Edward E. Wallach, M.D.
Associate Editor

Choosing a culture medium: making informed choices

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Objective: To analyze critically the reasons justifying the choice of two-step protocols requiring two media for the culture of human preimplantation embryos from the zygote to the blastocyst.
Design: Literature review.
Result(s): Two types of protocol are used for the culture of human preimplantation embryos from the zygote to the blastocyst, using either one medium (one-step protocol) or two media of different composition (two-step protocol). Two-step protocols are the most widely used, largely because all but one of the commercially available protocols are of this type. The reasons for the adoption of two-step protocols are described and critically analyzed. They are based on considerations of the functions of glucose, ethylenediaminetetraacetic acid (EDTA), glutamine, and amino acids that are included in the media. A reappraisal of the reasons for selecting two-step protocols is important because recent animal experiments and clinical observations have raised doubts as to whether the more complex, two-step protocols have any advantage over one-step protocols. The analyses show that all of conclusions reached should be considered equivocal.
Conclusion(s): Clinical embryologists should evaluate the justification for selecting two-step protocols for the culture of human preimplantation embryos from the zygote to the blastocyst. (Fertil Steril® 2008;90:473–83. ©2008 by American Society for Reproductive Medicine.)

Key Words: Embryo culture, one-step and two-step protocols, energy sources, EDTA, glutamine, amino acids

Historically, it was customary to use media that permitted the culture of human zygotes for 2 to 3 days to reach the four- to eight-cell stage, at which time the embryos were replaced in the patient (1). However, since 1997, the extended culture of zygotes to the blastocyst stage has attracted more attention (2). One reason for this interest was based on the argument that the extended culture of human embryos to the blastocyst stage may provide a means to select more robust embryos for replacement in the patient. This would permit the transfer of fewer embryos with higher implantation rates without, in turn, affecting pregnancy rates, while at the same time reducing the incidence of multifetal pregnancy. Later, a second reason was based on the more widespread use of preimplantation genetic diagnosis where, typically, blastomere biopsy is performed on day 3 cleavage stage embryos and requires the extended culture to day 5 so that the results of the biopsy are available before intrauterine blastocyst transfer. There is now another reason for the development in vitro of robust blastocysts: the generation of embryonic stem cells. We will focus in this review on the culture of human zygotes to the blastocyst stage. Nevertheless, some of our comments are equally valid for the culture of the one-cell zygote to day 3 embryos alone, and thus of relevance to those clinical IVF programs that mainly perform day 3 embryo transfers.

Three types of protocol can be used for the culture of zygotes to the blastocyst stage: [1] uninterrupted culture using one medium throughout the 5 days of culture (*nonrenewal single medium protocol*), [2] interrupted culture where one medium is used throughout but is renewed on the third day of culture (*renewal single medium protocol*), and [3] interrupted culture where two media of different composition are used sequentially (*sequential media protocol*). The renewal or change of medium occurs typically at the seven- to eight-cell stage of development. Surprisingly, all but one of the commercially available protocols for the culture of human preimplantation embryos to the blastocyst stage are of the sequential media type (Table 1).

Sequential media protocols have been actively promoted by a number of clinical embryologists involved with human IVF programs, most notably Gardner and Lane (2–4) and Pool (5, 6). We have previously commented that there is very little, if any, direct experimental evidence that sequential media protocols are better than one-step protocols (1, 8–11). It is thus instructive to consider why sequential media protocols have become so popular in human IVF. In general, arguments

Received August 5, 2008; revised and accepted August 5, 2008.
J.D.B. is a consultant to IVFOnline. M.C.S. has nothing to disclose.
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0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2008.08.010

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TABLE 1

Commercial systems for the culture of human preimplantation embryos.

Commercial sequential two-media systems:

Company	Sequential media
Cook IVF	Sydney IVF cleavage medium/ Sydney IVF blastocyst medium
CooperSurgical	Quinn's Advantage cleavage medium/Quinn's Advantage blastocyst medium
FertiPro N.V.	FERTICULT/FERTICULT G3
InVitro Care Inc.	IVC-ONE/IVC-TWO
Irvine Scientific	ECM/MultiBlast Medium
MediCult	EmbryoAssist/BlastAssist
Vitrolife	G-1 v5 PLUS/G-2 v5PLUS

Commercial single-medium system:

Company	Medium
IVFonline	Global

Biggers. Preimplantation embryo culture. Fertil Steril 2008.

in favor of sequential media protocols have focused on four areas: [1] the changing energy requirements of the preimplantation embryo and the inhibitory effect of glucose on early cleavage stage embryos, [2] the inhibitory effect of ethylenediaminetetraacetic acid (EDTA) on blastocyst development and the inner cell mass (ICM), [3] the chemical breakdown of L-glutamine (Gln) in aqueous solution and the accumulation of ammonia and its effects on embryo development, and [4] the role of amino acids on preimplantation embryo development. We shall consider each of these areas in turn, and show that all are equivocal reasons for justifying sequential media protocols for the extended culture of human preimplantation stage embryos. Finally, we will summarize recent results of studies on mice and on clinical IVF patients where the effectiveness of one-step and two-step protocols are directly compared. It should be noted that many of the studies that resulted in the use of different culture media for use in human IVF, such as HTF, P-1, G1/G2 types, and KSOM and derivatives (see below) were either largely or exclusively undertaken using mouse embryos. For this reason much of this review will focus on the outcome of studies involving mouse and human embryos. However, we will refer to the results of other animal studies when considered germane to the discussion.

ARE THE REASONS FOR USING SEQUENTIAL MEDIA UNEQUIVOCAL?

Energy Requirements and the Inhibitory Effect of Glucose

Studies by Whitten (12, 13) showed that glucose did not support the development of the preimplantation mouse embryo before the eight-cell stage, and that lactate did not support development before the two-cell stage. Detailed investigations by Biggers et al. (14) demonstrated that during oogenesis the energy pathways become restricted such that only pyruvate and three other closely associated intermediates in the energy pathway support oocyte maturation and division of the one-cell stage. During embryo cleavage the energy pathways become less restricted, and by the eight-cell stage the Embden-Meyerhoff pathway is fully functional. These observations led to the routine incorporation of pyruvate into media for the culture of the initial stages of development of all mammalian species, including humans (15). Leese and Barton (16), using sensitive microflouresence techniques to analyze metabolism in single embryos, showed that pyruvate was the preferred energy substrate used by the mouse mature oocyte and zygote, and that by the eight-cell stage glucose became the preferred carbon source. Later studies by Leese and coworkers (17) demonstrated in the human preimplantation embryo that the uptake of glucose relative to pyruvate increased after the eight-cell stage. However, the uptake of pyruvate by the human preimplantation embryo remained high throughout development to the blastocyst stage and does not sharply decline as in the mouse (18, 19). Based on these observations it was commented by Gardner (2):

> in order to support development of a competent zygote to the blastocyst stage, one needs to use more than one culture medium to take into account the significant changes in embryo physiology and metabolism which occur during the preimplantation period.

Similarly, as noted by Pool (5):

> Although the goals of blastocyst production are well defined the classic approach of using a single medium throughout the entire [sic] culture period has proven ineffective, as it fails to recognize that the physiology of preimplantation embryos is dynamic.

These quotes reflect the widespread belief that it is essential to use two media of different composition sequentially to mimic knowledge of the physiology of the mother and the embryo. We have previously argued that this belief is based on an heuristic argument that depends on the truth of three hypotheses (1, 7): first, that the environment of the preimplantation embryo changes as it travels from the ampulla of the oviduct to the uterus; second, the chemical compositions of the environments that an embryo encounters have evolved so as to be highly correlated with the embryo's metabolism; and third, that glucose in all species inhibits the first cleavage division of preimplantation embryos.

Extant evidence used to support the assumption that the environment of the human preimplantation embryo changes as the embryo traverses the oviduct to the uterus is weak (1, 7), because it is based on the analyses of glucose, lactate, and pyruvate in oviductal and in uterine fluid collected from nonpregnant women (20). Fluid was collected (typically 0.5

μL) from the ampulla of the oviduct and the uterus from women at different stages of the menstrual cycle undergoing routine diagnostic laparoscopy. The concentrations of glucose, pyruvate, and lactate in the samples were then measured. The assumption is then made that the difference between the concentrations of glucose, pyruvate, and lactate in the ampulla at midcycle, and the uterus in the luteal phase simulates the changes experienced by the embryo as it passes down the oviduct to the uterus. The observed changes in concentrations were: glucose 0.5 to 3.15 mmol/L, pyruvate unchanged at 0.24 mmol/L, lactate 10.5 to 5.87 mmol/L. The fact that these data have been determined on nonpregnant patients and that only two compounds have been shown to change hardly justifies the sweeping generalization made by Pool (6) on the results of the Gardner study (20) as:

> The most important study providing direct evidence that human embryos experience profoundly different nutritional environments during preimplantation embryogenesis…

Studies of oviductal fluid have been used, together with the information of changes in the preferential use by the preimplantation embryo of glucose and pyruvate, to support the second assumption that the chemical composition of the environments that an embryo encounters have evolved so as to be highly correlated with the embryo's metabolism. It should be recognized that this intuitively attractive assumption, however, is merely a speculation that cannot be confirmed or disproved experimentally. Furthermore, this speculation lacks rigor, because it overlooks the possibility that embryos in culture may adapt and selectively use what they require from a constant environment as long as the constituents fall within tolerable ranges (17).

The third assumption that has been advanced to support the use of sequential media is that glucose inhibits early development in several species, including the human embryo in culture. Indeed, this belief is now dogma (1, 7, 21). For example, Devreker and Englert (22) wrote:

> While pyruvate is essential for the early preimplantation embryo, glucose has been shown to compromise the viability in the embryo in vitro.

The same reviewers comment further that

> Glucose is also toxic for the preimplantation development of embryos of other species including outbred strains of mice, hamsters, cows, sheep.

Moreover, it has been recommended by Pool (5, 6) that for the culture of human embryos,

> In the first (culture) interval, glucose should be reduced to 0.5 mmol/L or omitted completely.

However, a careful analysis of the literature shows that the unqualified dogma that glucose is detrimental to early preimplantation embryos is untenable. We have previously reviewed in detail the effects of glucose on early mammalian embryo development (1, 7), and thus will limit the present discussion to a few salient points. The findings of Schini and Bavister (23) in 1988 that glucose and phosphate caused arrest of preimplantation development of the hamster at the two-cell stage greatly influenced the design of media for the culture in vitro of a large number of species of preimplantation embryos, including humans. Their observations were a major influence on the design of medium CZB, which does not contain glucose and inorganic phosphate (24). Subsequently, a modification of Quinn's medium HTF for the culture of human embryos was formulated in which glucose and phosphate were omitted and EDTA and glutamine added (25). Additional changes resulted in medium P1 which is, in fact, a modified form of Quinn's glucose-free and phosphate-free modified HTF medium in which taurine and citrate are substituted for glutamine and EDTA, respectively (26). The omission of glucose from culture media for the initial stages of preimplantation development, nevertheless, seems paradoxic, because the oviduct contains significant amounts (20, 27–29), and ignores the fact that media exist where glucose is not harmful to early preimplantation development (1, 7). It now seems increasingly likely that the concentrations of other components and their interactions in the culture media determines whether glucose inhibits early embryo development (1, 7, 21). Such interactions can be identified by the experimental determination of a concentration–response surface. For example, a two-dimensional concentration response surface was determined for the joint effects of glucose and phosphate in medium KSOM on the development of CF1 outbred mouse zygotes to blastocysts (21). The results showed no significant interaction between the concentrations of glucose and phosphate, demonstrating that the effects of glucose and phosphate are independent. No significant concentration–effects of glucose on blastocyst formation and hatching were observed, that is, the glucose concentration–response surface is flat for all concentrations of phosphate. In those cases where the concentration–response surface is flat it is not unreasonable to use the measured in vivo concentration of that component.

The need to exclude glucose and phosphate or use glucose in a low concentration is touted in the advertisements of several commercially advertised media. This precludes recognition of the possibility that media could be designed to support early human development in which glucose and phosphate are present in concentrations approximating their normal ranges. Evidence has been presented that glucose does not inhibit development of the early human embryo in some media (30–32). The findings from these studies highlight the fact that glucose does not always inhibit the initial stages of human preimplantation embryo development, and that the removal of glucose from the culture medium should be considered nonphysiologic. For reasons stated above, glucose should be included in media at the concentrations typically found in the human oviduct, approximately 1.0–2.0 mmol/L.

The Role of EDTA

Early studies of mouse preimplantation development were complicated by the two-cell block, typically seen in outbred and inbred mouse strains (33, 34). Abramczuk et al. (35) overcame this block by adding EDTA to Whitten's medium (13). Other studies soon confirmed this result using other media (36–39). Medium CZB (24), for example, contains 0.1 mmol/L EDTA. Gardner and Lane (40) described studies of mouse preimplantation embryo development using a two-step protocol supplemented with EDTA at a concentration of 0.1 mmol/L. They reported that 0.1 mmol/L EDTA caused decreased cell counts in the ICM of mouse blastocysts and that the body weights of fetuses following transfer to surrogate mothers were significantly lower than blastocyts not exposed to EDTA. They described similar effects of 0.1 mmol/L EDTA on the in vitro development of bovine blastocysts (41). Consequently, they adopted a two-step protocol in which EDTA was removed from the second of their two culture media to obtain more viable blastocysts. Earlier studies, however, had indicated that EDTA added at lower concentrations, typically 0.005–0.01 mmol/L, also overcame the two-cell block in most mouse strains (35, 37, 38). For example, the optimum concentration of EDTA needed in medium SOM (42) that was developed using the experimental strategy, sequential simplex optimization (and its derivative, KSOM), was determined to be 0.01 mmol/L, an order of magnitude less than that used in the studies of Gardner and Lane (40, 41). Furthermore, EDTA at a concentration of 0.01 mmol/L does not have a deleterious effect on either mouse preimplantation embryo development or postimplantation development following embryo transfer to surrogate mothers (10, 11) Consequently, by using a low concentration of EDTA it is unnecessary to adopt a two-step protocol to avoid the deleterious effects observed at higher concentrations of EDTA as the blastocyst develops.

L-Glutamine (Gln) and Ammonium

It was a cause for concern when Gardner and Lane (43) showed that ammonium accumulation in embryo culture media can compromise embryo development. In addition, they observed cases of exencephaly in mice whose preimplantation embryos had been cultured in vitro, and suggested that the congenital abnormality may be due to the presence of Gln in the medium (44). What is the likelihood that congenital abnormalities occur in human babies whose preimplantation embryos had been cultured in media containing Gln? Although we have previously discussed this topic in a number of reviews, articles and commentaries (7, 45, 46), there still seems to be considerable degree of confusion about the subject.

In summary, Lane and Gardner (44) suggested that the exencephaly was the result of the well-known instability of Gln in aqueous solution where it breaks down fairly rapidly into equimolecular amounts of ammonium and pyrrolidine-5-carboxylic acid. The putative toxicity of Gln on preimplantation embryos in vitro should not be exaggerated. First, Gln has been included in most chemically defined media designed for the culture of preimplantation embryos, following the studies of Daniel (47), Naglee et al. (48), Kane and Foote (49) on rabbit embryos, and Menezo (50, 51) on bovine and human embryos. Chatot et al. (24) included Gln at a concentration of 1 mmol/L in medium CZB to help overcome the two-cell block seen in outbred mouse strains, based on the work of Carney and Bavister (52) on the hamster embryo. Gln, at a concentration of 1 mmol/L, was also found to be the optimum concentration that overcame the two-cell block in the development of SOM using sequential simplex optimization (42), and this concentration was used in medium KSOM (53). The same concentration of 1 mmol/L Gln was used by Gardner and Lane in their studies of sequential media, including G1/G2 type media (2), DM1/DM2 (40), and DM2/DM3 (54). In none of these studies has the occurrence of fetal aberrations been reported. Second, exencephaly has not been confirmed in mice in studies performed since the first reports of Gardner and Lane (44). We believe that concerns about ammonium derived from the chemical breakdown of Gln and to a lesser extent other amino acids that occurs during the culture of preimplantation embryos have been overstated (see below).

To study the putative toxicity of Gln further, Lane and Gardner (44) simulated the effect of Gln with exogenous ammonium. The addition of ammonium in the form of ammonium chloride to a modified mouse tubal fluid medium (29) resulted in a concentration-dependent relationship between the ammonium concentration in the medium and embryo viability that included decreased blastocyst cell counts, reduced implantation rates, retarded fetal growth, and an increase in the incidence of exencephaly after transfer of cultured embryos to surrogate mothers. Lane and Gardner (55) subsequently described additional studies on the effects of added ammonium on mouse preimplantation embryo development. We have previously questioned this strategy of simulating the effects of Gln breakdown in a culture medium with high concentrations of exogeneous ammonium (7, 11, 45). Nakazawa et al. (56) provided direct analytical evidence that under conditions used for the culture of preimplantation embryos the buildup of ammonium is principally derived from the spontaneous breakdown of Gln, showing that the rate of breakdown of Gln in aqueous solution is largely independent of the culture system. Thus, for a simulation study using ammonium to be valid it is important that the ammonium be added in a way that duplicates the kinetics of the spontaneous breadown of Gln in aqueous solution. By using the published description of the chemistry and kinetics of the chemical breakdown of Gln in aqueous solution we concluded that the conditions used by Lane and Gardner (7, 45, 46) did not meet this requirement.

The putative toxicity of Gln was one of the reasons that Gardner and Lane (43, 44) introduced their use of a two-step protocol in which the medium is renewed half way through the culture period to remove the accumulated ammonium. The entire controversy is rendered irrelevant, however, by replacing Gln with a stable dipeptide of Gln,

such as L-alanyl-L-glutamine (AlaGln) or glycyl-L-glutamine (GlyGln) (46). Using these compounds it becomes unnecessary to use a second medium, thus eliminating a primary reason for using a two-step protocol.

Role of Amino Acids

It is important to recognize that amino acids are not required for the morphologic development of mouse zygotes into blastocysts in vitro (57, 58). However, these early studies used simple chemically defined media supplemented with a protein source, bovine serum albumin (BSA), that may also be a source of amino acids (58). Fissore et al. (38) showed high rates of development of mouse zygotes to blastocysts in a simple salt solution with no added protein, but supplemented with EDTA. Subsequently, Mehta and Kiessling (59) obtained viable offspring following embryo transfer of mouse embryos cultured in amino acid-free, protein-free simple chemically defined media containing EDTA. Caro and Trounson (60) reported successful development of two-cell mouse embryos to blastocysts in protein-free media. Later they described the fertilization of human oocytes and their development to cleavage stage embryos in a simple culture medium containing no fixed nitrogen source with subsequent successful pregnancies (61). Nevertheless, the beneficial effects of amino acids on preimplantation embryo development have been recognized for many years in several species, beginning with the work of Brinster (58) in the mouse (1, 7, 26, 62–64). Daniel (47), at about the same time as Brinster, studied the effects of varying the concentrations of single amino acids on the rates of blastocoel expansion in rabbit embryos in medium Ham's F10 (65) supplemented with 15% normal rabbit serum. In 1995, Ho et al. (66) presented findings on the culture and development of mouse zygotes in medium KSOM supplemented with half the concentrations of amino acids used by Eagle (67) for the culture of human cancer cells. At this concentration, the inclusion of all 20 amino acids throughout the preimplantation period gave the highest rates of blastocyst development. They noted that these results were obtained without a change of media. Importantly, the rigorous proof that amino acids influence the development of preimplantation embryos in vitro requires that they be tested in a base medium that does not contain other proteins, such as BSA (58, 68). Toward this end, Biggers et al. (69, 70) studied the effect of varying the concentrations of amino acids on the development of mouse preimplantation embryos in the medium KSOM in which BSA was replaced with polyvinyl alcohol. These experiments showed that raising the concentrations of the set of all 20 common amino acids had little effect on the rate of blastocyst formation, but significantly increased the rate of hatching, and, more importantly, significantly increased the total numbers of cells in the blastocysts, particularly in the ICM. Further, the extracellular matrix in the blastocysts was more normally organized when amino acids were present. The dose–response studies showed that the overall maximum effect was attained when using half the concentrations of AAs in Eagle's medium (67).

What are the optimum concentrations of individual AA needed in a medium for the culture of preimplantation embryos? Is only a subset of the 20 natural amino acids necessary? Two experimental strategies have been used to determine the concentrations of amino acids to include in media. Either the effects of each amino acid can be examined by supplementing a base medium with each AA individually (47), or by supplementing a base medium with the complete set or subset of all 20 common AAs. The concentrations of the individual AAs used have been chosen in several ways by reproducing: [1] the AA composition of a natural protein, such as BSA (58); [2] the AA composition of follicular fluid (56), where the levels are typically one-half to one-third that described by Eagle (67); [3] the AA compostion of oviductal fluid, for example, sheep (71), humans (28, 72–74). These approaches are, however, approximate at best because the choice of BSA is arbitrary, and measurements of the composition of oviductal fluid can be subject to significant sampling errors (1, 7).

A different approach for determining the AA requirements of mouse preimplantation embryos in vitro has been used by Gardner and Lane (26, 43, 75, 76). They used the so-called *essential* (EAA) and *nonessential* (NEAA) amino acids defined by Eagle in 1959 (67). An understanding of the studies of Gardner and Lane and of their interpretation of the results is important because it has played a large part in the introduction of sequential two-step methods for the culture of human preimplantation embryos. We have previously reviewed in detail the AA studies of Gardner and Lane (1, 7). The essential feature emerging from their AA studies is that the first component in their sequential media protocol, G1 contains only NEAA, whereas the second component, G2, contains both NEAA and EAA. A number of caveats need to be reiterated, however, regarding work using experiments based on EAA and NEAA; EAA and NEAA are commercially available mixtures based on experimentally determined AA requirements of human cancer cells (67). Because mouse zygotes develop into blastocysts in media that do not contain amino acids (13, 57, 59, 68), none of the 20 amino acids can be considered absolutely essential. Consequently, the classic nutritional sets of EAA and NEAA have little relevance to the absolute amino acid requirements of preimplantation embryos (1, 7, 23). Moreover, the concentrations of AAs in EAA and NEAA are markedly higher than the measured levels in both human follicular fluid (56, 77) and human oviductal fluid (28), two biologic fluids that have been analyzed to assist in the formulation of chemically defined media for the culture of human preimplantation embryos. Furthermore, these natural fluids contain all 20 natural amino acids. Perhaps, more importantly, have been the data recently accrued by Leese and colleagues on amino acid turnover in preimplantation embryos from a number of species. Lamb and Leese (78) first described in 1994 the results of studies that demonstrated the feasibility of analyzing the net turnover of most of the common amino acids simultaneously during different stages of mammalian preimplantation embryo development in vitro. Leese et al. (73, 74) have used the measured concentrations of amino acids in human oviductal

fluid to assess the use and metabolism of amino acids by human preimplantation embryos in vitro. They showed that different patterns of use of individual amino acids by day 2/3 cleavage-stage embryos was predictive of subsequent blastocyst development (73). A later study showed a correlation between amino acid turnover of day 1/2 cleavage stage human embryos and subsequent pregnancy following embryo transfer (74). It was further noted that there were changes in the patterns of amino acid turnover throughout human preimplantion embryo development in vitro, including changes in net amino acid turnover of those amino acids grouped under EAA the most notable of which are L-methionine, L-leucine, L-isoleucine and L-lysine. Leese and colleagues (72, 73) have questioned, as have others (1, 7), the recommendation of Gardner and Lane (75, 76) to culture cleavage-stage human embryos in media supplemented only with NEAAs. Leese (73) notes that of the amino acids that show a net depletion during human preimplantation embryo development none fall into the NEAA category. Leese et al. (72) further comment,

> … This requirement would obviously not be fulfilled by culture media which included only nonessential amino acids during the early preimplantation phase . . . Our data lead us to favour including all 20 amino acids in human embryo culture at concentrations believed to be physiological, and let the embryo choose which to consume or release and in what quantities

Leese and coworkers (79–81) have described similar AA turnover studies on both bovine and porcine embryos. Parenthetically, Leese has recently argued in favor of "quiet" embryo metabolism during preimplantation embryo culture and development to produce the most viable embryos (82–84). This condition would be met by using all 20 common amino acids at low concentrations throughout preimplantation embryo development in vitro. How best do we determine the optimal concentration range of each of the 20 common amino acids for use in embryo culture media? The direct experimental determination of the concentrations of each of the common amino acids to include in a medium is a formidable task, because these can be determined only by taking into account the interactions between their different effects. These joint effects can be theoretically represented by a concentration–response surface in 21-dimensional space (42, 85). To explore this surface with a factorial experiment using three concentrations of each amino acid would require the comparison of $3^{20} = 3{,}486{,}784{,}401$ media! Seeking the combination of concentrations that give a maximum response using sequential simplex optimization would be logistically feasible, although the procedure becomes less efficient as the number of factors to optimize increases. To date, the strategies that have been employed to determine the concentrations of each of the amino acids to use in media for the culture of preimplantation embryos do not take account of possible interactions between the effects of these compounds. Thus, it is certain that no currently used media contain optimal concentrations of the different amino acids. Available media for the culture of human preimplantation embryos that only use NEAA during the first culture period may be particularly unbalanced.

In summary, the experimental findings and reasons that have hitherto been used to justify the use of a variety of different sequential media are in fact equivocal.

SINGLE MEDIA AND SEQUENTIAL MEDIA PROTOCOLS AND THE CULTURE OF HUMAN PREIMPLANTATION EMBRYOS

Over the years a number of different sequential protocols have been used for the in vitro culture of mammalian embryos, including human embryos (1, 7). However, sequential media protocols did not attract widespread interest until a pair of media was introduced, denoted G1 and G2, for the extended sequential culture of human zygotes into blastocysts (2, 63, 86). Other sequential protocols for the culture of human embryos soon followed. For example, Jones et al. (87) recommended an alternative protocol in which human zygotes were cultured in medium IVF-50 (Scandinavian IVF Sciences AB [now Vitro Life, Gothenburg, Sweden]) for 2 or 3 days, followed by culture in medium G2 for a further 2 to 4 days. A number of companies are now promoting sequential media for the culture of human preimplantation embryos (Table 1).

It has been stated many times in the scientific and clinical literature that a sequential media protocol is superior to a protocol in which a single medium is used throughout without renewal, despite the fact that very few studies have actually been undertaken to test this assertion. Recently, Biggers et al. (10) compared directly the in vitro development of mouse embryos using two sequential media protocols, G1.2/G2.2 and DM1/DM2, with a nonrenewal single medium protocol, $KSOM^{AA}$. No significant differences were observed in the proportion of blastocysts, rates of hatching, and differential cell counts when these different media were compared. Fetal viability after embryo transfer was, however, higher in embryos cultured in $KSOM^{AA}$ when compared with G1/2.G2.2. Recently, Perin et al. (88) compared commercially available $KSOM^{AA}$ (Millipore Corp, Billerica, MA) using a renewal single medium protocol and G1/G2 using a two-step sequential media protocol for the culture of mouse one cell embryos in an in vitro fertilization system. Enhanced blastocyst development was observed when $KSOM^{AA}$ was used. However, no embryo transfer experiments were done in this study. Consequently, although sequential media protocols are sufficient for the support of preimplantation mouse embryos in vitro from the zygote to the blastocyst, they are not necessary, and can be replaced by a nonrenewal single medium protocol. It is thus important to know if this finding applies to culture media used in human IVF.

One of the difficulties in the interpretation of clinical outcomes data from human IVF is the heterogeneous nature of the patient population, which includes the age of the patient, infertility diagnosis, clinical protocols used for ovarian stimulation, and so on. Moreover, there is the confounding issue

TABLE 2

The percentage human zygotes that develop into blastocysts over 5 to 6 days in global medium and in several sequential media observed in several clinics.

	Single medium (Global)		Sequential media		
Reference	**Protocol**	**Blastocysts**	**Protocol**	**Blastocysts**	***P***
Freeman and Rieger[a]	Renewed	36% (71/198)	IVC-1/G2	26% (52/199)	.036
Greenblatt et al.[b]	Renewed	53% (29/55)	G1/G2	38% (21/55)	.180
Angus et al.[c]	Renewed	58% (197/337)	GIII	50% (131/261)	.047
Kumagai et al.[d]	Renewed	54% (79/149)	Quinn's cleavage/Multiblast Irvine	48% (71/147)	.486
Matsubara et al.[e]	Renewed	46% (73/187)	BAS Medicult	39% (90/195)	.179
Sepulveda et al.[f]	Renewed	44% (117/268)	IrvineECM/Multiblast	33% (93/283)	.011
Zech et al.[g]	Renewed	44% (290/667)	G1.3/G2.3	35% (170/491)	.003

Note: Fisher's exact test for a 2×2 contingency table used to compute *P*.

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of the quality control procedures used by each clinical IVF laboratory. Consequently, the choice of media for the culture of human embryos represents only one part of a quality control process that is designed to reduce the incidence of pathophysiologic stresses during embryo culture (1, 7). Gardner and Lane (3, 89), in particular, have emphasized the importance of establishing good quality control and quality assurance measures in human IVF. These various factors must be considered when undertaking and comparing studies that involve human IVF subjects.

A preliminary study by Biggers and Racowsky (9) showed that human blastocysts develop from zygotes with high efficiency in 5 days of culture using medium KSOM^{AA} without renewal of the medium. The yield did not differ from the efficiency of a sequential media protocol used routinely in the human IVF laboratory. Further, Macklon et al. (32) compared in a prospective randomized study a nonrenewal single medium protocol (17:3 mixture, Earle's balanced salt solution: Ham's F10 medium supplemented with albumin, [the so-called Rotterdam medium]), and a sequential media protocol (G1/G2) for the culture and development of human blastocysts derived from IVF. They reported no difference between the effects of the media on blastocyst formation, implantation rates, or pregnancy.

A commercially available version of KSOM^{AA} (Global medium; see Table 1), has frequently been used in a renewable single-medium protocol for the culture of human preimplantation embryos. The use of this protocol is justified mainly as a means of removing toxic substances that accumulate in the medium derived from contaminants in the plastics used. Recently, several preliminary studies have been undertaken comparing the development of human preimplantation embryos using Global in a renewable single-medium protocol and several other commercially available sequential media protocols. The results are summarized in Tables 2 and 3. Although all of these results have only been reported in preliminary abstract form, and have not been subject to peer review, they consistently suggest that the use of a renewable single medium (Global) results in the development of blastocysts at the same or higher rate than the several sequential media protocols (Table 2). Furthermore, four of these

TABLE 3

The percentage of ongoing pregnancies that arise from blastocysts cultured from human zygotes.

Reference[a]	Single medium (Global)		Sequential media		P
	Protocol	Pregnancies	Protocol	Pregnancies	
Greenblatt et al.[b]	Renewed	43% (?)	G1/G2	42% (?)	—
Angus et al.[c]	Renewed	40% (12/30)	GIII	28% (8/29)	.412
Matsubara et al.[e]	Renewed	41% (7/17)	BAS Medicult	40% (6/15)	1
Zech et al.[g]	Renewed	49% (21/43)	G1.3/G2.3	40% (9/22)	.606

[a] For reference numbers, see Table 2.

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studies also reported on the pregnancy rates following embryo transfer (Table 3). The findings are that the pregnancy rates using Global and sequential media were not significantly different.

Thus, on the basis of the currently available evidence we tentatively conclude that sequential protocols have no advantage over single renewable protocols for the culture of human preimplantation embryos. We wish to emphasize that we are not stating that single medium culture methods are superior to sequential methods for this purpose, but that the latter may not be needed given our current state of knowledge of the needs of the human preimplantation embryo in culture.

Eventually, each human IVF clinic will need to decide which protocols best fit their particular practice. Single media protocols have several important advantages over sequential media protcols. For example, single media protocols offer the advantage of cost and simplified laboratory management when compared with sequential protocols. Table 4 summarizes some of the characteristics and practical differences between single media, renewable single media and sequential media protocols.

In conclusion, the field of clinical human IVF is dominated by the view that a two-step, sequential protocol is necessary to imitate the metabolic milieu encountered by preimplantation embryos in vivo. Pool (5) comments, for example, that,

> the maximum number of viable, late stage human embryos is produced through the use of a sequential culture system.

The advantages of a single medium protocol together with doubts about the validity of the scientific reasons for recommending sequential media protocols, and the increasing evidence that single-media protocols results in development as good as that obtained with sequential media should cause clinical embryologists to question this unsubstantiated statement and critically reexamine the way in which they select protocols for the culture of human embryos.

Future research on the design of media for the culture of preimplantation embryos will focus mainly on the avoidance

TABLE 4

Comparison of some general characteristics of a nonrenewal single medium, a renewal single medium, and a sequential medium.

Characteristic	Single medium (nonrenewed)	Single medium (renewed)	Sequential media
Leaves embryos undisturbed	Yes	No	No
Accumulated endogenous growth factors	Left in place	Lost	Lost
Replacement of essential nutrients	No	Yes	Yes
Accumulated toxins	Left in place	Removed	Removed
Relative environmental stress to embryos	Low	Moderate	High
Required quality control	One medium	One medium	Two media
Relative labor intensity	Low	Moderate	High
Relative cost	Low	Low	High

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of epigenetic effects (7, 90–92). Advances in this area will require replacing the classical way in which we look at embryos in terms of morphology and biochemistry, and focusing more on the effects of culture media on gene regulatory networks, the chronicity of gene expression patterns during early embryonic development and the fluxes they control through networks of metabolic reactions (91). Such studies involve the techniques being developed in systems biology. The study of the effects of glucose starvation in yeast, *Saccharomyces cerevisiae*, where genes of the glycolytic cycle are down regulated and the genes of the TCA cycle are upgraded as metabolism switches from anaerobic to aerobic metabolism may be a useful initial model for metabolic studies in preimplantation embryos (93). A beginning has been made on analyzing these networks in preimplantation mouse embryos. It is known that up-regulation and down-regulation of genes in mouse preimplantation embryos are affected by the composition of culture media (94). Solter et al. (95) have recently used the systems biology approach to study such phenomena in the two-cell mouse embryo.

Acknowledgments: We thank Dr. Betsey S. Williams for helpful comments on the manuscript.

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ADDENDUM

The preliminary data of Sepúlveda et al. (see Table 2) has been published in full: Sepúlveda S, Garcia J, Arriaga E, Diaz J, Noriega-Portella L, Noriega-Hoces L. In vitro development and pregnancy outcomes for human embryos in either a single medium or in a sequential media system. Fertil Steril 2008. Published online 25 April 2008 [Epub ahead of print].