage data were compared using Fisher's exact test.

Results

The data show that the transfer of human embryos on day 5 of development results in a significant increase in implantation rate compared to embryos transferred on day 3 ($P < 0.01$), when assessed as both fetal sac and fetal heart development per embryo transferred (Table I). The patients were allocated
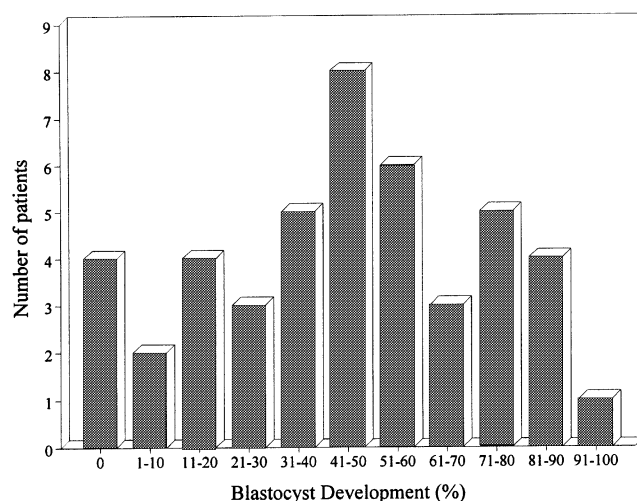
Figure 2. Patient variation in percentage blastocyst development.

to either arm of the trial on day 8 of their cycle and there were no cancellations. There were no failed fertilizations. Implantation rates were calculated using all patients irrespective of pregnancy outcome. Furthermore, equivalent pregnancy rates could be obtained with day 3 and day 5 transfer but with significantly fewer embryos transferred on day 5 ($P < 0.01$), this despite the fact that patients having a transfer on day 3 had undergone significantly fewer previous IVF cycles than those having a day 5 transfer.

Blastocyst development and pregnancy rate

The mean percentage blastocyst development from 2-pro-nuclear embryos for all patients was 46.5%. The majority of blastocysts were obtained on day 5 (43%; i.e. 92% of all blastocysts formed), the remaining 3.5% being formed by day 6. The mean number of blastocysts formed per patient was 5.4. Eighty percent of patients had two or more blastocysts transferred on day 5, while two-thirds of patients had super-numerary blastocysts frozen (Table I). Blastocyst development varied significantly from patient to patient, and the embryos from four patients of the 45 in the study failed to form any blastocysts (Figure 2). However, in two of these, the patients had morulae that were transferred on day 5. Both patients have ongoing pregnancies as a result. So in all, only 4.5% (2/45) of patients having blastocyst culture did not have a transfer.

Despite the variation in blastocyst development (Figure 2), the pregnancy rate when blastocysts were transferred was similar, irrespective of the percentage of embryos within a cohort forming blastocysts (Figure 3). Although there was a significant linear relationship between the number of pronucle-ate embryos from a given patient and the total number of blastocysts (Figure 4a; $P < 0.01$), this did not correspond to an increase in the percentage of blastocyst development (Figure 4b). Age had no significant effect on either the number of blastocysts obtained (Figure 4c) or the percentage of pronucleate embryos becoming blastocysts (Figure 4d). The pregnancy rate was equivalent across all age groups (Table II), although such a general comparison necessitated small sample sizes.

The majority of patients (89%) received either two or three

blastocysts. The effect of the number of blastocysts transferred on pregnancy outcome is shown in Table III. The implantation rate was not affected whether two or three blastocysts were transferred. Consistent with observations on the cleavage stage

Figure 3. Pregnancy rate as a function of percentage blastocyst development. Data include only those 43 patients who had an embryo transfer.

Table II. Effect of patient age on pregnancy rate after blastocyst transfer

Age (years)	No. of patients	No. pregnant	% pregnant
<30	14	9	64
30–34	13	11	85
35–39	13	8	62
≥40	5	4	80

embryo, pregnancy rates were higher when more blastocysts were replaced. Ten of the first 13 patients having blastocyst transfers received three embryos. When the high implantation became evident, the majority of the remainder of the patients received only two blastocysts at transfer.

Effect of ICSI on blastocyst development and pregnancy rate

There was no apparent effect of ICSI on either implantation rate or pregnancy rate. The percentage blastocyst and pregnancy rates for 30 patients who had conventional IVF were 47 and 73% respectively. For the 15 patients having ICSI, the respective figures were 45 and 67%.

Frozen–thawed blastocyst transfers

Of the 12 patients who did not conceive with fresh blastocyst transfers, three returned for the replacement of thawed blastocysts. Ten blastocysts were thawed, of which nine survived and re-expanded and two of these started hatching. One of the three patients subsequently conceived and has an ongoing pregnancy.

Discussion

This prospective randomized trial has demonstrated that it is feasible to culture human embryos to the blastocyst stage in serum-free sequential culture media. Furthermore resultant blastocysts have a significantly higher implantation rate than embryos transferred on day 3 of culture at the 4- to 8-cell stage after culture in Ham's F-10 and fetal cord serum. It is therefore possible to attain high pregnancy rates without having to transfer three or more embryos, at least in patients who
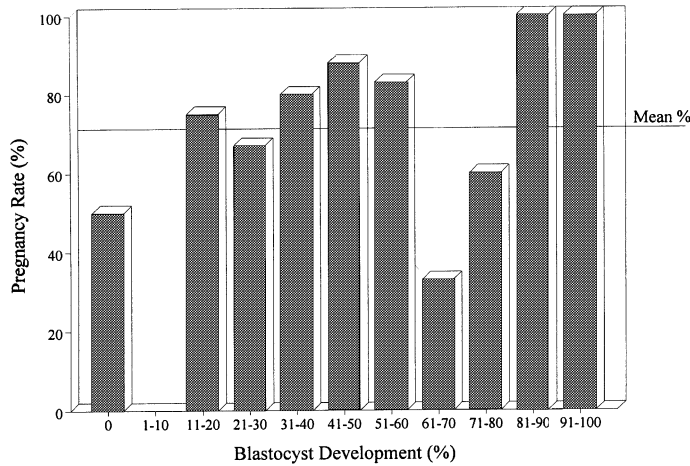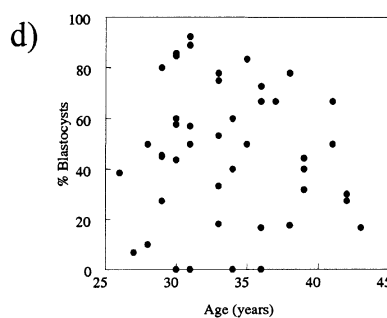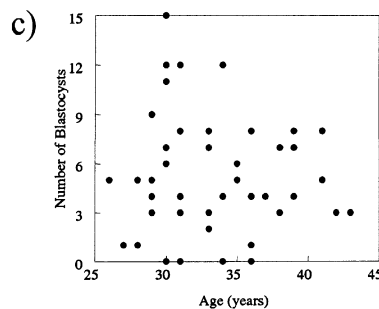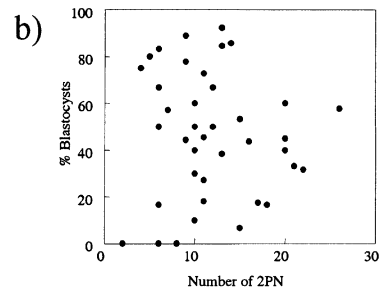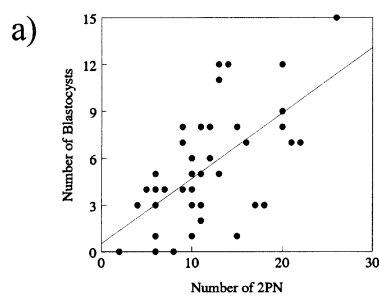

Figure 4. Effect of the number of pronuclear embryos and age on blastocyst development. (**a**) There was a significant linear relationship between the number of pronuclear embryos and the number of blastocysts formed ($P < 0.01$). (**b**) Percentage blastocyst development was not related to the number of pronuclear embryos, i.e. ~50% of pronuclear embryos formed blastocysts. (**c**) There was no significant relationship between age and the number or (**d**) percentage of blastocysts formed.

Table III. Effect of number of blastocysts transferred on implantation and pregnancy rates

No. of blastocysts transferred (no. of patients)[a]	Age (years) (mean ± SD)	Implantation rate (% fetal heart)	Pregnancy rate (%)	Singletons[b] (%)	Twins[b] (%)	Triplets[b] (%)
2 (25)	33.3 ± 0.8	52	68	47	53	–
3 (15)	34.7 ± 1.5	49	87	61	8	31

[a]Of the 45 patients in the blastocyst group two did not have an embryo transfer. Two patients received morulae; both became pregnant. One patient received one blastocyst and did not conceive.
[b]Percentage of ongoing pregnancies.

have a moderate to high response to gonadotrophins. Ongoing investigations will determine the efficacy of extended culture in sequential media for patients with a low response to gonadotrophins.

There are two reasons for the higher viability of blastocysts compared to cleavage stage embryos; first, the human cleavage stage embryo normally resides in the oviduct and does not enter the uterus until after compaction (Croxatto *et al.*, 1978). The oviduct and uterus provide different nutritional environments for the embryo (Gardner *et al.*, 1996), so it is therefore plausible that development of cleavage stage embryos is compromised by premature transfer to the uterus. In support of this hypothesis, in other mammalian species, the transfer of cleavage stage embryos to the uterus results in lower pregnancy rates than the transfer of morulae or blastocysts (Bavister, 1995). Secondly, by culturing the embryo for 4 days to the blastocyst, embryos with limited, if any, developmental potential may be identified and avoided (Bolton *et al.*, 1989; Dawson *et al.*, 1995). Certainly at this time, embryos are assessed after the initiation of embryonic genome activation (Braude *et al.*, 1988). Furthermore, some chromosomally abnormal embryos fail to develop in culture (Munné *et al.*, 1995).

The percentage of blastocyst development from all pro-nucleate embryos was 46%, with >90% of blastocysts being formed by day 5. However, there was considerable inherent patient variation with respect to blastocyst formation. Such percentage blastocyst development is similar to that reported when co-culture has been used (Ménézo *et al.*, 1990; 1992b; Wiemer *et al.*, 1993; Bongso *et al.*, 1994; Olivennes *et al.*, 1994), and to those obtained when a single culture medium was employed (Hardy *et al.*, 1989; Bolton *et al.*, 1989; 1991; Dokras *et al.*, 1993; Desai, 1997). However, it appears that blastocysts obtained in this study had a higher viability than those in previous studies. It is proposed that the high viability of the blastocysts in this study is due to the use of sequential culture media in combination with optimal laboratory and clinical procedures, demonstrated by the relatively high implantation rate of the embryos in the control group. One of the key findings of research on mouse embryos is that it is possible to obtain morphologically good-looking blastocysts using different culture conditions; however, the resultant blastocysts have very different viabilities when transferred to recipients, i.e. an expanded blastocoelic cavity may coexist with poor inner cell mass development (Gardner *et al.*, 1997; Lane and Gardner, 1997). This reinforces the fact that blastocysts developed in culture may not necessarily be viable.

The experimental data supporting the use of a two-step culture system have been discussed extensively (Gardner and Leese, 1990; Gardner and Lane, 1993; 1997; Gardner and Sakkas, 1993; Gardner, 1998). It is evident that the culture conditions that support the highest rates of cleavage in the mouse zygote do not support optimum blastocyst development and differentiation. Conversely, those conditions that best support blastocyst development and differentiation from the 8-cell stage actually reduce mouse zygote development and subsequent viability. The latter point is a most important concept, as it helps to explain the study of Bolton *et al.* (1991), in which 40% blastocyst development was obtained, but the resultant implantation rate was only 7%. The culture system used by Bolton *et al.* (1991) was Earle's balanced salt solution supplemented with pyruvate and 10% maternal serum. Clearly such simple media are not suitable for the extended culture of the human embryo. In contrast, co-culture systems have employed more complex media resulting in implantation rates of ~20% (Olivennes *et al.*, 1994). Interestingly, Ménézo *et al.* (1992b) calculated the blastocyst implantation rate from only those women who became pregnant and did not take into account the number of embryo transfer procedures that failed to give rise to a pregnancy. With this approach, Ménézo *et al.* (1992b) found that the implantation rate of human blastocysts after co-culture with Vero cells was 52.8%. Similarly, Bertheussen *et al.* (1997) who used sequential media (Universal IVF Medium and M3; MediCult) reported an implantation rate (per successful pregnancy) of 36%. Using the same criterion to calculate implantation rate, using sequential culture media in this study the fetal sac rate would be 71% per embryo transferred in pregnant patients only, with a fetal heart rate of 66%.

Of the 45 patients in the blastocyst group, two produced embryos which did not develop past the 8-cell stage, and two produced embryos which compacted but failed to form a blastocoel on day 5. The latter two patients had morulae transferred on day 5 and subsequently became pregnant, confirming the observations of Huisman *et al.* (1994) and Bertheussen *et al.* (1997) that morulae have a high viability. In studies using either co-culture (Ménézo *et al.*, 1992b) or a single culture medium with serum (Scholtes and Zeilmaker, 1996), 8.5 and 28% of patients respectively produced embryos which failed to form a blastocyst. In the present study, 8.9% of patients produced embryos that failed to reach the blastocyst, while only 4.5% of patients did not have a transfer. Eighty-nine per cent of patients had two or more blastocysts transferred, the mean number of blastocysts formed per patient being 5.4. This

figure is in contrast to Scholtes and Zeilmaker (1998) who observed that only 59% of cases had two blastocysts for transfer. However, in the latter study, only a single medium was used for embryo culture and the study was based on an unselected group of patients.

In the present study it appeared that, provided a patient had blastocysts to transfer, she had the same chance of becoming pregnant no matter how many embryos reached the blastocyst stage. Initially patients received three blastocysts. However, it soon became apparent that the blastocysts had a high implantation rate and the number transferred was subsequently reduced to two, irrespective of patient age. The implantation rate was not affected by the reduction, although the pregnancy rate decreased from 87 to 68% respectively.

Although the absolute number of blastocysts obtained from a given patient was related to the number of pronucleate embryos, a finding previously reported by Janny and Ménézo (1996), the percentage of blastocyst development was not dependent upon the initial number of embryos. Furthermore, the number of blastocysts obtained was not affected by patient age, nor was the percentage of blastocyst formation. It has to be remembered, however, that the patient base in this study consisted of patients with a moderate to good response to gonadotrophins. It has been shown that in women over 40 years, a good ovarian response is consistent with improved outcome in IVF (Roest *et al.*, 1996; Widra *et al.*, 1996). Therefore the findings reported here are not necessarily in contrast to those of Janny and Ménézo (1996), who observed a significant decrease in blastocyst formation with patient age. This emphasizes the need to extend prospective studies on blastocyst culture and transfer, using sequential media, to other patient groups.

Finally, it is important that supernumerary blastocysts developed using sequential culture media can be adequately cryopreserved. This is especially true as the number of blastocysts required for transfer is decreasing. Ménézo *et al.* (1992a) and Kaufmann *et al.* (1995) have conclusively demonstrated that it is possible to freeze human blastocysts, which can subsequently give rise to viable pregnancies.

In conclusion, an acceptable percentage of blastocyst development can be obtained using appropriate serum-free sequential culture media. Importantly, the viability of such blastocysts was significantly higher than cleavage stage embryos in the group of patients entering the prospective randomized trial. The efficacy of blastocyst culture and transfer in patients who exhibit a poor response to gonadotrophins needs evaluating in further prospective trials.

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