A prospective randomized comparison of sequential versus monoculture systems for in-vitro human blastocyst development

N.S.Macklon[1,3], M.H.E.C. Pieters[1], M.A.Hassan[1], P.H.M.Jeucken[1], M.J.C.Eijkemans[2] and B.C.J.M.Fauser[1]

[1]Division of Reproductive Medicine, Department of Obstetrics and Gynaecology and [2]Department of Public Health, Centre for Clinical Decision Sciences, Erasmus Medical Centre, Rotterdam, The Netherlands

[3]To whom correspondence should be addressed at: Department of Obstetrics and Gynaecology, Erasmus Medical Center, Dr. Molewaterplein 40, 3015GD Rotterdam, The Netherlands. E-mail: macklon@gyna.azr.nl

BACKGROUND: Extending the period of in-vitro culture to the blastocyst stage may improve implantation rates in IVF treatment. Recognition of the dynamic nature of early embryo metabolism has led to the development of commercially available sequential culture systems. However, their improved efficacy over monoculture systems remains to be demonstrated in prospective studies. METHODS: Embryos obtained from 158 women undergoing IVF treatment were randomized by sealed envelopes to culture in one of three systems: (A) culture for 5 days in our own monoculture medium (Rotterdam medium); (B) culture for 3 days in Rotterdam medium followed by 2 days in fresh Rotterdam medium; (C) culture for 5 days using the commercially available G1/G2 sequential culture system. RESULTS: There were no significant differences in blastulation, implantation or pregnancy rates between the three tested culture systems. CONCLUSION: The employed monoculture system is as effective as the G1/G2 sequential system for the culture of blastocysts for IVF.

Key words: blastocyst/culture medium/IVF/pregnancy rates/randomized controlled trial

Introduction

An increasing number of IVF centres now offer transfer of blastocysts as a means of improving pregnancy rates. However, the true value of culture to the blastocyst stage of development remains a matter of debate (Jones and Trounson, 1999; Plachot *et al.*, 2000). Earlier transfer of the embryo into the uterus reduces the need for rigorous quality control of laboratory conditions or complex culture media. Moreover, the risk of disappointing the patient whose embryos fail to develop is reduced. On the other hand, the benefits most frequently cited by proponents of blastocyst transfer include the selective effect of prolonged culture whereby genetically defective embryos may fail to survive, improving embryo selection for transfer (Munné *et al.*, 1995; Gardner *et al.*, 2000a; Sandalinas *et al.*, 2001). In addition, improved synchrony between the embryo and intrauterine environment at the time of transfer may have a beneficial effect on implantation rates (Milki *et al.*, 2000; Gardner and Lane, 2001). It is also proposed that improving the selection process and embryo–uterine developmental synchrony may encourage the transfer of fewer embryos, thus leading to a reduction in multiple pregnancies without reducing overall pregnancy rates (Scholtes and Zeilmaker, 1996; Fong *et al.*, 1997, 1998; Fauser *et al.*, 1999; Marek *et al.*, 1999; Milki *et al.*, 1999; Karaki *et al.*, 2002).

Early reports of human blastocysts grown in culture employed culture medium alone (Steptoe *et al.*, 1971; Edwards *et al.*, 1981). Using simple defined media supplemented with serum, other workers obtained high blastocyst formation rates (Hardy *et al.*, 1989a,b; Fitzgerald and di Mattina, 1992). However, implantation rates remained poor (Bolton *et al.*, 1991) until co-culture systems in which trophoblast cells were included in the culture environment were developed and implemented (Ménézo *et al.*, 1990, 1992; Ménézo and Ben Khalifa, 1995; Fong *et al.*, 1997). At the same time, culture media capable of sustaining embryo development to the blastocyst stage without the use of co-culture systems were developed (Huisman *et al.*, 1994, 2000). Increasing understanding of the dynamic nature of early embryo metabolism (Leese *et al.*, 1986; Hardy *et al.*, 1989b; Gott *et al.*, 1990) and the changing environment encountered by the preimplantation embryo *in vivo* (Gardner *et al.*, 1996; Gardner, 1998) led to the formulation of sequential culture media. These were designed to support the growth of human pronuclear embryos to the blastocyst stage (Barnes *et al.*, 1995; Gardner *et al.*, 1996; Gardner and Lane, 1997; Ménézo *et al.*, 1998; Behr *et al.*, 1999; Milki *et al.*, 1999).

Central to the design of sequential media is the switch from pyruvate (the preferred nutrient of the early cleavage stage when low levels of oxygen are consumed) to glucose to meet the increased energy demands as development to the blastocyst

stage proceeds (Hardy *et al.*, 1989b). Moreover, the complex structure of the blastocyst is considered to merit recognition in the design of culture media since the inner cell mass requires the essential group of amino acids, whereas the trophectoderm utilizes the non-essential amino acids and glutamine (Lane and Gardner, 1997a). Consistent with this notion is the supposition that conditions supporting optimal development of the early cleavage stage embryo are unlikely to support optimal blastocyst development and differentiation (Gardner and Lane, 1997) and vice versa (Lane and Gardner, 1997b).

Addressing these changes in the requirements for both carbohydrates and proteins during early embryonic development, two commercially available culture media were formulated to support the growth of human pronuclear embryos to the blastocyst stage (Gardner *et al.*, 1996, 1998a,b). The first medium (G1.1) is based on the levels of carbohydrate present in the human Fallopian tube at the time when the cleavage stage embryo is present. It also contains amino acids, which have been shown to stimulate development of the cleavage stage embryo (non-essential amino acids and glutamine). The chelator EDTA is also present to sequester any toxic divalent cations present in the system. It also helps to minimize glycolytic activity of the embryo thus minimizing the metabolic perturbations (Lane and Gardner, 1997b). The second medium is based on the levels of carbohydrate present in the human uterus and contains both essential and non-essential amino acids to facilitate both blastocyst formation and differentiation. However, EDTA is not present as it may selectively impair inner cell mass development and function (Hewitson and Leese, 1993).

Initial experience with sequential culture systems designed to address the complexities and dynamic nature of embryo metabolism was very encouraging and high rates of blastulation and implantation were reported (Gardner and Lane, 1997b; Gardner *et al.*, 1998a; Ménézo *et al.*, 1998). A subsequent prospective study comparing outcomes from blastocysts produced by sequential culture systems with those from day 3 embryos produced from monoculture systems confirmed the potential of this approach (Gardner *et al.*, 1998b). The compelling theoretical advantages of sequential culture systems and number of retrospective studies suggesting their superiority over monoculture and co-culture techniques (Jones *et al.*, 1998a), have led to the widespread use of sequential culture systems which are now commercially available. However, most studies of blastocyst culture published so far are uncontrolled, based on small numbers and with highly selected patients, making comparisons of results between different culture systems difficult. Moreover, the superiority of sequential culture of embryos over monoculture systems for producing blastocysts for transfer in IVF treatment awaits confirmation in prospective studies. We set out to address this by prospectively comparing blastulation, implantation and viable pregnancy rates from embryos randomized to culture in sequential and non-sequential culture systems using our own complex medium or the commercially available sequential G1/G2 medium.

Materials and methods

Subjects and study design

Between September 1999 and September 2000, 158 patients with an indication for IVF were recruited to the study. Written informed consent

was obtained from all patients according to the requirements of the local medical ethics committee. No patients were excluded from the study for reasons of age, medical history, indication or response to IVF treatment. The only exclusion criterion for the study was an indication for ICSI. Subjects were randomized for one IVF cycle only.

Earlier studies have indicated that following sequential culture with G1/G2, implantation rates per blastocyst of 40–60% may be achieved (Gardner *et al.*, 2000a). The mean implantation rate per blastocyst derived from monoculture systems in our centre is ~20% (Huisman *et al.*, 2000). In order to show with 80% power at $P < 0.05$ that G1/G2 sequential culture as opposed to monoculture with the Rotterdam medium could increase implantation rates per blastocyst from 20 to 40%, 100 transferred embryos were required in each arm. Since these two culture systems were also to be compared with the Rotterdam medium in a serial culture system, and a maximum of two embryos were transferred per cycle, a total of 150 completed cycles was required.

Pituitary down-regulation was achieved by administering the GnRH agonist triptorelin (Decapeptyl®; Ferring, Hoofddorp, The Netherlands) 0.1 mg/day s.c. starting in the mid-luteal phase of the preceding cycle ('long protocol'). Following pituitary down-regulation, multiple follicle development was induced with 150–300 IU/day recombinant FSH (Puregon®; NV Organon, Oss, The Netherlands) administered s.c. Follicular growth was monitored by ultrasonography until three or more dominant follicles measuring 17 mm in diameter were observed. A single i.m. injection of 10 000 IU of hCG (Pregnyl®; NV Organon) was administered to induce final stages of oocyte maturation. Transvaginal ultrasound-guided follicular aspiration was performed 34–36 h later. Following oocyte retrieval, patients were randomized by sealed envelopes to one of three groups for embryo culture. In group A, embryos underwent non-sequential culture in our own complex medium. This culture system was termed the 'Rotterdam monoculture system'. In group B, embryos were cultured in the same medium but were transferred to a fresh drop of this medium on day 3. This was referred to as the 'Rotterdam serial culture system'. In group C the commercially available G1.2/G2.2 sequential medium (Vitrolife, Gothenburg, Sweden) referred to as the 'G1/G2 sequential system' was employed (Table I).

Culture systems and embryo scoring

Organ culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) were prepared, each with three drops of 100 µl medium laid out under oil (Allegiance, Zutphen, The Netherlands) and numbered 1, 2 and 3. Drops for embryos randomized to undergo culture in group A and B consisted of our own laboratory medium. The medium was prepared weekly and consisted of a 17:3 mixture of Earle's balanced salt solution and Ham's F-10 medium supplemented with a pasteurized plasma protein solution (CLB, Amsterdam, The Netherlands) (Table II). The osmolarity of the medium was 280–285 mOsm (Zeilmaker, 1986; Huisman *et al.*, 1992). The drops for group C differed as follows: the first drop for oocyte incubation prior to fertilization was formed of Rotterdam culture medium, the second drop consisted of G1.2 medium which is designed to support embryo development to the 6–8 cell stage (day 3) and the third was G2.2 medium which is designed to support embryo development from the 6–8 cell stage onwards (Gardner *et al.*, 1998a).

Oocytes from all patients were identified in the laboratory and flushed in Rotterdam culture medium to remove follicular fluid and blood cells. Consistent with general clinical practice, a maximum of four oocytes was incubated in the first of the three drops of the system to which they had been randomized, and stored in a closed incubator (model 3336; Forma, Scientific, Marietta, Ohio, USA) at 37°C in 5% CO_2 in air atmosphere. Motile sperm separated by 45:90 discontinuous colloidal silica gel double gradient (PureSperm;

Table I. Protocols for transfer of embryos between culture drops for the different culture systems in a randomized comparative trial in 146 IVF patients undergoing blastocyst (day 5) embryo transfer

Group (culture system)	Day 1	Day 3	Day 5
A (Rotterdam monoculture)	Transfer to fresh RM	No fresh RM	ET
B (Rotterdam serial culture)	Transfer to fresh RM	Transfer to fresh RM	ET
C (sequential G1.2 + G2.2)	Transfer to G1.2	Transfer to G2.2	ET

RM = Rotterdam medium; ET = embryo transfer.

Table II. An overview of the sub-constituents of the Rotterdam medium for embryo culture in IVF

Constituent	Per litre	Concentration (mmol/l)	Source
NaCl	5.494 g	94.0	Merck (Darmstadt)
KCl	0.336 g	4.51	Merck
$MgSO_4 \cdot 7H_2O$	0.173 g	0.70	Merck
$NaHCO_3$	2.096 g	24.9	Merck
$NaH_2PO_4 \cdot 2H_2O$	0.135 g	0.87	Merck
Glucose	0.846 g	4.70	Merck
Na pyruvate	0.020 g	0.18	Merck
Ca-L (+) lactate	0.557 g	2.64	Fluka (Steinheim)
Ham's F-10	1.731 g/l		Gibco (Paisley)
Streptomycin	10 IU/ml		Gibco
Pencillin	10 IU/ml		Gibco
Plasma protein substitute	175 ml		CLB

Nidacon, Göteborg, Sweden) were used to inseminate the oocytes after 3–6 h preincubation (day 0). Inspection of fertilization (pronuclei) was performed 16–20 h after insemination. Following inspection, zygotes were transferred into medium drop 2. On day 3, those embryos randomized to groups B and C were transferred to fresh medium (drop 3). Those in group A were left to continue culture in medium drop 2. The transferring scheme is summarized in Table I. All embryos were cultured for 5 days.

Embryo morphology scoring was performed on days 3 and 5 for all study groups. Scoring of day 3 embryos was performed according to previously published criteria including cell number, regularity of the blastomeres, fragmentation and morphological aspects such as granulation (Veeck, 1998; Huisman *et al.*, 2000). Day 3 embryos were thus scored on a scale of 1 (high grade) to 4 (low grade). On the day of embryo transfer all embryos were scored again according to strict day 5 criteria including embryonic stage, cavitation, inner cell mass and cell morphology (Veeck, 1998; Huisman *et al.*, 2000). When no fragmentation was evident and the developmental stage was appropriate for their age, embryos were described as 'high grade' (score 1). Embryos showing developmental delay and >50% fragmentation were described as 'low grade' (score 4). Embryos of better and worse intermediate quality were scored as grade 'intermediate high' and 'intermediate low' respectively (score 2 and 3).

Blastulation rate was calculated from the number of blastocysts available for embryo transfer and cryopreservation divided by the total number of oocytes fertilized. Pregnancy was identified by a positive urinary hCG test result (sensitivity of >25 IU/l) (Clearview; Unipath Ltd, Bedfordshire, UK) performed 17 days after ovum retrieval. Pregnancy rates per embryo transfer procedure and implantation rates per transferred blastocyst were based on the detection of fetal heart beats by ultrasound carried out 5 weeks after embryo transfer.

Data analysis

Differences in blastulation, pregnancy and implantation rates per blastocyst between the three groups were analysed using the χ^2-test. Differences in embryo quality for the studied protocols on day 3 and day 5 were also evaluated using the χ^2-test. Data related to the cryopreservation of supernumerary embryos derived from study treatment cycles were not included in the analysis.

Results

In nine cases total fertilization failure occurred. In three cases protocol violations occurred: two when transfer to fresh drops was not performed and the third when a patient requested transfer earlier than day 5. A total of 146 treatment cycles was therefore finally subject to analysis. There were no differences between the groups in patient characteristics (Table III), or cycle drop-out rates.

The total number of oocytes obtained in groups A, B and C were 450, 426 and 461 respectively. The mean embryo score on day 3 and on day 5 was not different between the groups (Figure 1). Blastulation rates in groups A, B and C were 35, 36 and 40% respectively (Table IV). Similarly, no significant differences in the percentage of grade 1 blastocysts obtained, implantation rates per blastocyst or ongoing pregnancy rates were observed between the study groups (Table IV). There was no significant difference between the study groups A, B and C in the percentage of women who had at least one score 1 embryo transferred (Table IV). Moreover, pregnancy rates in these women were similar at 58, 52 and 48% for study groups A, B and C respectively. For embryos scoring 2, 3 and 4, the respective pregnancy rates for the total study population were 40, 12 and 10%.

Discussion

The present study was designed to compare the rate of viable blastocyst formation following monoculture using the Rotterdam culture medium with that obtained using the Rotterdam culture medium in a serial system and the G1/G2 sequential culture system. Previous observations from our own group indicate that viable blastocysts could successfully be produced after 5 days in monoculture, where day 0 is the day of oocyte retrieval (Huisman *et al.*, 2000). Extended culture beyond 5 days is advocated by some to achieve the blastocyst stage in retarded embryos (Gardner and Lane, 2001). However, studies have indicated that the rate at which the blastocyst is formed is an important indicator of quality (Shoukir *et al.*,

Table III. Patient characteristics of 146 women participating in a randomized comparison of three culture systems for blastocyst transfer in IVF

	Rotterdam monoculture	Rotterdam serial culture	G1/G2 sequential
Patients (*n*)	53	45	48
Age (years)	35 ± 4 (27–42)	34 ± 4 (25–40)	35 ± 4 (27–43)
Oocytes (*n*)	9.7 ± 6.1 (2–31)	9.8 ± 6.5 (1–26)	9.4 ± 5.5 (1–26)
Fertilization rate (%)[a]	63 ± 23 (13–100)	66 ± 23 (7–100)	62 ± 19 (25–100)
Embryo transfer [*n* (%)][b]	43 (81)	41 (91)	43 (90)
Embryos transferred	1.6 ± 0.8 (0–2)	1.8 ± 0.6 (0–2)	1.7 ± 0.7 (0–2)

Values are means ± SD (range) unless otherwise stated. There are no significant differences in the given parameters between the groups.
[a]Percentage of oocytes fertilized.
[b]Number (*n*) and percentage of patients who underwent embryo transfer.
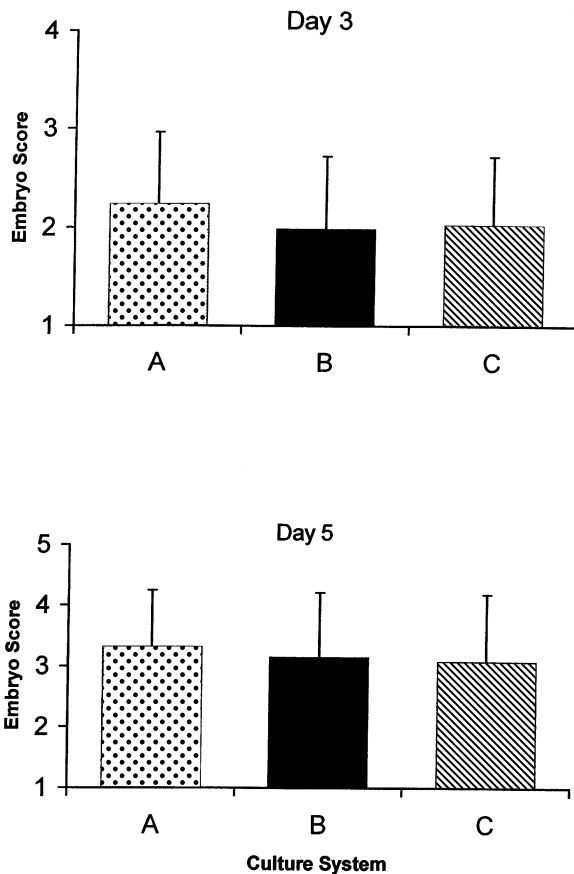

Figure 1. The mean embryo score (+ SD) at day 3 (upper panel) and day 5 (lower panel) are given for embryos cultured in the three tested media systems. (**A**) Rotterdam monoculture; (**B**) Rotterdam serial culture; (**C**) G1/G2 Sequential. There were no significant differences in any of the outcomes shown between the three culture systems.

1998) and the best blastocysts are likely to be those that have developed after 5 days of culture. In a recent study, embryos that developed to the expanded blastocyst stage and were transferred on day 5 after retrieval were twice as likely to implant as those in which transfer was delayed until day 6 (Shapiro *et al.*, 2001).

The viability of the blastocysts obtained is an important measure of quality since a morphologically normal blastocyst is not

Table IV. Outcomes following embryo culture for IVF in the three tested media systems

	Rotterdam monoculture	Rotterdam serial culture	G1/G2 sequential
Embryos obtained	304	283	269
Blastocysts (% of embryos)	107 (35)	103 (36)	107 (40)
Grade 1 blastocysts (% of blastocysts)	46 (43)	48 (47)	41 (38)
Embryo transfers performed	43	41	43
Embryos transferred	85	79	82
Embryos implanted (% of transferred)	16	20	17
Ongoing pregnancies	9	11	10
Ongoing pregnancy rate per ET (%)	21	27	23
Ongoing pregnancy rate per high score ET (%)	58	52	48

There were no significant differences in any of the outcomes shown between the three culture systems.
ET = embryo transfer.

necessarily viable. This concept may explain why previous attempts to culture the human blastocyst have resulted in a low implantation rate after transfer (Bolton *et al.*, 1991). Indeed, Behr *et al.* concluded from their study that the sequential media approach may not necessarily result in a higher number of blastocysts being obtained but in an increase in the number of viable blastocysts as evidenced by high implantation rates (Behr *et al.*, 1999). We therefore used implantation rates per blastocyst (as represented by the presence on ultrasound examination of intrauterine gestational sacs and fetal poles with visible heart beats) as a means of comparing viability of the blastocysts formed by the different media and systems studied.

Previous studies have reported implantation rates for transferred blastocysts of between 25 and 70% (Jones *et al.*, 1998b; Gardner *et al.*, 1998b, 2000b; Milki *et al.*, 2000;). The implantation potential observed of transferred blastocysts in all three groups of the present study was lower than those previously published. In addition to a possible reduced performance in our laboratory, this difference may also be explained by the lack in our study of any selection of patients or embryos for culture to the blastocyst stage, as was also the case in our previous report on blastocyst transfer (Huisman *et al.*, 2000). Moreover, the blastulation rate was based on the entire cohort of the patient's

fertilized oocytes and not on the spare embryos as in other studies (Bolton *et al*., 1989; Desai *et al*., 1996). The present study therefore represents a comparison of the two media and culture systems as may be used in everyday clinical practice. When the analysis was restricted to those women in whom at least one top quality embryo was transferred, implantation rates of up to 30% of blastocysts transferred and ongoing pregnancy rates of up to 40% per embryo transfer procedure were observed in all groups. These high rates are similar to those reported by other groups analysing selected patients (Gardner *et al*., 2000b; Milki *et al*., 2000).

The success of our monoculture system in producing viable blastocysts may be due in part to fact that the mixture of Earle's balanced salt solution and Ham's F-10 medium used under our conditions contains all three energy substrates (pyruvate, lactate and glucose) required. The latter is not the case in many routinely used culture media (Gardner and Lane, 2001). Furthermore, none of the nutrients appears to be supplied in a concentration likely to lead to developmental arrest or retardation at any stage from the zygote to the blastocyst stage. In the present study, embryos were cultured in groups (with a maximum of four embryos) in microdrops of medium. Both human studies (Moessner and Dodson, 1995; Amalgor *et al*., 1996), and studies in sheep and mice have indicated that culturing embryos in groups has beneficial effects on development, in part by improving cell allocation to the inner cell mass (Gardner *et al*., 1994, 1997). This effect is considered to be due to a relative decrease in the volume of culture medium (Paria and Dey, 1990; Amalgor *et al*., 1996) and a relative increase in the concentration of paracrine and autocrine factors stimulating embryo development (Gardner *et al*., 1997).

The serial culture protocol involving transfer to a new drop of medium on day 3 revealed slightly favourable results over the monoculture system when comparing biochemical and ongoing pregnancy rates. Although these differences did not reach statistical significance, they would appear to be consistent with improved blastocyst development and viability following the elimination of waste products from embryonic metabolism. Refreshment itself may also have a beneficial effect, simply due to renewal of the metabolic nutrients in a new medium drop. However, the corollary to this is the possible negative impact on embryo development of the additional handling required.

The benefits of continued improvements to blastocyst culture conditions would appear to be limited by genetic factors, since the presence of oocyte and sperm genetic abnormalities will prevent a proportion of the pronuclear embryos from reaching the blastocyst stage, independent of culture conditions. The majority of these abnormalities are chromosomal, as ~25% of oocytes are aneuploid (Kola *et al*., 1993; van Blerkom, 1994). Since the number of blastocysts that develop in culture reflect the quality of the gametes from which they were derived (Behr *et al*., 1998), further improvements in media used for blastocyst culture may only allow limited improvements in blastulation rate. In a recent prospective study comparing sequential blastocyst culture with co-culture with Vero cells, no significant difference in take-home baby rate was observed (Giscard d'Estaing *et al*., 2001). Moreover, in a comparison of two commercially available sequential culture systems, no

difference in outcomes in the blastocyst programme was observed (Van Langendonckt *et al*., 2001). Further development towards optimal culture conditions for viable blastocyst development may therefore require a new approach.

At present however, the design of blastocyst culture media continues to focus primarily on the successful production of viable embryos. In this, the first randomized prospective study comparing sequential culture with monoculture of human blastocysts, no significant differences in blastulation and implantation rates were observed between the Rotterdam medium used in monoculture and serial culture or the sequential G1.2 and G2.2 media system. It may be concluded that the Rotterdam medium contains the nutrients required by the preimplantation embryos at the different stages of development. The applicability of our results to other laboratory settings is inevitably limited by other local factors which may have affected the study outcome. However, the results of this study indicate that human embryos do not necessarily have to be cultured in specially designed sequential media to fully develop into viable blastocysts capable of resulting in ongoing pregnancy.

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