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Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues

Michael C.Summers[1] and John D.Biggers[2,3]

[1]Reproductive Science Center, Lexington, MA and Department of Obstetrics and Gynecology, University of Massachusetts Medical School, Worcester, MA and [2]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

[3]To whom correspondence should be addressed at: Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA. E-mail: john_biggers@hms.harvard.edu

Considerable advances in media development for the culture of preimplantation mammalian embryos have been made since mouse embryos were first cultured and successfully transferred to foster mothers. The purpose of this review is to detail the history of the development of chemically defined media for the culture of preimplantation embryos. Two approaches have been used to determine the composition of chemically defined media: the 'back-to-nature' approach and 'let the embryo choose' or empirical optimization approach. Recent developments, including the supplementation of media with amino acids and the use of sequential media for the extended culture of preimplantation embryos, are critically assessed. Importantly, it is recognized that even the best media currently used are not optimal and inevitably cause imbalances and stress to the embryos. Consequently, preimplantation embryos must adapt to the culture environment in order to survive. The adaptations to stress that occur when embryos are placed in a chemically defined environment are reviewed. The implications of these various stresses on the patterns of gene expression in the early embryo and their potential long-term effects are also emphasized. The scientific and ethical issues raised by the commercialization of human embryo culture media are briefly addressed.

Key words: amino acids/culture media/extended culture/KSOM/sequential media

Introduction

A relatively short span of time elapsed between the pioneering studies (Harrison, 1907) in which a biological medium was used for tissue culture and further studies by Lewis and colleagues (Lewis and Lewis, 1911a; b; 1912) on embryonic chick tissues using a chemically defined medium. The potential value of chemically defined media was recognized by Lewis and Lewis who commented: ''It is to be hoped that an artificial medium will be found as satisfactory as the plasma, for the advantages are obvious if one can work with a known medium in the investigation of the many new problems, which suggest themselves.''

The design of chemically defined media accelerated in the 1940s with the publication of the composition of media for plant cells (White, 1946) and animal cells (Fischer, 1947). During the following years, a number of other chemically defined media were introduced (for reviews, see Biggers *et al*., 1957; Waymouth, 1965; 1972).

The culture of early mammalian embryos was studied principally in two species: the rabbit and the mouse. The rabbit was almost used exclusively in the early studies using media composed of ill-defined biological fluids, such as the studies of Brachet and Pincus (for a review, see Biggers, 1987). The possibility of experimentally studying the mammalian preimplantation embryo *in vitro* was realized by Whitten (Whitten, 1956), who reported that 8-cell mouse embryos would develop into blastocysts when cultured in a simple chemically defined medium containing only nine components, including water. In 1958, this medium was used (McLaren and Biggers, 1958) to produce blastocysts that later developed into outwardly normal young after being transferred into the uterus of surrogate mothers. By the use of genetic markers, it was possible to rule out that the surrogate mothers produced young from their own oocytes. In 1957, it was reported (Whitten, 1957) that the 2-cell stage mouse embryo, but not the zygote, would develop into a blastocyst if calcium lactate replaced calcium chloride in the initial medium. Subsequently, Brinster undertook a systematic study of the preimplantation mouse embryo in Whitten's medium (Brinster, 1963; 1965a; b; c; d; Biggers and Brinster, 1965; Brinster and Thomson, 1966). Further studies eventually showed that the mouse oocyte and 1-cell embryo stage were dependent on the presence of pyruvate in the medium (Biggers *et al*., 1967). The dependence on pyruvate seems to be

universal among mammalian species, and it is now generally accepted that pyruvate is an essential component of all media for preimplantation embryo development.

The reasons for utilizing chemically defined media are pragmatic: they are reproducible at different times and in different laboratories; they can be varied in a controlled manner; and they are free of unknown biological activities, such as enzymes and growth factors, which may affect the responses being studied. The reasons are, thus, not dependent on any physiological principles.

The development of chemically defined media for the culture of mouse preimplantation embryos has been an active area of research since the studies of Whitten. Moreover, studies on the mouse, which has been recommended as a model for the human (Quinn and Horstman, 1998) has had a significant impact on the design of media for the culture of human preimplantation embryos. Most notable amongst these is the belief that glucose inhibits early preimplantation embryo development and the use of paired media in sequence—so-called sequential media—that has been promoted by Gardner and colleagues (Gardner and Lane, 1997; Gardner, 1998; Gardner and Schoolcraft, 1999). It has been advocated however that other species are perhaps better models for the human, particularly in relation to human IVF (Betteridge and Rieger, 1993; Menezo and Herubel, 2002). An enormous advantage of undertaking experimental studies on the mouse, apart from ready availability, is the large number of strains and mutants available for study. This, along with the recent report on the sequence of the mouse genome (Mouse Genome Sequencing Consortium, 2002) and of a preliminary comparative analysis of the mouse and human genomes, confirms that the mouse will be the model system of choice for a long time in studies of the regulation and control of early embryonic development at the molecular level. As a model for the human, the most appropriate choice for study is a cross between two strains of different genotype, such that after fertilization and zygotic activation all embryos are outbred. For example, over the years the bulk of the present authors' studies of mouse preimplantation development have been carried out on (B6D2F1×CF1) zygotes.

The purpose of this review is to: (i) detail developments in the design of chemically defined media for the culture of preimplantation embryos; (ii) discuss the adaptation and stress that occurs when preimplantation embryos are placed in chemically defined media; (iii) review the potential long-term effects of preimplantation embryo culture; and (iv) consider the scientific and ethical issues raised by the commercialization of human embryo culture media. Particular emphasis will be placed on recent work on mouse and human preimplantation embryos, but other species will be included when considered appropriate. No attempt will be made to review the extensive literature on the development of media for the culture of human and non-human embryos (for reviews, see Biggers, 1987; 1993; 1998; 2002; 2003; Gardner, 1994; Bavister, 1995; Gardner and Lane, 1999; 2002; 2003; Gardner *et al*., 2000b; Martin, 2000; Lane, 2001; Loutradis, 2000; Quinn, 2000; Smith, 2002).

The design of chemically defined media

The following questions had previously been asked (Biggers, 1991): ''Can a single culture medium be developed which supports development throughout the preimplantation period?'', and

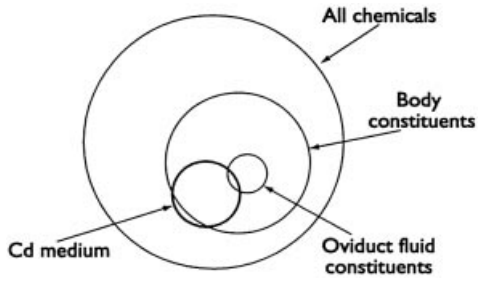

Figure 1. The classes of compounds included in media for the culture of preimplantation embryos. Reproduced from an article in *Reproductive BioMedicine Online* by Biggers (2002), with permission from Reproductive Healthcare Ltd.

''Should the composition of the medium be changed to correspond with the physiological changes which occur as development proceeds?'' These questions were prompted by an earlier theoretical model that summarized the interactive physiological changes that occur in early embryonic development and implantation (Biggers, 1981). Two major problems must be addressed in the design of chemically defined media: the first is the selection of compounds to include in the medium; and the second is the determination of the concentrations of each of the selected compounds.

The Venn diagram (Figure 1) shows three classes of compounds that are found in chemically defined media for the culture of mammalian embryos. The compounds consist mainly of components common to all body fluids. A few of them may be specific to the oviduct fluid, and a few are artificial chemicals not found naturally. The number of compounds may be small to give the arbitrarily defined 'simple' media which only contain ~12 constituents, or 'complex' media containing many more than 12 compounds. The existence of these two groups is the result of historical quirks.

The compositions of most media are still largely based on one of three physiological salines: Earle's balanced salt solution (Earle, 1943), Krebs-Ringer bicarbonate (Krebs and Henseleit, 1932), and Tyrode's solution (Tyrode, 1910). Tyrode's solution is the basis of medium M16 (Whittingham, 1971), which has been used for many years in the culture of mouse preimplantation embryos.

The choice and concentrations of the compounds selected for inclusion in a chemically defined medium is not a simple issue. Two approaches have been used to determine the concentrations used in a medium: (i) the 'let the embryo choose' principle; and (ii) the 'back-to-nature' principle. Using the former approach, a bioassay is carried out in which the response of cells to several concentrations of a component is measured. The results are used to estimate a concentration–response line. The concentration selected for use in a medium is usually that which gives a maximum response. The 'back-to-nature' approach uses the concentration of a substance that is present in the natural environment of the embryo.

'Let the embryos choose' principle

A traditional approach has been to vary the concentrations of each compound separately, keeping the concentrations of the other components constant. The design of a culture medium, however,

involves the simultaneous choice of all the concentrations in a mixture because the effects of each component in the medium may depend on the concentrations of the other components. The problem can be illustrated geometrically by representing the joint action of two components as a concentration–response surface in three-dimensional space (Figure 2). The graph shows clearly that the response to Compound 1 is very dependent on the concentration of Compound 2. Thus, the study of each compound in isolation would provide very incomplete information on which concentrations to select in order to stimulate a maximal response. For a medium containing more than two constituents, say *n* components, the concentration–response surface can be represented in (*n+1*)-dimensional space. This model was proposed (Biggers *et al*., 1957) in a discussion of the design of media for cell and organ culture and was based on a similar model (Box, 1957) to approximate the yields in a complex chemical engineering process.

To explore a concentration–response surface, experiments varying the concentrations of only a single factor must be replaced by other experimental strategies, such as factorial, fractional factorial, rotatable and shell designs, in which the number of concentrations of each component is >2. For example, a 3^3 factorial design was used to investigate the joint actions of NaCl, glutamine and glucose (Lawitts and Biggers, 1992). These experimental designs become intractable when the number of components in a medium is more than three. An alternative strategy is to use a method that locates the maximum point on the concentration–response surface; this point is sometimes called the 'optimum response'. It should be noted, however, that the maximum response is not necessarily the natural response. Several procedures have been described for finding the maximum of a regression surface (Everitt, 1987), one of which was sequential simplex optimization (Spendley *et al*., 1962; Walters *et al*., 1991).

Sequential simplex optimization was used to design a medium for the culture of mouse preimplantation embryos through a block to development, called the 2-cell block, which frequently interfered with studies on early development (Lawitts and Biggers, 1991; 1992). This medium was called medium SOM, and was subsequently modified as the result of measurements of the intracellular concentrations of some inorganic ions in the blastomeres of 2-cell mouse embryos exposed to SOM (Biggers *et al*., 1993) to give the medium called KSOM (Lawitts and Biggers, 1993). Although KSOM medium was based on the proportion of embryos that passed through the 2-cell block, it fortuitously proved very effective for the production of blastocysts from the zygote (Erbach *et al*., 1994).

'Back-to-nature' principle

The 'back-to-nature' approach proposes that the concentration of a substance incorporated into a medium should approximate the concentration to which the embryo is naturally exposed. The principle was first invoked to design a medium for the culture of the sheep preimplantation embryo (Tervit *et al*., 1972). Others (Quinn *et al*., 1985) subsequently used this principle to design a medium for the culture of the human preimplantation embryo called medium human tubal fluid (HTF). Similarly, others (Gardner and Leese, 1990) developed a medium called mouse tubal fluid (MTF) by changing medium M16 (Whittingham, 1971)

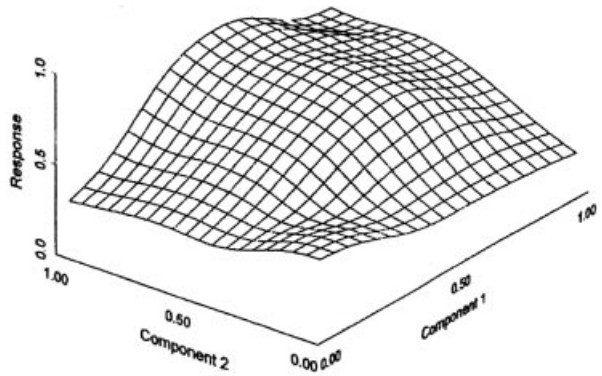

Figure 2. Two-dimensional concentration–response surface. Reproduced from an article in *Reproductive BioMedicine Online* by Biggers (2002), with permission from Reproductive Healthcare Ltd.

to include the concentrations of glucose, lactate and pyruvate present in mouse oviductal fluid.

The 'back-to-nature' approach depends on prior knowledge of the compositions of the female genital tract fluids in the oviduct and uterus. Unfortunately, knowledge of the composition of the oviductal and uterine fluids *in situ* is far from complete (for reviews, see Leese, 1987; Leese *et al*., 2001) because of the limited volumes available for chemical analysis. The difficulties associated with some of the early methods for collecting oviductal fluid have been listed (Restall, 1966) in an attempt to avoid the difficulties of collecting oviductal fluid by using a cannulation technique, by collecting the fluid over several hours. This method, unfortunately, would obliterate any regional differences of the secretions along the oviduct and be subject to degradation of the constituents. Another group (Holmdahl and Mastroianni, 1965) attached a small refrigeration unit to the collection catheter to minimize such degradation. Similar difficulties arise in the collection of uterine fluid for analysis.

More recently, ultramicrochemical methods have allowed sampling of small volumes of oviductal fluid for chemical analysis using micropuncture or microsampling techniques in the mouse (Roblero *et al*., 1976; Borland *et al*., 1977; Gardner and Leese, 1990) and human (Borland *et al*., 1980; Gardner *et al*., 1996). Microsampling of genital tract secretions at known times overcomes many of the objections to the older techniques, and has provided information on the concentrations of Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}, glucose, pyruvate and lactate in human oviductal fluid (Table I). Microsamples of human uterine fluid have been aspirated from the uterine cavity (Casslén and Nilsson, 1984; Gardner *et al*., 1996), enabling the measurement of the concentrations of Na^+, K^+, $Ca^{2+,}$ Cl^-, pyruvate, lactate and glucose.

Ideally, the concentrations of constituents in the local microenvironments of the developing preimplantation embryos, as they journey from the ampullary region of the oviduct to the uterus, need to be known for the design of suitable chemically defined media. This requirement has been met in studies conducted in mice (Borland *et al*., 1977). Such samples cannot be obtained from women, largely for ethical reasons. As a substitute, an heuristic approach has been used in which samples of fluid from patients with normal menstrual cycles are obtained at different stages of the cycle. The assumption is then made that the concentrations found

Table I. Comparison of the composition of medium HTF with published analyses of the composition of human oviductal fluid. (Modified from Biggers, 2001.)

Compound (mmol/l)	Lippes *et al.* (1976)[a]	Lopata *et al.* (1976)[b]	Medium HTF[c] (1985)	David *et al.* (1973)[d]	Borland *et al.* (1980)[e]	Gardner *et al.* (1996)[f]
Na^+	139–140	149.2	148.3	142–148	130	–
K^+	7.7–9.9	4.5	5.06	6.7	21.2	–
Cl^-	117–120	–	108.3	112–127	132	–
Ca^{2+}	3.8–4.8	1.38	2.04	–	1.13	–
Mg^{2+}	–	0.19	0.2	–	1.42	–
Glucose	2.39–3.04	–	2.78	–	–	2.32
Pyruvate	–	0.18	0.33	–	–	0.16
Lactate	–	2.52	21.4	–	–	6.19

[a]Samples collected over 24 h by cannulation (n = 16).
[b]Samples collected at laparoscopy (n = 2).
[c]Concentrations of ions calculated from the concentrations of constituents in Table 7 from Quinn *et al.* (1985).
[d]Samples collected at laparotomy (n = 33).
[e]Microsamples collected by microsampling (n = 7).
[f]Microsamples collected during the luteal phase at laparoscopy (n = 9).

in the luteal phase approximate those that the preimplantation embryo experiences. Thus, microsamples of ampullary fluid were obtained from seven patients undergoing hysterectomy and bilateral salpingo-oophorectomy for menometrorrhagia (Borland *et al.*, 1980). High concentrations of K^+ and Cl^- and low concentrations of Ca^{2+} compared to concentrations found in serum were observed, but no differences were detected in the levels of Na^+ and Mg^{2+}. Microsamples of ampullary and uterine fluids obtained from patients undergoing laparotomy during treatment for infertility (Gardner *et al.*, 1996) showed that the concentrations of pyruvate and lactate were higher in fluids recovered from the ampullary region of the oviduct than in fluids obtained from the uterus, while the concentration of glucose was lower. Others (Casslén and Nilsson, 1984) obtained microsamples of uterine fluid from 129 patients in the proliferative, midcycle and luteal phases who 'desired contraceptive counseling'. The concentration of glucose was not different from that in serum, and did not change with the phases of the menstrual cycle. The concentration of K^+ was higher, and that of Ca^{2+} lower, than in serum. Both varied cyclically, being lower during the midcycle phase than in the proliferative and luteal phases. The Na^+ concentration was lower than in serum and did not change with the phases of the menstrual cycle.

In designing medium HTF, one group (Quinn *et al.*, 1985) based the concentrations of some constituents of this medium on concentrations in the oviduct reported by others (Lippes *et al.*, 1972; Lopata *et al.*, 1976). The analyses of the former group (Lippes *et al.*, 1972) were on samples obtained over an extended period of time from cannulated oviducts, while those of the latter group (Lopata *et al.*, 1976) were based on microsamples from two human patients. The concentrations of only three ions (Na^+, K^+, Ca^{2+}) were measured in both of these studies. There were considerable differences in the concentrations of all three ions in the two studies (Table I). The composition of HTF is also shown in Table I, together with other published analyses of human oviductal fluid. It is clear that, with few exceptions, there is only marginal agreement between the composition of HTF and the composition of human oviductal secretions. Furthermore, there are only eight constituents in HTF that form a very small subset of the compounds in oviductal fluid. Although HTF supports early development of human preimplantation embryos *in vitro*, it is potentially misleading to name it 'human tubal fluid'. Similar arguments can be presented for two other media that are claimed to mimic the concentrations of components found in oviductal fluid: medium synthetic oviductal fluid (SOF) and medium mouse tubal fluid (MTF).

There is evidence from studies on mice that the concentration of substances in oviductal fluid does not necessarily support the development of preimplantation embryos as seen in a chemically defined medium. This fact was first observed in studies on K^+. Although the mouse oviductal fluid contains a high concentration of K^+ (~25 mmol/l) (Roblero *et al.*, 1976; Borland *et al.*, 1977), it has been reported that mouse zygotes do not develop in concentrations ranging from 12 to 48 mmol/l (Whittingham, 1975). In contrast, others (Wales, 1970) found that 2-cell mouse embryos would develop in K^+ concentrations ranging from 1 to 48 mmol/l. Another group (Roblero and Riffo, 1986) reported that a concentration of 25 mmol/l K^+ would support preimplantation development in the mouse. Others (Quinn *et al.*, 1985) found maximum development when the concentration of K^+ ranged from 2.3 to 5.1 mmol/l, whilst another group (Wiley *et al.*, 1986) found that mouse embryos developed best when the K^+ concentration was <6 mmol/l. The concentration of K^+ in KSOM is 2.85 mmol/l (see Table II; Lawitts and Biggers, 1993). These different observations could be due to differences in the concentrations of other components in the various media being used. Two additional examples of the lack of correspondence between the conditions that give a maximal response *in vitro* and the natural environment are osmolarity and pH. These topics are discussed later.

It is important to recognize, however, that both the 'let the embryos choose' and the 'back-to-nature' approaches to media development have limitations. The first principle determines the concentrations of a finite set of constituents that leads to a maximum response. The combination of concentrations may well be changed if the medium is supplemented with further constituents. Further, there is no guarantee that the maximum response is

Table II. Compositions of media HTF (Quinn *et al*., 1985), modified HTF (Quinn *et al*., 1995), P1 (Gardner *et al*., 2000) and KSOM (Lawitts and Biggers, 1993)

Compound (mmol/l)	HTF	Modified HTF	P1	KSOM
NaCl	101.6	101.6	101.6	95.0
KCl	4.69	4.69	4.69	2.50
KH_2PO_4	0.37	–	–	0.35
$CaCl_2$	2.04	2.04	2.04	1.71
$MgSO_4$	0.20	0.20	0.20	0.20
$NaHCO_3$	25.0	25.0	25.0	25.0
Glucose	2.78	–	–	0.20
Na pyruvate	0.33	0.33	0.33	0.20
Na lactate	21.4	21.4	21.4	10.0
Citrate	–	–	0.5	–
Glutamine	–	1.0	-	1.0
Taurine	–	–	0.05	-
EDTA	–	0.1	–	0.01
Penicillin (U/ml)	100	100	–	–
Streptomycin SO_4 (50 μg/ml)	100	100	–	–
Phenol red (μg/ml)	10	10	5	–

the natural response. The 'back-to-nature' principle is limited by the difficulty of determining the concentrations of substances in the environment in which the embryos develop naturally. Caution should be employed in using estimates of compounds in the natural environment, as there are several examples where apparent optimum concentrations *in vitro* differ from the concentrations found naturally. It seems likely, however, that a combination of approaches will be needed to obtain preimplantation embryos cultured *in vitro* that best approximate to those found *in vivo* (see Concluding remarks).

Energy requirements

The findings during the late 1950s (Whitten, 1956, 1957) that glucose would not support the development of the preimplantation mouse embryo before the 8-cell stage and that lactate would not support development before the 2-cell stage, led one group to investigate the requirements of the maturing oocyte of the mouse and the 1-cell embryo stage (Biggers *et al*., 1967). The combined results showed that, at sometime during oogenesis, the energy pathways become restricted such that only pyruvate, and three other closely associated intermediates in the energy pathway, would support oocyte maturation and division of the 1-cell stage. As development proceeds, the energy pathways seem to open up so that by the 8-cell stage the Embden–Meyerhoff pathway is fully functional again. It was these investigations which led to the routine incorporation of pyruvate into media for the culture of the initial stages of development of all mammalian species. The subsequent demonstration using ultrafluorometric analytical methods which are sufficiently sensitive to study single preimplantation embryos (Leese *et al*., 1984) showed that pyruvate was the preferred source of energy by the mouse mature oocyte and zygote, and that by the 8-cell stage glucose became the preferred source (Leese and Barton, 1984). Later, it was demonstrated in the human preimplantation embryo that the uptake of glucose relative to pyruvate increased after the 8-cell stage (Leese *et al*., 1993). The uptake of pyruvate by the human preimplantation embryo, however, remained high throughout development to the blastocyst stage and does not sharply decline as in the mouse (Hardy *et al*., 1989; Conaghan *et al*., 1993).

Does glucose inhibit early preimplantation development?

The observation made during the late 1980s (Schini and Bavister, 1988) that glucose and phosphate caused arrest of preimplantation development of the hamster at the 2-cell stage have had a major impact on the design of media for the culture *in vitro* of a large number of species of preimplantation embryos, including human. The observation was soon confirmed for other species, including mouse (Chatot *et al*., 1989; 1990; Diamond *et al*., 1991; Lawitts and Biggers 1991; Brown and Whittingham, 1992; Scott and Whittingham, 1996; Haraguchi *et al*., 1996), rat (de Hertogh *et al*., 1991; Reed *et al*., 1992a; Miyoshi *et al*., 1994), sheep (Thompson *et al*., 1992), cow (Takahashi and First, 1992) and human (Conaghan *et al*., 1993; Hardy, 1994; Quinn *et al*., 1995; Coates *et al*., 1999). Moreover, these observations were a major influence on the design of medium CZB for the culture of the mouse zygote to the blastocyst (Chatot *et al*., 1989). Medium CZB does not contain glucose and inorganic phosphate. Soon after, a modification of Quinn's medium HTF was formulated in which glucose and phosphate were omitted and EDTA and glutamine added (Table II) (Quinn *et al*., 1995). More recently, another medium for the culture for human preimplantation embryos was described called P1 (Table II) (Gardner *et al*., 2000a). This medium is, in fact, a modified form of Quinn's glucose-free and phosphate-free modified HTF medium in which citrate and taurine are substituted for glutamine and EDTA.

The need to omit glucose from culture media for the initial stages of preimplantation development seems paradoxical since the oviduct contains significant amounts (Gardner and Leese, 1990; Gardner *et al*., 1996). The attention paid to glucose inhibition has obscured the reports in which glucose was not found to inhibit early development *in vitro*. In 1985, it was reported that cleavage of the sheep zygote was stimulated by glucose (Betterbed and Wright, 1985). Further studies on the preimplantation development *in vitro* of sheep failed to show any inhibitory effect of glucose (Thompson *et al*., 1989; McGinnis and Youngs, 1992). No inhibitory effect on preimplantation development *in vitro* has been reported in the pig (Petters *et al*., 1990; Hagen *et al*., 1991; Swain *et al*., 2002), except in the case of one experiment out of several reported later (Reed *et al*., 1992b). The claim by others (Seshagiri and Bavister, 1991) that glucose inhibits the preimplantation development of the pig appears to be due to their misinterpretation of the published analysis of variance (Petters *et al*., 1990). Strong evidence now exists to suggest that the background composition of the media used determines whether glucose inhibits early mouse development. It was reported (Lawitts and Biggers, 1992) that the addition of glucose to a medium called SOM, in a concentration as high as 5 mmol/l, did not inhibit the development of outbred CF1 mouse zygotes to blastocysts. Medium KSOM supports the development of the mouse zygote to the blastocyst when the glucose concentration is raised to the level found in blood (5.56 mmol/l). Phosphate only slightly inhibits development in KSOM, and its effects are independent of the concentration of glucose (see Figure 3; Biggers and McGinnis, 2001).

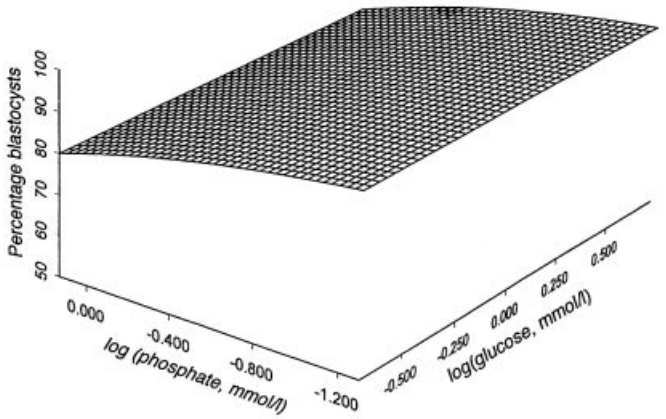

Figure 3. Concentration–response surface showing the joint action of glucose and phosphate on the development of mouse zygotes into blastocysts cultured in medium KSOM. Reproduced from Biggers and McGinnis (2001), © European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/*Human Reproduction*.

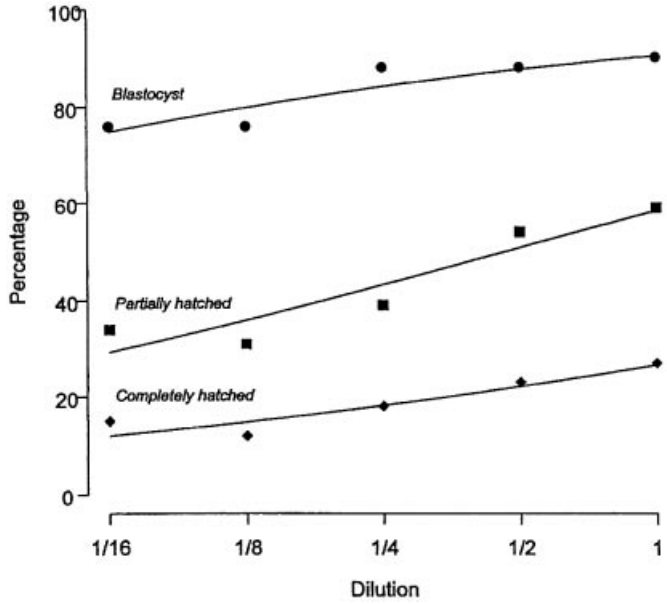

Figure 4. Effect of different dilutions of Eagle's amino acids in KSOM on percentage of zygotes developing at least into zona-enclosed blastocysts, and partially hatched or completely hatched blastocysts. Reproduced from Biggers *et al.* (2000), with permission from the Society for the Study of Reproduction, Inc.

The belief that normal levels of glucose and phosphate inhibit early human embryo development in culture is now dogma. The need to exclude glucose and phosphate or to use glucose in a low concentration is touted in the advertisements of several commercially advertised media. The dogma 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 range. There is also evidence that glucose does not inhibit development of the early human embryo in some media (Barak *et al*., 1998). Others (Coates *et al*., 1999) compared the development of preimplantation human embryos in Earle's balanced salt solution with and without 5.5 mmol/l glucose, the concentration normally found in blood. The pregnancy rates using the two media were not significantly different. These authors did find, however, statistically lower cleavage rates and grades of embryo in the medium containing glucose. They concluded that: ''... a reduction of the glucose concentration of the medium used for embryo culture from the pronucleate stage to embryo transfer on day 2 or 3 is prudent''. The statistical differences were marginal and based on large numbers of embryos; thus, the results may have little clinical significance as previously discussed (Biggers and McGinnis, 2001). More recently, others (Macklon *et al*., 2002) have described a complex medium containing 4.7 mmol/l glucose that supports complete preimplantation development of the human zygote to the blastocyst stage without a change of medium. It is now generally accepted that glucose does not absolutely inhibit the initial stages of human preimplantation development. Consequently, the removal of glucose from the culture medium should be considered non-physiological, and attempts to improve a suboptimal culture medium by removal of glucose are to be discouraged. Glucose should be included in media at the concentrations typically found in the human oviduct, approximately 2.0–3.0 mmol/l (Gardner *et al*., 1996; Bavister, 1999; Coates *et al*., 1999; Gardner and Lane, 1999).

Requirement for amino acids

In the following discussion, 'amino acids' is a generic term denoting collectively all 20 common naturally occurring amino acids. Amino acids are not necessary for complete preimplantation development of mouse embryos (Whitten and Biggers, 1968; Cholewa and Whitten, 1970). Nevertheless, amino acids have beneficial effects that have been recognized for many years in several species, beginning with early studies in mice (Brinster, 1965c; for reviews, see Biggers 1987; Gardner, 1994; Bavister, 1995; Gardner *et al*., 2000b; Devreker *et al*., 2001). 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 bovine serum albumin (BSA), which may also be a source of amino acids (Brinster, 1965c; Cholewa and Whitten, 1970). The effect of varying the concentrations of amino acids on the development of mouse preimplantation embryos in a base medium in which BSA was replaced with polyvinyl alcohol, was studied by others (Biggers *et al*., 1997; 2000) (Figure 4). Varying the concentrations of the set of all 20 common amino acids had little effect on the rate of blastocyst formation, but increasing the concentration of all 20 amino acids significantly increased the rate of hatching and, more importantly, significantly increased the total numbers of cells in the blastocysts, particularly in the inner cell mass (ICM). Further, the extracellular matrix in the blastocysts that developed was more normally organized when amino acids were present. However, there was little benefit in using full-strength Eagle's amino acids (AA) when compared to half-strength Eagle's AA (Biggers *et al*., 2000). The concentrations of AA used in KSOM and derived media is half-strength Eagle's AA (Biggers, 2002). These results confirmed many of the results of other studies on the combined effects of amino acids on the development of mouse preimplantation embryos *in vitro* where BSA was retained in the media used. On reflection, it is not surprising that amino acids exert beneficial effects on the development of the preimplantation embryo, as they are known to have multiple functions including: precursors for

biosynthesis, energy sources, regulators of energy metabolism, osmolytes, buffers of intracellular pH, and chelators of heavy metals (for a review, see Gardner *et al*., 2000b).

The determination of the concentrations of each of these amino acids to include in a medium that optimizes the development of preimplantation embryos is a formidable task. Their joint effects can be theoretically represented by a concentration–response surface in 21-dimensional space. 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.

Two methods have been used to determine the concentrations of amino acids to include in media. First, the effects of each amino acid can be examined by supplementing a base medium with each compound individually. Second, the base medium can be supplemented by either the complete set or subsets of all 20 amino acids. The concentrations of amino acids used in early studies (Brinster, 1965c) were based on the amino acid composition of bovine plasma albumin. Others (Nakazawa *et al*., 1997) described studies on mouse embryo development using all 20 amino acids at the concentrations found in follicular fluid where the levels are typically one-half to one-third that described previously (Eagle, 1959). The most recent studies on the amino acid requirements of preimplantation embryos have utilized the so-called 'essential' and 'non-essential' amino acids, mixtures that are available commercially. The terms were first coined in nutritional studies of whole animals (Rose, 1938), with essential amino acids being required in the diet while the non-essential amino acids were not. The same terms were adopted to describe findings on the nutritional requirements for amino acids of several human cell lines cultured *in vitro* (Eagle, 1959). These studies led to the identification of two subsets of the common natural amino acids: 13 'essential' amino acids that had to be included in the culture medium, and seven 'non-essential' amino acids that were not required. The subset of essential amino acids was included in the widely used 'minimum essential medium' (MEM), while the non-essential subset was not included in MEM. The generic use of the terms 'essential' and 'non-essential', including specified concentrations of the amino acids to use in all systems, as proposed by Eagle, was soon challenged, largely on the grounds that they ignore the fact that concentration–response surfaces vary according to the cells being cultured. In an extensive discussion of the term, one investigator (Waymouth, 1965) wrote: ''The terms 'essential' and 'non-essential' amino acid, rather commonly used, are not, therefore, strict designations and should be accepted with scepticism unless carefully defined in a particular context...''.

As mouse and human zygotes develop into blastocysts in media that do not contain amino acids (Whitten and Biggers, 1968; Cholewa and Whitten, 1970; Conaghan *et al*., 1993), none of the 20 amino acids can be considered to be absolutely essential. Recently, another group (Houghton *et al*., 2002) also pointed out that the classification is over-simplistic and may mask important subtle effects. The terms essential and non-essential, therefore, have little significance in the field of preimplantation embryo culture, and ideally their use should be dropped.

The reason that the terms essential and non-essential have become ingrained in the literature is because mixtures of Eagle's essential and non-essential amino acids have been commercially available for some time. A severe limitation in their use in experimental work, however, is the restriction that the relative concentrations of amino acids within each of the two subsets of amino acids cannot be changed.

Gardner and Lane have undertaken the most extensive studies on the role of amino acids on the development of mouse preimplantation embryos *in vitro* (for reviews, see Gardner, 1994; Gardner and Lane, 1999; Gardner *et al*., 2000b). An understanding of these authors' work is important as it has formed the basis in part for the introduction of the sequential two-step method for the culture of human preimplantation embryos (see below). In initial studies (Gardner and Lane, 1993), these authors exposed mouse zygotes of an F_1 hybrid strain to medium mMTF (Gardner and Leese, 1990) supplemented with all 20 amino acids or non-essential amino acids with or without glutamine. The concentration of AA used was full-strength Eagle's amino acids. After 96 h in culture, the best response of the embryos, assessed both morphologically and by cell number, was obtained using the non-essential amino acids. In contrast, essential amino acids were inhibitory. Also, when culture was extended for more than 96 h, development was inhibited even in the presence of non-essential amino acids. The delayed development was attributed to the accumulation of ammonium in the medium produced by the deamination of amino acids (see below), and it was thus found necessary to change the medium after 48 h to reduce the build-up of ammonium ions. In subsequent studies (Lane and Gardner, 1997), it was shown that improved blastocyst development is obtained when zygotes are cultured in medium mMTF supplemented with non-essential amino acids and glutamine to the 8-cell stage, followed by culture to the blastocyst stage in medium mMTF supplemented by all 20 amino acids (Lane and Gardner, 1997).

In support of the two-step method, the same authors (Lane and Gardner, 1997) reportedly showed a statistically significant positive correlation between blastocyst cell counts, the number of cells in the ICM, the ability of the ICM to form outgrowths, and fetal development. By contrast, no correlation was reported between either blastocyst formation or hatching and fetal development. However, it is not possible to determine simultaneously differential cell counts or to produce outgrowths and assess the ability of a single blastocyst to implant and undergo fetal development as the former methods require destruction of the embryo. Although it seems reasonable to examine trends within a cohort of embryos cultured in a specific manner, it is not possible to obtain statistical correlations. For this reason, the estimated correlations are flawed. Recently, one group (Lane *et al*., 2001) showed that mouse preimplantation development was enhanced if the concentration of Eagle's essential AA was reduced to half-strength. This is consistent with the present authors' previous studies that showed no advantage in using Eagle's AA at full strength for the culture and development of preimplantation mouse embryos (Biggers *et al*., 2000).

The same group (Gardner and Lane, 1996) have also investigated the use of sequential culture methods using an outbred mouse strain that typically undergoes cleavage arrest at the 2-cell stage of development. This phenomenon, which was first described

in the mid-1960s (Cole and Paul, 1965), is referred to as the 2-cell block (for reviews, see Biggers, 1987; 1993; 1998). The media designated DM1 and DM2 consist of a modified MTF, supplemented with full-strength non-essential Eagle's AA. DM1 differs from DM2 in that the latter is also supplemented with 0.1 mmol/l EDTA (Abramczuk *et al*., 1977). These studies showed that both ammonium accumulation and EDTA inhibit blastocyst development. Blastocysts derived from culture in DM2 showed decreased implantation rates and delayed fetal development after transfer into pseudopregnant recipients. Acceptable results were obtained by first culturing early cleavage stages in DM2 for 48 h, followed by transfer into DM1 for an additional 48 h. More recently, others (Gardner *et al*., 1999) have described a new media combination DM2/DM3 for the culture of mouse preimplantation embryos. Medium DM2 is the same as described above, except that the EDTA concentration is reduced to 0.01 mmol/l. DM3 contains both full-strength essential and non-essential Eagle's AA. It is perhaps worth noting that EDTA was included as one of the original 10 components in the simplex optimization procedure that led to the development of KSOM (Lawitts and Biggers, 1991; 1993; Erbach *et al*., 1994). This resulted in the use of EDTA at a concentration of 0.01 mmol/l.

In 1995, one group (Ho *et al*., 1995) presented their findings on the culture and development of mouse zygotes in medium KSOM supplemented with half-strength Eagle's AA. At this concentration, the inclusion of all 20 amino acids throughout the preimplantation period gave the best rates of blastocyst development. It was noted that these results were obtained without a change of media, and this prompted a comment on the inhibitory effect of ammonium described previously (Gardner and Lane, 1993). Particular emphasis was placed on the higher amino acid concentrations used in these studies and on the ratio of number of embryos to volume of culture medium. Similarly, others (Nakazawa *et al*., 1997) noted that in their studies all 20 amino acids at the concentrations used were needed throughout the preimplantation period to enhance blastocyst development.

It is perhaps both attractive and persuasive to argue that sequential media are more physiologically and better able to meet the changing metabolic needs of the developing preimplantation embryo (Gardner 1994; 1998; Gardner and Lane, 1999; Gardner *et al*., 2000b). In the mouse, at least, the original formulations of these media, based on blastocyst development, blastocyst differential cell counts and embryo viability after transfer, inhibited development of the embryo requiring a change of medium to ensure good blastocyst development and viability. We are not persuaded that presently formulated sequential media offer any advantage over a single medium such as KSOM supplemented with amino acids, for the continuous culture of mouse preimplantation embryos. However, based on the types of extended sequential media studies described above for the mouse and other species, notably the sheep (Gardner *et al*., 1994) and cow (Steeves and Gardner, 1999; Gardner *et al*., 2000b; Lane *et al*., 2003a), modifications were soon applied to the extended culture of human preimplantation embryos.

Recent attempts have been made to elucidate the role of amino acids on the development of human preimplantation embryos *in vitro*. Varying the concentration of glutamine increased the yield of blastocysts (Devreker *et al*., 1998). Other work has shown that taurine and glycine are organic osmolytes (Devreker *et al*., 1999; Hammer *et al*., 2000). These results, however, do not help in the determination of the optimum concentrations to use in culture media.

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.

L-Glutamine and ammonium

Recently, the amino acid L-glutamine (Gln) has been the subject of considerable scrutiny. Gln has been included in the majority of chemically defined media since preliminary studies were conducted (Chatot *et al*., 1989), in which Gln at a concentration of 1 mmol/l in medium CZB was used to overcome the 2-cell block seen in outbred mouse strains. Gln at a concentration of 1 mmol/l was also found to be the optimum concentration that overcame the 2-cell block in the development of SOM using sequential simplex optimization (Lawitts and Biggers, 1991), and was subsequently used at the same concentration in medium KSOM (Lawitts and Biggers, 1993). The same concentration of 1 mmol/l Gln has been used by Gardner and Lane in their studies of sequential media described above; this includes G1/G2-type media (Gardner and Lane, 1997), DM1/DM2 (Gardner and Lane, 1996), and DM2/DM3 (Gardner *et al*., 1999). The role of Gln in early preimplantation embryo development is probably complex given its many physiological and biochemical functions (for a review, see Bulus *et al*., 1989). For example, one group (Lawitts and Biggers, 1992) showed that Gln displays differential, concentration-dependent effects in simple, chemically defined media. Thus, Gln at 3 mmol/l inhibits blastocyst development in the presence of 85 mmol/l NaCl, but stimulates blastocyst development when the NaCl concentration is raised to 125 mmol/l (Lawitts and Biggers, 1992). In this setting, Gln is probably functioning as an organic osmolyte, as opposed to its biochemical role as a glucogenic amino acid, metabolic precursor and nitrogen source.

As noted above, the decreased development *in vitro* of mouse F_1 zygotes to blastocysts was attributed to the toxic effects of ammonium that resulted from the spontaneous breakdown of amino acids (Gardner and Lane, 1993). Subsequently, the same authors (Lane and Gardner, 1994) using the same F_1 hybrid mouse strain, reported that the addition of ammonium in the form of ammonium chloride to a modified mouse tubal fluid medium (Gardner and Leese, 1990) 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 into the uteri of surrogate mothers. A deleterious effect of Gln in media on mouse preimplantation embryos *in vitro* was also suggested (Devreker and Hardy, 1997). These authors described studies in which zygotes of an F_1 hybrid mouse strain were cultured in KSOM (Lawitts and Biggers, 1993) which contains Gln (see Table II) but no other amino acids, or Gln-free KSOM. No differences in the proportion of zygotes developing to blastocysts in the two media were demonstrated, but the use of Gln-free KSOM resulted in an increase in the numbers of cells that developed in the ICM and trophectoderm. Others (Nakazawa *et al*.,

1997) provided analytical evidence that, under conditions used for the culture of preimplantation embryos, the build-up of ammonium was principally derived from the spontaneous breakdown of Gln.

The chemical instability of Gln was recognized shortly after its isolation and structure determination undergoing spontaneous breakdown to ammonium and 5-pyrrolidonecarboxylic acid (for a review, see Greenstein and Winitz, 1961). The terminal amide of Gln is not, however, inherently chemically unstable, but is subject to a kinetically driven intramolecular ring-closure reaction to give a 5-membered pyrrolidone structure in the presence of a free α-amino group. The terminal amide is completely stable if, for example, Gln is derivatized by N^{α}-acetylation or by peptide formation. This finding was of considerable historical interest because it helped explain the observation that, whereas free Gln was unstable in solution, ammonium was not easily released when proteins—which presumably contained Gln—were heated in solution (see Greenstein and Winitz, 1961). Another group (Tritsch and Moore, 1962) drew attention to the breakdown of Gln in cell culture media and noted that the decomposition followed a first-order rate in a cell culture medium called '213' medium. A report was also made on the levels of ammonium in commercial cell culture media, and the rate of decomposition of Gln was assessed at 37°C in RPMI 1640, MEM, DMEM and Ham F12 (Heeneman *et al*., 1993); these authors obtained similar rates of decay in all media, with similar first-order rate constants to those described elsewhere (log $k = -0.95$) (Tritsch and Moore, 1962). Others (Gardner and Lane, 2003) have reported that ammonium accumulates significantly in $KSOM^{AA}$, and for this reason they have asserted that this medium is unsuitable for many studies. However, it is important to understand that the rate of decay of Gln in aqueous solution follows a first-order rate constant that is largely determined by a pH- and temperature-dependent chemical reaction (Vickery *et al*., 1935; Gilbert *et al*., 1949). The exception seems to be when very high levels of salts (up to 0.2 mol/l) such as phosphate and arsenate are present (Hamilton, 1945; Gilbert *et al*., 1949). Thus, there is nothing exceptional about KSOM with regard to the degradative breakdown of Gln. The chemistry of the ring-closure reaction, which is almost certainly accelerated as a result of intramolecular catalysis through hydrogen-bonding, is in all likelihood relatively insensitive to the concentration ranges of components typically present in simple chemically defined media that are used for the culture of preimplantation embryos. For example, the data of two groups (Gardner and Lane, 2003; Lane and Gardner, 2003) have been taken by us on the reported breakdown of Gln in $KSOM^{AA}$, and kinetic parameters obtained of similar value (e.g. log $k = -0.97$) to that described above for other cell culture media (M.C.Summers, L.K.McGinnis, J.A.Lawitts and J.D.Biggers, unpublished). Consequently, the chemical instability of Gln in media used for the culture of preimplantation embryos needs to be addressed for all culture media, as it is now clear that the concentration of Gln currently used is too high.

More recently, the same authors (Lane and Gardner, 2003) described additional studies on the effects of added ammonium on preimplantation embryo development. They reported that the addition of 0.3 mmol/l ammonium chloride to medium G1.2/G2.2 had numerous detrimental effects on the preimplantation development of (CF1×CF1) zygotes. The effects observed included reduced blastocyst cell counts, decreased ICM and increased apoptosis, perturbed cell metabolism, abnormal expression of the imprinted gene H19, and decreased embryo viability after embryo transfer. In order to assess critically the effects of ammonium on both preimplantation and post-implantation development, it is necessary to consider both the strain of mouse used in the study and the choice of culture medium. The sensitivity of preimplantation embryos from different mouse strains to variations in culture conditions is well known. From an historical perspective, it is perhaps best exemplified by the effect of culture media on the 2-cell block seen with embryos from outbred mouse strains. Moreover, others (Suzuki *et al*., 1996) studied the preimplantation development of embryos in Whitten's medium from 55 strains of mice after fertilization *in vitro* of ova using a single strain of male as the source of spermatozoa. These authors noted considerable strain differences in development *in vitro* and emphasized the need to consider the strain of mouse used when interpreting data from reproductive studies with mouse embryos. Recent studies using molecular markers to study embryo development and viability have also emphasized the role of mouse strain and culture medium. One group (Kamjoo *et al*., 2002) described studies on the incidence of apoptosis in the ICM (apoptotic index) of two different strains of mice cultured in medium M16 (Whittingham, 1971) or KSOM (Lawitts and Biggers, 1993), and noted that the level of apoptosis was influenced both by genetic strain and the culture medium used. Others (Steeves *et al*., 2001) noted differences in the ability of 2-cell embryos derived from ova of different mouse strains to respond to a shift in intracellular pH following exposure to ammonium chloride in medium KSOM. In particular, embryos derived from CF1 ova were more sensitive to an ammonium chloride load compared to Balb/c mouse embryos. These researchers also emphasized the dangers of generalizing on the basis of findings from a single mouse strain. The significance of the increased level of expression of the imprinted gene H19 in (CF1×CF1) embryos described elsewhere (Lane and Gardner, 2003) is difficult to assess as their study did not include an examination of the allelic DNA methylation patterns. Even *in-vivo*-derived mouse blastocysts can exhibit varying levels of expression of imprinted genes (Mann *et al*., 2003b). It has also been shown that B6(CAST-H19)×B6F1 hybrid 2-cell embryos, when cultured to blastocysts in $KSOM^{AA}$ (which in this study was supplemented with 1 mmol/l Gln) display an essentially normal monoallelic expression of H19 (there was ~10% leakage seen with the paternally expressed H19 gene) and appropriate DNA methylation (Doherty *et al*., 2000). By contrast, others (Lane *et al*., 2003b) reported that when CAST females were crossed with F_1 males, almost 70% of the resulting blastocysts following culture in G1/G2 supplemented with 0.3 mmol/l ammonium chloride demonstrated abnormal expression of the paternal H19 allele. There are perhaps a number of explanations for these observed differences, including culture methods, 1-cell versus 2-cell stage of culture, and perhaps a more detrimental effect from immediately creating artificially high levels of ammonium throughout the culture in the latter studies (Lane *et al*., 2003a). Also, it is necessary to take into account the relative lability of the H19 imprinted gene. One group (Sasaki *et al*., 1995) first noted the instability of the mouse H19 gene to *in-vitro* manipulations when compared with other mouse imprinted genes. Similarly, others (Doherty *et al*., 2000) showed that there was approximately 40%

loss of imprinting of H19 when B6(CAST-H19)×B6F1 embryos were cultured in Whitten's medium, which contains neither Gln nor amino acids. By contrast, embryos derived from the reciprocal cross display a normal pattern of H19 imprinting when cultured under identical conditions. Thus, the genetic background of the mouse strain strongly influences the pattern of imprinting and DNA methylation, particularly for a relatively unstable locus such as H19. Similarly, with some genotypes, leakage in H19 expression is even seen with *in-vivo*-derived embryos (Mann *et al*., 2003a; R. Schultz, personal communication). At present, there is much we do not know regarding the control and regulation of expression of the H19 gene (for example, see Arney, 2003). The role of culture medium and the genetic strain of mouse used for study are, nevertheless, important variables in describing the results of any study. Over the years, many embryo culture and embryo transfer studies have been carried out using either KSOM-based media, CZB or Whitten's medium. The present authors are unaware of any reports describing abnormal rates of implantation, decreased fetal growth or anomalies associated with these media.

Interestingly, it has been shown that bovine follicular fluid contains appreciable amounts of ammonium, and that exposure of bovine oocytes to high levels of ammonium (up to 0.35 mmol/l) did not affect their fertilization, subsequent cleavage, or development to hatching blastocysts (Hammon *et al*., 2000). Another group (Hagemann *et al*., 1998) also obtained similar rates of development of bovine embryos in media supplemented with either Gln or a heat-stable Gln-dipeptide derivative.

The additional variable factor of ammonium accumulation in the culture medium from the chemical breakdown of Gln is thus probably best avoided. A recommendation was made to change the medium after 48 h of culture in order to prevent ammonium build-up (Gardner, 1994). However, subculture at 48 h may add extra stress to the embryos, along with the potential adverse effect of diluting out any putative embryo-derived growth factors that may have accumulated in the medium. Consequently, an *in-situ* enzymatic method was described for recycling ammonium that did not require a change of medium (Lane and Gardner, 1995). Others (Nakazawa *et al*., 1997) were able to obtain mouse development in a single medium containing 0.18 mmol/l Gln that resulted in decreased ammonium accumulation. These approaches nevertheless still require the presence of Gln and thus do not take into account the potential adverse effect of 5-pyrollidonecarboxylic acid that is produced in equimolar amounts with ammonium from the breakdown of Gln and has been reported to be toxic to cells in culture (Heller *et al*., 1967; Roth *et al*., 1988). One approach, therefore, is to eliminate Gln from the culture medium (Devreker and Hardy, 1997; J.D.Biggers, L.K.McGinnis and J.A.Lawitts, unpublished). Another approach is to replace Gln with a heat-stable peptide derivative of Gln, such as L-alanyl-L-glutamine (abbreviated, AlaGln) or glycyl-L-glutamine (abbreviated, GlyGln). The last approach has been successfully used in the development of chemically defined media for cell culture (Roth *et al*., 1988; Brand *et al*., 1989; Ollenschlager *et al*., 1989; Atanassov *et al.*,1998; Christie and Butler, 1998), and is based on the finding that dipeptides can be utilized by cells *in vitro* (Eagle, 1959). There are, however, few published detailed studies on the use of dipeptide Gln derivatives for the culture and development of preimplantation mouse embryos. In general, the published studies involve a simple substitution of 1 mmol/l Gln with 1 mmol/l AlaGln. For example, it has been shown that lowering the concentration of essential amino acids in which Gln was replaced with AlaGln reduced the accumulation of ammonium and resulted in increased blastocyst development and cell numbers (Lane *et al*., 2001). Other than our own studies (J.D.Biggers, L.K.McGinnis and J.A.Lawitts, unpublished), we are unaware of any detailed dose–response studies of the effects of Gln-dipeptides on preimplantation embryo development. Based on these findings and those of others (Devreker and Hardy, 1997; M.C.Summers, L.K.McGinnis, J.A.Lawitts and J.D.Biggers, unpublished), Gln-supplemented KSOM-based media have not been used by us for the past 3–4 years.

Sequential media and embryo culture

The first use of a stepwise protocol for the culture of mouse preimplantation embryos was in the description of medium CZB (Chatot *et al*., 1989; 1990). This medium, which contains no glucose, was used to support development to the 8-cell/morula stage, at which time glucose is added to the microdroplet containing the embryos to support further development to the blastocyst stage. Similarly, others (Brown and Whittingham, 1992) showed that 1-cell MF1 embryos can successfully develop to the blastocyst stage in high yield by *in-vitro* culture in glucose-deficient M16 for 48 h, followed by transfer into M16 medium.

Sequential protocols did not receive close attention until a pair of media were introduced, denoted G1 and G2, for the extended sequential culture of human zygotes into blastocysts (Gardner, 1994; Barnes *et al*., 1995; Gardner and Lane, 1997). Subsequently, a recommendation was made (Jones *et al*., 1998) for an alternative protocol in which human zygotes were cultured in medium IVF-50 for 2–3 days followed by culture in medium G2 for a further 2–4 days. Medium IVF-50 is a commercial medium sold by Scandinavian IVF Sciences AB (now Vitro Life, Gothenburg, Sweden). A number of companies are currently promoting sequential media for the culture of human preimplantation embryos (for a listing, see Biggers, 2000).

Media G1 and G2 differ in six respects: (i) lactate is reduced from 21 to 11.74 mmol/l; (ii) pyruvate is reduced from 0.32 to 0.10 mmol/l; (iii) glucose is increased from 0.50 to 3.15 mmol/l; (iv) taurine is reduced from 0.1 to 0 mmol/l; (v) the seven Eagle's non-essential amino acids only are present in G1, while all 20 natural amino acids are present in G2; and (vi) EDTA is reduced from 0.1 to 0 mmol/l. The justification for the first three changes is based on the 'back-to-nature' principle, while the last three changes are based on 'let the embryos choose' principle. Later, a modification was described to the original G1/G2 media designated G1.2 and G2.2 (Gardner *et al*., 1998). Recently, others (Lane *et al*., 2003b) provided a listing of the earlier G1/G2 formulations. The amino acids formulations are based largely on their studies in the mouse, described above, with selected vitamins added to the G2 component. The latter is based on studies of the effect of culture conditions on the metabolic profile and viability of cultured mouse and sheep blastocysts (Gardner *et al*., 1994; Lane and Gardner, 1998).

Studies using the non-essential and essential amino acid mixtures have resulted in two-step techniques for the culture of human embryos similar to that used for the culture of mouse

preimplantation embryos (Giscard d'Estaing *et al*., 2001). Only non-essential amino acids are included in the medium for culture to the 8-cell stage, followed by all 20 amino acids for culture to the blastocyst stage. Recently, it has been shown (Devreker *et al*., 2001) that supplementing Earle's balanced salt solution with non-essential or 20 amino acids to form two media that were used sequentially to culture human zygotes stimulated the total number of cells in the blastocysts that developed.

One reason for renewing a medium during a period of culture is to remove any toxic substances, such as ammonia, that may have accumulated (as discussed above). The need to replace a culture medium with another of different composition at about the 8-cell stage of human development has been outlined (Gardner, 1998) as follows: ''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''.

This intuitively reasonable assertion is based on the 'back-to-nature' principle. It is, however, an heuristic argument depending on the truth of two hypotheses. The first hypothesis is that the environment of the preimplantation embryo changes as it travels from the ampulla of the oviduct to the uterus. As shown earlier, extant evidence that this hypothesis is true is based on weak information from analyses of glucose and pyruvate in oviductal and uterine fluid collected from non-pregnant women (Gardner *et al*., 1996). The second hypothesis is that the chemical compositions of the environments which an embryo encounters have evolved so as to be highly correlated with the embryo's metabolism. This reasoning is based on the well-documented observations in animal models and humans that, in early development, the embryo preferentially utilizes pyruvate over glucose and that around the time of compaction this preference is reversed so that the embryo uses glucose rather than pyruvate. These changes in the preferential use of glucose and pyruvate appear complementary to observed changes in the composition of fluids in the oviduct and uterus during the menstrual cycle. Gardner's explanation lacks rigor as it overlooks the possibility that embryos in culture may adapt and selectively utilize what they require from a constant environment as long as the concentrations of the chemical constituents fall within tolerable ranges.

It has been stated many times that a two-step culture protocol is superior to a protocol in which a single medium is used throughout without renewal. Reports have been published comparing some of these commercial protocols (Devreker *et al*., 2001; Langendonckt *et al*., 2001). Few, if any, studies have been conducted asking the more fundamental, practically important, question: ''What is the experimental evidence that justifies the use of two-step protocols instead of a one-step protocol where the medium is left unchanged for a culture period of 5 days?'' Good data exist to show that KSOM-derived media support complete mouse preimplantation embryo development of a wide variety of strains of mice (Summers *et al*., 1995; 2000). Moreover, high implantation rates and normal fetal development based on measurements of fetal weights, interval fetal growth and normal anatomy are observed after embryo transfer into pseudopregnant recipients (Summers *et al*., 1995; 2000; 2003; Biggers *et al*., 2003). KSOM-type media have also been shown to support preimplantation embryo development in the cow (Liu and Foote, 1996; 1997; Elhassan *et al*., 2001), rabbit (Liu *et al*., 1996; Deng and Yang, 2001), pig (Machaty *et al*., 1998) and rhesus monkey (Weston and Wolf, 1996). A supplemented form of KSOM has been used for the culture of human blastocysts derived either from poor-quality, normally discarded multicell embryos (Lavoir *et al*., 1998) or donated cryopreserved embryos (Summers *et al*., unpublished results) to produce human stem cells. Others (Wiemer *et al*., 2002) showed that day 3, 6- to 8-cell stage human preimplantation embryos cultured in KSOM supplemented with amino acids develop to blastocysts in high yield (about 60%) after a further 2 days of culture in a select group of patients. It has also been shown (Biggers and Racowsky, 2002) that human blastocysts develop from zygotes with high efficiency in 5 days of culture using medium KSOM^{AA} (Biggers *et al*., 2003), without renewal of the medium. The yield did not differ from the efficiency of a two-step protocol used routinely. More recently, others (Macklon *et al*., 2002) have compared in a prospective randomized study a single complex medium (without the need for co-culture) and a sequential medium (G1/G2) for the culture and development of human blastocysts derived from IVF. These authors reported no difference in either blastocyst formation, implantation rate or pregnancy. Further investigations comparing development using either two- or one-step protocols may show that although two-step procedures are sufficient to support development, they may not be necessary.

Cellular homeostasis

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Stresses are inevitably placed on preimplantation embryos grown *in vitro* in chemically defined media. The stresses arise because chemically defined media provide only a partial representation of the natural environment in which the embryos develop. The fact that embryos will grow under these conditions suggests that they can compensate and/or adapt to the imposed stresses (Biggers, 1993; 1998; Leese, 1995; 2002; Lane, 2001). For example, metabolic processes in the embryos continually deplete or add substances to the medium which affect the intercellular pH and cell volume of cells, thereby imposing minor stresses. The embryo counteracts these perturbations so as to maintain the intracellular pH and cell volume at the normal set points of these parameters (for reviews, see Biggers, 2002; Biggers *et al*., 1991; Lane and Gardner, 2000). If the imposed pH and osmotically related stresses are too great, then these responses will fail and the embryos will die.

Regulation of cell volume

Delicately balanced mechanisms are brought into play to maintain a normal cell volume after cells are exposed either to hypotonic or hypertonic solutions (Lang *et al*., 1998; Lange, 2000). Three sequential responses occur when a cell is placed in a hypertonic solution (Figure 5). An initial rapid response involves the movement of water out of the cell, causing it to shrink. A second, slower, response follows this initial response in which ions and/or organic osmolytes move from the environment into the cell, thereby restoring the cell volume. If the cell volume is still not restored, a very slow third response occurs in which genes are activated to stimulate the synthesis of organic osmolytes

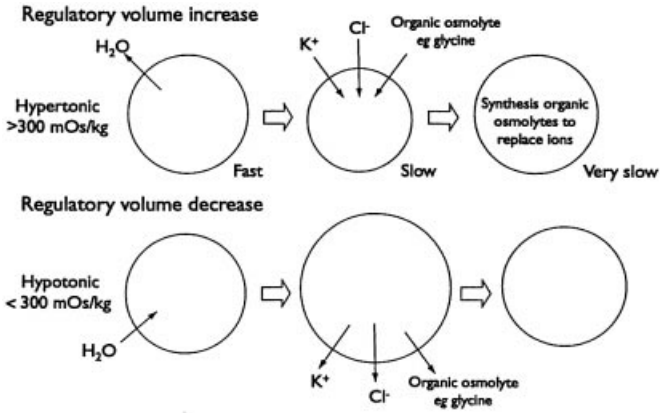

Figure 5. Control of regulatory volume increase and regulatory volume decrease in response to hypertonicity and hypotonicity. Reproduced from an article in *Reproductive BioMedicine Online* by Biggers (2002), with permission from Reproductive Healthcare Ltd.

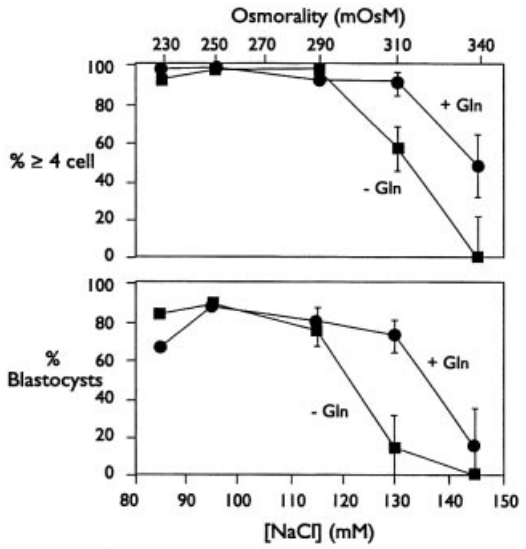

Figure 6. Development of mouse zygotes in KSOM in media of different osmolarity produced by varying the concentration of NaCl with and without glutamine. Reproduced from Dawson and Baltz (1997), with permission from the Society for the Study of Reproduction, Inc.

intracellularly. Exposure to a hypotonic solution results in the same sequence of responses, each in reverse.

Considerable advances have been made in recent years in understanding the regulation of cell volume in the preimplantation embryo of the mouse (Baltz, 2001). Figure 6 illustrates the effect of varying the concentration of NaCl in a medium on the development of a mouse zygote into a blastocyst *in vitro*. When the NaCl concentration exceeds ~115 mmol/l (290 mOsm), the development of the embryos is severely impaired; development is not impaired if the NaCl concentration is as low as 80 mmol/l (~230 mOsm). The organic osmolyte, Gln, significantly protects the embryo from the injurious effects of a high concentrations of NaCl when added to a medium. Other organic osmolytes such as glycine, betaine, proline, alanine and hypotaurine, also protect the mouse preimplantation embryo against hypertonicity (Van Winkle *et al*., 1990; Biggers *et al*., 1993; Dawson and Baltz, 1997). Three transporters, located in the cell membrane, are known to regulate the cell volume of preimplantation embryos, namely GLY, β and VSOAC. GLY imports glycine, glutamine and other compounds into the cell, β imports taurine, alanine and other amino acids, and VSOAC exports organic osmolytes (Figure 5). The joint action of these transporters, which are sensitive to changes in cell volume, maintains the intracellular ionic strength at levels that do not impair cellular functions and embryonic development in the face of high external osmolarity.

The latest estimate of the osmolarity of mouse oviductal fluid is ~300 mOsm (Collins and Baltz, 1999). Yet, paradoxically, the osmolarity of many effective media for the culture of preimplantation embryos is significantly lower, at 250–270 mOsm. It has been proposed that preimplantation embryos, particularly the zygote, maintain normally an intracellular ionic strength sufficient to offset an external tonicity of 250–270 mOsm (Baltz, 2001). When the embryos are in an environment with higher tonicity—as *in vitro* or in media with higher tonicity—the balance is made up by importing organic osmolytes. Thus, the beneficial effects of adding certain amino acids to media for the culture of preimplantation embryos may be due to their potential roles as organic osmolytes. In the design of media it is clearly wrong to add inorganic salts to a medium to raise its osmolarity to that present in oviductal fluid.

Regulation of pH

The normal functions of cellular proteins depend on the degree of ionization of their component amino acids. The ionization in turn depends on the presence of a stable acid–base status in the cells (Tyler-Jones and Taylor, 1999). Preimplantation embryos are usually cultured in a closed system. A stable acid–base status in the cells of the embryo depends on the exchanges between the three compartments of the culture system: the embryo, the medium and the gas phase.

The acid–base status of a mixture of solutes in water is determined by the relative activities of hydrogen (H^+) and hydroxide (OH^-) ions. The control of this ratio is complex. In biological fluids, such as arterial blood plasma, it depends on three factors that act independently: the strong ion difference; the carbon dioxide partial pressure; and the total weak acid present (Stewart, 1978). The strong ion difference is the difference in the concentrations of strong cations and anions, the most important of which are sodium, potassium, magnesium, chloride and lactate. In a culture system using a chemically defined medium, all three of these factors are held constant and the solution is buffered using a CO_2/bicarbonate system. Thus, the pH—and hence the $[H^+]$—can be approximately estimated using the Henderson–Hasselbach equation:

$$pH = pK_a + \log [HCO_3]/[CO_2]. \quad (1)$$

where pK_a is the pH when $[HCO_3]$ and $[CO_2]$ are equal, which depends on other factors such as ionic strength and temperature. A practically useful form of this equation is:

$$pH = 6.1 + \log [[NaHCO_3]/(0.03 \times PaCO_2)] \quad (2)$$

where $PaCO_2$ is the partial pressure of CO_2 in mmHg, $[NaHCO_3]$ is the concentration of $NaHCO_3$ in mmol/l, and $pK_a = 6.1$. $pK_a = 6.1$ is the value when the solution is the ionic strength of blood and

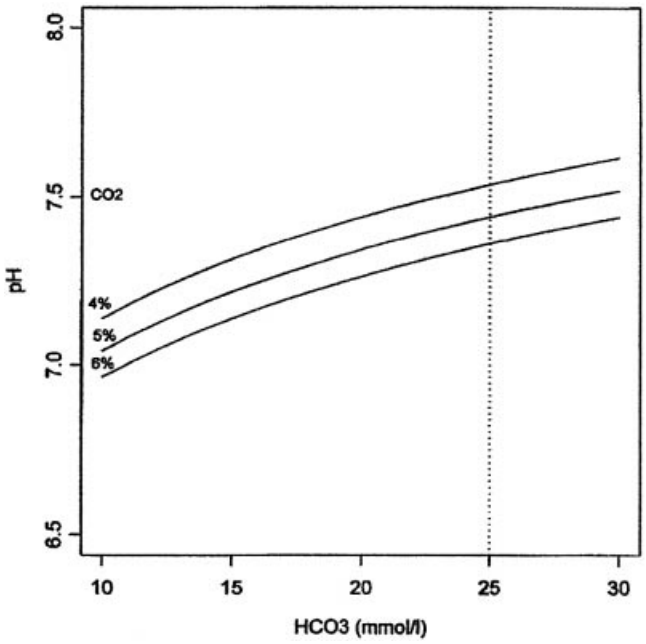

Figure 7. Effect of varying the concentrations of HCO_3^- and CO_2 on pH, calculated from the Henderson–Hasselbach equation.

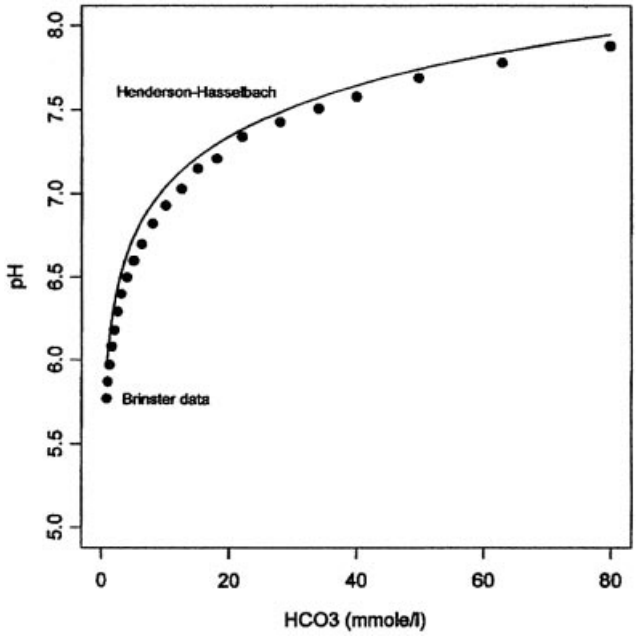

Figure 8. The relationship between (a) the concentration of HCO_3^- under a gas phase of 5% CO_2 and pH calculated using the Henderson–Hasselbach equation; and (b) the experimental measurement by Brinster (1965a) of the pH of a base medium containing different concentrations of HCO_3^- under a gas phase of 5% CO_2.

the temperature is 37°C. For example, if [$NaHCO_3$] is 25 mmol/l, and if the medium is gassed with 5% CO_2 in an atmospheric pressure of 740 mmHg where the $PaCO_2$ is 740×5/100 = 37 mmHg, the estimated pH is 7.45. These calculations are readily performed on the website http://medcalc3000.com/HendersonHasselbach.htm.

Figure 7 shows the effect of different [$NaHCO_3$] and [CO_2] on pH using Eqn (2). There is considerable latitude in the choice of concentrations of these two substances in order to maintain the pH between 7.0 and 7.5. The adoption of 25 mmol/l $NaHCO_3$ and 5% CO_2, which is widely used for the culture of preimplantation embryos began with the work of Whitten (Whitten, 1956). The choices were based on the concentration of $NaHCO_3$ in Krebs-Ringer solution (24.88 mmol/l), described by Krebs and Henseleit (Krebs and Henseleit, 1932), on which Whitten's medium was based. It is important to recognize that the Henderson–Hasselbach equation only approximates the pH that is found in culture media. This fact is illustrated in Figure 8, based on the data of Brinster (Brinster, 1965a) who measured the pH of a simple medium containing 10.15 mmol/l sodium lactate gassed with 5% CO_2 in which [$NaHCO_3$] was varied. The Henderson–Hasselbach equation slightly overestimated the pH at all values of [$NaHCO_3$].

From a physiological point of view, pH is an unsatisfactory parameter to evaluate the acid–base status of a system (Tyler-Jones and Taylor, 1999). It is more realistic to use the actual H^+ concentration, rather than pH which can distort reality since it is a non-linear transformation of [H^+]. There is about 36 nmol/l [H^+] (pH = 7.44) in a medium containing 25 mmol/l [$NaHCO_3$] gassed with 5% CO_2 at 760 mmHg. This value is almost one-half the [H^+] concentration of 63 nmol/l (pH = 7.2) in human embryonic cells (Phillips *et al*., 2000).

Knowledge of the control of intracellular pH in the cells of the preimplantation embryo has increased rapidly in recent years from studies that permute the extracellular environment (for reviews, see Biggers *et al*., 1991; Lane and Gardner, 2000). These investigations have been conducted mainly on mouse embryos, supplemented by other studies on hamster and human embryos. The intracellular pH of the cells of preimplantation embryos is controlled locally and rapidly by intrinsic buffers such as the zwitterionic amino acids. More persistent changes in the intracellular pH are regulated more slowly by the activities of ionic transport systems in the cell membrane. Studies on the human preimplantation embryo have shown that intracellular alkalosis and acidosis are compensated by transporters analogous to those found in the mouse (Phillips *et al*., 2000). Recovery from alkalosis is mediated by a Cl^- HCO_3^- exchanger that is activated when the pH is >7.2. Recovery from acidosis involves two transporters: a tentatively identified Na^+, $Cl^-HCO_3^-$ exchanger which is activated when the pH is <7.0; and a Na^+-H^+ antiporter which is activated when the pH is <6.8. These three transporters collectively maintain the pH_i of blastomeres at a set point that falls between 7.0 and 7.3, provided that HCO_3^- and CO_2 are present.

It has been shown (Brinster, 1965a) that mouse embryos can develop over a wide range of pH values, from 5.87 to 7.78. The implication of this result is clarified by plotting the results of the percentage development against the [H^+] (Figure 9). The results show that mouse embryos develop in a range of [H^+] from to 16.6 nmol/l (pH 7.78) to 1349 nmol/l (pH 5.87), with a maximum at 151 nmol/l (pH 6.82). The concentration–response line is not symmetrical, being much steeper on the left of the maximum than the right. Thus, relatively small reductions in [H^+] from the optimum [H^+] have a pronounced inhibitory effect on development. In contrast, comparable increases in [H^+] above the optimum [H^+] have relatively little inhibitory effect. There is considerable

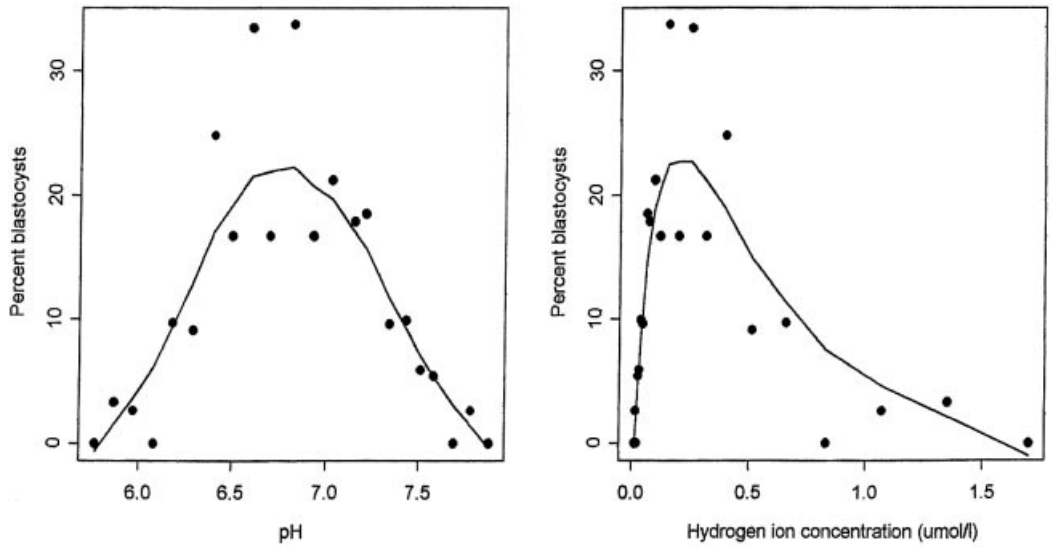

Figure 9. Development of 2-cell mouse embryos into blastocysts *in vitro* in a base medium containing different concentrations of H^+. [Calculated from the data of Brinster (1965a).]

evidence from several species that the pH of oviductal fluid is alkaline in the range pH 7.7–8.2. Thus, the $Cl^-HCO_3^-$ exchanger in the blastomeres of the early embryo developing *in vivo* would be continually active. Buffering a medium to maintain a pH ~7.2 using a HCO_3/CO_2 system would remove the naturally occurring stimulation of the $Cl^-HCO_3^-$ exchanger.

Pathophysiology arising from stresses due to culture

Do the conditions used for the culture of preimplantation embryos have long-term deleterious effects? Stresses occur when homeostatic mechanisms are overcome, and under extreme conditions, the embryos die. Of particular interest are those conditions where the embryos survive but may be seriously impaired. The concerns range from short-term effects such as generation of high concentrations of free radicals, particularly reactive oxygen species (ROS) (Johnson and Nasr-Esfahani, 1993; Guérin *et al*., 2001) to long-term effects that are only manifested later in development after the cultured embryos are transferred to surrogate mothers (Rieger, 1998; Khosla *et al*., 2001a). Many of these topics fall outside the scope of this review. Three areas, however, are briefly summarized here: the effects of ROS; the abnormal activation of genes; and some general aspects of the cellular response to stress.

ROS and preimplantation development in vitro

Under certain conditions, dioxygen (O_2) can give rise to the ROS: superoxide anion (O_2^{*-}), hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^*) (Cadenas and Davies, 2000). OH^*, the most reactive of these species that can damage DNA, RNA and proteins, is produced when homeostatic mechanisms that maintain the balance between O_2^{*-} and H_2O_2 are insufficient. O_2^{*-} can also react with nitric oxide (NO^*), a reactive nitrogen species, to produce peroxynitrite ($ONOO^-$) which can damage lipids, proteins and DNA (Ghafourifar and Richter, 1997; Vinten-Johansen, 2000). ROS are produced by preimplantation mouse embryos only in culture and only at the G_2/M stage of the second cell cycle (Nasr-Esfahani and Johnson, 1990). Also, the embryo is sensitive to NO^* only at the mid- to late 2-cell stage of development (Warren *et al*., 1990).

In the pioneer studies on the culture of mouse preimplantation embryos, the media were gassed with 5% CO_2 in air which contains about 20% O_2 (Whitten, 1956; Brinster, 1963). Later (Whitten, 1970), claimed that mouse zygotes would not cleave unless the cultures were gassed with 5% CO_2, 5% O_2 and 90% N_2. Over the years, considerable variation occurred between different laboratories as to which of these two gassing mixtures should be used. Since the oxygen tension in the mammalian oviduct is low, typically in the range of 7 to 70 mmHg (Fischer and Bavister, 1993), it has been argued that a gas mixture with a lower oxygen tension is preferable. Using an oxygen tension above normal could favour the production of excess ROS. In fact, minimization of ROS production might explain the recent finding that gassing a medium with 5%, rather than 20% O_2, for the culture of preimplantation mouse embryos results in a higher yield of blastocysts with larger cell numbers (Orsi and Leese, 2001). Similar studies on human preimplantation embryos have not shown any significant advantage of a low oxygen tension over the first 2–3 days of development *in vitro* (Noda *et al*., 1994; Dumoulin *et al*., 1995, 1999). Although the effects of oxygen tension on preimplantation development are still not clear, it seems that the use of a low oxygen tension for the gassing of culture media may be preferable.

Gene expression

There is ample evidence that genes control the development of preimplantation embryos (for a review, see Warner and Brenner, 2001). The advent of ultra-sensitive methods has demonstrated the expression of a large number of active genes during the preimplantation period of development. (Ko *et al*., 2000; Latham *et al*., 2000). After mapping 798 genes in the mouse it was concluded that: (i) ''a significant fraction of the genome is dedicated to genes expressed specifically in early development; ...

(ii) genes co-expressed in the same stage tend to cluster in the genome; and (iii) the expressed genes include cohorts acting in a stage-specific manner...'' (Latham *et al*., 2000). These changes are also observed at the level of protein synthetic activity in early cleavage-stage mouse embryos (Latham *et al*., 1991; 1993). Similar observations were made some years ago on the ontogeny and chronicity of protein expression patterns in early embryos of *Drosophila melanogaster* (Summers *et al*., 1986). Thus, there is considerable opportunity for the disruption of the activity of these genes when embryos are removed from their natural environment and manipulated *in vitro* (Niemann and Wrenzycki, 2000).

A number of observations have been made on the effects of culture conditions on patterns of gene expression in mouse embryos. For example, there are comparisons of the activities of genes in embryos developing *in vivo* with those developing *in vitro*. One group (Shim *et al*., 1996) found that the development of laminin chain-specific mRNA transcripts was less in embryos developing in medium M16 (Whittingham, 1971) than in those developing *in vivo*. Similarly, others (Uechi *et al*., 1997) estimated that the level of expression of GLUT-1 in 2-cell embryos grown in medium BWW (Biggers *et al*., 1971) was one-tenth of that in embryos which developed *in vivo*. Another group (Fleming *et al*., 1997) found no difference in the appearance of some members of the growth arrest genes (gas and gadd family) in preimplantation embryos that developed *in vivo* compared with those cultured in medium KSOM. A significant increase in Hsp 70.1 gene was reported in embryos cultured in medium M16 compared with the increase that occurs *in vivo* (Christians *et al*., 1995). Others (Vernet *et al*., 1993) described studies of transgenic mouse embryos expressing the reporter gene lacZ; these authors observed that transgene expression was induced at the 2-cell stage in embryos cultured in medium M16, but not *in-vivo*-derived embryos. However, an apparent inhibitory effect of culture conditions compared with natural development *in vivo* needs to be assessed carefully, as the effect may be due to a generalized slowing down of development arising from stressful culture conditions.

Comparisons have been made on the expression of genes in embryos cultured in different media. One group (Ho *et al*., 1995) analysed the expression of nine genes: five of the genes (actin, G3PDH, Na^+/K^+-ATPase, Sp1, Tata box binding protein) were expressed in the same manner when cultured in Whitten's medium (Whitten, 1970) and medium KSOM supplemented with half-strength Eagle's amino acids ($KSOM^{AA}$), whereas four genes (IGF-I, IGF-IR, IGF-II, IGF-IIR) were expressed significantly higher in the embryos cultured in $KSOM^{AA}$ than those in Whitten's medium. Previous studies have shown that both IGF-I and IGF-II have specific stage-dependent effects on protein synthetic activity in cultured preimplantation mouse embryos (Shi *et al*., 1994). Others (Doherty *et al*., 2000) have also examined the expression of the H19 gene (which is concerned with imprinting) in embryos cultured in Whitten's medium and $KSOM^{AA}$. The gene was found to be expressed normally by only the maternal allele in embryos cultured in $KSOM^{AA}$, but abnormally in embryos cultured in Whitten's medium where it was expressed in both the maternal and paternal alleles. The pattern of expression of imprinted genes is presumably a reflection of the epigenetic control of DNA methylation. In this regard, KSOM-based media both for IVF and culture *in vitro* have been used to obtain developmentally synchronized mouse zygotes to study DNA methylation/demethylation patterns in early mouse embryos (Santos *et al*., 2002). Three phases of epigenetic modification of nuclear DNA were determined: (i) active demethylation of the male pronucleus; (ii) passive demethylation during cleavage stages; and (iii) *de-novo* methylation in the ICM at the blastocyst stage.

An important study which is often overlooked (Ho *et al*., 1994) showed that the expression of mRNAs of IGF-I, IGF-IR, IGF-II and IGF-IIR in embryos cultured in medium SOM (Lawitts and Biggers, 1993) was reduced when the concentration of NaCl was increased from 85 to 125 mmol/l. It is essential to recognize that the expression of genes in preimplantation embryos cultured in the same medium can be altered by changing the concentration of a single constituent.

The results of studies in mice demonstrate that the composition of chemically defined media can significantly alter the expression of genes in the preimplantation embryo. Other studies have determined that culture conditions affect gene expression in both sheep (Young, L.E. *et al*., 2001) and cow (Niemann and Wrenzycki, 2000; Wrenzycki *et al*., 1996; 1999; 2001). Recently, one group (Rizos *et al*., 2002) studied patterns of mRNA expression in bovine embryos generated under four different conditions, and demonstrated major differences in the differential expression of a number of developmentally important genes.

No detailed comparative studies have been carried out on the effect of culture media on the expression of genes during development of the human preimplantation embryo. A paucity of material and obvious ethical restrictions make such studies very difficult to undertake. It seems likely, however, that patterns of gene expression in the human embryo are culture-dependent. The last point is of particular concern when considering the recent proliferation in media for the extended culture of human preimplantation embryos. Studies on human preimplantation embryos have described the expression of a number of imprinted genes (Hemmings *et al*., 1992; Lighten *et al*., 1997a,b; Huntriss *et al*., 1998; Salpekar *et al*., 2001; for a listing of imprinted genes, see Morrison and Reeve, 1998).

Other aspects of stress

The effects of stress on cells at the molecular level represents a particularly active area of research at the present time. Substances such as the heat shock proteins (HSP), CCAAT/enhancer binding protein homologous protein-10 (CHOP-10) and ceramide are involved. For example, inhibiting the HSP Hsp70 by its complementary antisense oligonucleotide sensitizes the 4-cell mouse embryo to arsenite (Dix *et al*., 1998). Hsp70 is produced constitutively in the mouse from the zygote to the 8-cell stage (Bensaude *et al*., 1983), and can be induced in response to stress only after the 4-cell stage is reached (for a review, see Luft and Dix, 1999). Blocking translation of Hsp70 mRNA inhibits blastocyst development of mouse embryos, indicating a normal functional role for Hsp70 in early development (Dix *et al*., 1998). Hsp70 acts as a molecular chaperone ensuring the normal tertiary structure of newly synthesized protein.

Recently, attention has turned to another stress protein, Hsp90, not only as a molecular chaperone (Young, J.C. *et al*., 2001), but also as a buffering agent against genetic variation and morpho-

logical evolution (McLaren, 1999; Mitchell-Olds and Knight, 2002; Rutherford, 2003). The principle of buffering the genotype or of developmental homeostasis is the basis of Waddington's model of the canalization of development (Waddington, 1942). Studies in *Drosophila* (Rutherford and Lindquist, 1998) have shown that by modifying Hsp90 function, either by using Hsp90 mutants or pharmacological manipulations, specific variants are produced, depending on the genetic background. When Hsp90 function is compromised by stress, cryptic variants are produced that are selected for and can be continually expressed even after Hsp90 function is restored. It was concluded that the phenotypic changes represent cryptic genetic variation that is normally prevented unless Hsp90 function is compromised. More recent studies on the *D. melanogaster* segmentation mutant, *Kruppel(Kr)* have provided evidence that changes in the level of Hsp90 can result in an heritably altered chromatin state (Sollars *et al*., 2003), thus providing a molecular mechanism for the role on Hsp90 as a capacitor for morphological evolution. Similar findings have recently been reported for *Arabidopsis thaliana* (Queitsch *et al*., 2002). The effects of stress on phenotypic variability in different strains of mice in light of the recent findings on Hsp90 have been discussed (McLaren, 1999; for an historical perspective on stress and phenotypic variability in the mouse, see Biggers, 2001). Preimplantation stage embryos in culture are in a fixed microenvironment in which the response of each embryo to stress would presumably be dependent on its genetic background. It is possible that, under certain circumstances, the stress induced by *in-vitro* culture compromises the homeostatic mechanisms, resulting in the expression of cryptic genetic variations leading to phenotypic variability. In the mouse, the homologues of Hsp90 are Hsp84 and Hsp86, and both of these are expressed in preimplantation stage mouse embryos (Barnier *et al*., 1987; Bensaude and Morange, 1983; Morange *et al*., 1984; Latham *et al*., 1992).

The gene CHOP-10 is over-expressed in mouse and bovine blastocysts exposed to stresses such as arsenite or the alkylating agent methyl methane-sulphonate (Fontanier-Razzaq *et al*., 1999; 2001). The levels of ceramide, a compound involved in sphingolipid metabolism, are increased in cells exposed to stress (Mathias *et al*., 1998; Ronai, 1999; Hannun and Luberto, 2000), but as yet this compound has not been studied in the preimplantation embryo. CHOP-10 and ceramide have each been shown to be involved in the control of cell cycle arrest and apoptosis. Might it be that these substances are produced by stresses induced by unbalanced chemically defined media, and that they potentially affect the later development of the fetus? Some further recommendations have recently been made on reducing embryo stress when considering the design of chemically defined media for the culture of preimplantation embryos (Leese, 2002).

Preimplantation embryo culture and its long-term effects

In 1984, Iannaccone described the results of studies in which cultured cleavage-stage mouse embryos were briefly exposed (1 h) to the chemical mutagen, *N*-methyl-*N*-nitrosourea (MNU) (Iannaccone, 1984). The time of exposure to MNU did not change the proportion of embryos progressing to blastocysts when compared with control embryos. However, offspring derived from MNU-exposed embryos had a 3-fold higher crude mortality rate in the first year when compared with controls. No gross morphological or histological abnormalities were detected; neither were abnormal karyotypes in the offspring demonstrated in either the treated or control groups. The findings of this study did demonstrate, however, that brief exposure to a chemical insult early in preimplantation embryo development can have effects which persist long after the exposure.

Earlier studies showed that mouse embryos cultured *in vitro* in Brinster's medium cleaved at a slower rate than *in vivo* embryos (Bowman and McLaren, 1970a). Embryo transfer experiments also confirmed that fetuses from *in-vitro*-cultured blastocysts weighed less than control, uterine blastocysts transferred at the same time into foster mothers (Bowman and McLaren, 1970b). Other studies also reported low viability of *in-vitro*-cultured blastocysts after embryo transfer using culture media available at the time (Biggers *et al*., 1965). More recently, other groups have confirmed decreased viability after *in-vitro* culture of mouse embryos prior to embryo transfer (Caro and Trounson, 1984; Arny *et al*., 1987; Van der Auwera *et al*., 1999). These studies, however, used chemically defined media supplemented with serum. Others have described studies on the effects of adding serum to medium M16 on the culture and post-implantation development of mouse embryos (Khosla *et al*., 2001a). After embryo transfer, fetuses derived from blastocysts cultured in M16 with serum weighed less than control embryos or M16 cultured embryos; they also were shown to have a different pattern of expression of several imprinted genes (Khosla *et al*., 2001a).

Previous studies in mice have shown that epigenetic events in the preimplantation embryo can affect the adult phenotype (Reik *et al*., 1993; Dean *et al*., 1998). Similarly, studies of cloned mice derived from adult somatic cell nuclear transfer have demonstrated an association with an obese phenotype that is not transmitted to the offspring suggesting epigenetic changes due to cloning (Tamashiro *et al*., 2002). Others have also shown that developmental abnormalities seen in cloned mice are not transmitted to the offspring (Shimozawa *et al*., 2002). Interestingly, evidence has been presented that cloned embryos have different media requirements when chemically defined media are used (Heindryckx *et al*., 2001; Chung *et al*., 2002). By way of explanation, others (Gao *et al*., 2003) have shown that cloned mouse embryos display donor nuclei somatic cell-like characteristics suggesting incomplete nuclear reprogramming. The cloned embryos displayed improved development in typical somatic cell culture media (for example, Ham's F10/DMEM). Recent studies in the cow (Enright *et al*., 2003) and mouse (Mann *et al*., 2003b) have focused on DNA methylation and histone acetylation in the donor somatic cell nucleus.

Recent studies have also focused on the role of culture media in early preimplantation embryo development, epigenetic events and its effect on genomic imprinting (for reviews, see Young and Fairburn, 2000; Khosla *et al*., 2001b; Rycke *et al*., 2002). Much of the stimulus for these investigations is based on findings in ruminants in which unusually large offspring are born following embryo culture and manipulation, the so-called large offspring syndrome (LOS). In general, LOS is associated with the use of complex media, sera or the use of co-culture. There is an extensive literature on the effects of *in-vitro* culture on embryos of domestic ruminants (for reviews, see Sinclair *et al*., 2000; Walker *et al*., 2000; Khosla *et al*., 2001b), and the topic will not be further reviewed here. Interest, however, is based on some common

phenotypic features of LOS, aberrant expression of imprinted genes and human fetal overgrowth syndromes, such as Beckwith–Wiedemann syndrome (BWS). BWS is associated with abnormal expression of a cluster of imprinted genes on the short arm of chromosome 11 (11p15.5; Morrison and Reeve, 1998). There are a number of human diseases and syndromes that have been associated with aberrant expression of imprinted genes, including Prader–Willi syndrome and Angelman syndrome (Morrison and Reeve, 1998). However, the occurrence of similar phenotypes between different species does not necessarily indicate similar underlying molecular mechanisms or patterns of expression of imprinted genes (Young, 2002). Several articles have been published recently (Powell, 2003; Gosden *et al*., 2003) emphasizing the need for continued vigilance on the possible relationship between *in-vitro* technologies and human genomic imprinting disorders, particularly BWS and Angelman syndrome. Attention is also directed to a recent commentary (Edwards and Ludwig, 2003). A large number of imprinted genes occur in clusters, the regulation and expression of which in different culture media have not been studied. Present evidence suggests that epigenetic regulation and modification of imprinted genes is through DNA methylation of CpG islands (Arney *et al*., 2001; Carlone and Skalnik, 2001; Dean and Ferguson-Smith, 2001). Interest is also directed at histone modification and the role of the nucleosome (Ahmad and Henikoff, 2002) and non-coding RNAs (Anderson and Panning, 2003). It is a sobering thought that even our current understanding of the mechanism and regulation of imprinting at H19/IGF-II, which is a well-studied experimental model of genomic imprinting, is far from complete (Arney, 2003).

Studies on the effects of manipulating preimplantation mouse embryos on post-natal growth and adult phenotypes using genetically identical (CS7/BL/6J×SJL/J) F_1 mice have been described (Papaioannou *et al*., 1989; Biggers and Papaioannou, 1991a). The growth and development of three groups of mice were compared: (i) controls, the natural offspring of timed matings; (ii) transferred controls, the offspring from 2-cell embryos transferred to recipients; and (iii) transferred half embryos, the offspring developing from one blastomere from the 2-cell stage transferred to recipients. In general, the differences observed were attributed to the smaller litter sizes in the transferred groups rather than to the effects of the manipulations or brief culture of embryos to the 2-cell stage. Subsequently, the mice were tested for the ability to escape from water (using the Morris Water test, which is a spatial navigation test; Crawley, 2000) as a test of the functional integrity of the nervous system (Biggers and Papaioannou, 1991b). No significant differences were detected in either escape time or learning time in male and female mice in the three groups. It remains to be seen if extending the culture time of preimplantation embryos to include blastocyst transfers will produce similar results when using specific behavioural paradigms. Recently, one group (Fernandez-Gonzalez *et al*., 2003) studied mouse blastocysts derived from culture in either KSOM or KSOM supplemented with 10% fetal calf serum. Blastocysts derived from each group were transferred into CD1 pseudopregnant recipients and allowed to go to term. Post-natal weight gain was reported to be higher in females derived from embryos cultured in the presence of serum. Adult mice derived from the embryo transfers were assessed using a variety of behavioural tests, and differences were reported in the adults derived from embryos cultured in KSOM supplemented with serum. Richard Schultz and coworkers (Schultz, 2003; R.Schultz, personal communication) have also presented preliminary results of behavioural studies of adult mice derived from *in-vitro* culture of blastocysts in two different media: Whitten's medium (Whitten, 1970) and KSOM (Lawitts and Biggers, 1993). These authors also have noted differences between the control and experimental groups depending on the particular behavioural paradigm used. These preliminary experimental findings are important—particularly if confirmed—as they are derived from the extended culture of mouse preimplantation embryos. It is, however, essential to confirm rigorously that there are no potential confounding variables that may have arisen between the control and treatment groups. For example, in previous studies (Biggers and Papaioannou, 1991a,b; Papaioannou *et al*., 1989) the observed post-natal differences between the control and experimental mouse groups were attributed to embryo transfer, birth weight and litter size, and likely environmental factors.

Much of our current understanding of the effects of the *in-vitro* culture of human embryos is based on pregnancy outcome data after the transfer of 2- to 3-day cultured embryos, typically at the 2- to 8-cell stage. Early studies by Edwards and colleagues established that human zygotes could be cultured in several different media, usually supplemented with inactivated serum (Edwards, 1981; Steptoe and Edwards, 1978; Edwards *et al*., 1970; 1980; 1981). Subsequently, others (Menezo *et al*., 1984) described a chemically defined medium B3 that would support *in-vitro* development of human embryos without added serum. Shortly thereafter, the medium HTF was described (Quinn *et al*., 1985). The extended culture of human embryos to the blastocyst stage in high yield was initially achieved using co-culture (Menezo, 1987; Menezo *et al*., 1990). Methods involving the sequential use of two media now dominate the field. Thus, current outcome data are based on pregnancies derived from embryos that typically were cultured in media that are probably no longer in widespread use in human IVF clinics. Some outcomes data on blastocyst transfer have been published (Menezo *et al*., 1999; 2000), but few clinical data exist on sequential media, except for pregnancy rates. One group (Kausche *et al*., 2001), in a retrospective study, reported no differences in the sex ratio and birth weight of infants resulting from the transfer of blastocysts derived from sequential culture, compared to cleavage-stage embryos. A full review of this topic is beyond the scope of this article, but some general points are worth mentioning.

Studies have reported a higher rate of low birth weight and obstetric complications in singleton pregnancies derived from assisted reproductive technology (ART) when compared with natural conceptions (Doyle *et al*., 1992; Tan *et al*., 1992; FIVNAT, 1995; Verlaenen *et al*., 1995; Bergh *et al*., 1999; Tarlatzis and Grimbizis, 1999; Schieve *et al*., 2002). However, not all studies of pregnancy outcome agree (Menezo *et al*., 2000). In addition, it is not clear if the low birth weight of singleton infants conceived through ART is due to the methodology used or to other factors related to infertility (Williams *et al*., 1991; Olivennes *et al*., 1993; Wang *et al*., 1994; Henriksen *et al*., 1997; McElrath and Wise, 1997; Sundstrom *et al*., 1997; Kallen *et al*., 2003). Interpretation of the data is also compounded by the heterogeneous nature of the patient population; this includes infertility diagnosis, patient age, medications and clinical protocols used for controlled ovarian stimulation, oocyte quality, and perhaps also differences in human

embryology laboratory practices and protocols. The last subject has recently been reviewed in detail (Geber, 2002; Jones *et al*., 2002; Gardner and Lane, 2003). Recent epidemiological studies suggesting a causal relationship between impaired growth during early life and adult-onset diseases that include cardiovascular disease, hypertension, type II diabetes and metabolic changes associated with insulin resistance syndrome raises some concerns (for reviews, see Desai and Hales, 1997; Newnham, 1998; Betteridge, 2001). This relationship has typically been presented under the rubric of the 'Barker Hypothesis' (see Newnham, 1998; Young, 2002), notwithstanding the considerable confusion and history of what constitutes low birth weight (Wilcox, 2001). From an heuristic standpoint it is not clear if one can apply the Barker hypothesis, which is based largely on retrospective epidemiological studies on low birth weight, *in-utero* growth restricted (IUGR) fetuses, and extend it to include the preimplantation embryo as some reviewers have done. This does not preclude the possibility of a relationship between the *in-vitro* culture of human preimplantation embryos and subsequent abnormal placental function causing IUGR, but there is at present a paucity of direct experimental evidence to support this position. Moreover, human studies are complicated by the finding that women with low fertility who conceive without the use of ART are still at increased risk for IUGR and obstetric complications, such as pre-eclampsia and premature birth (Li *et al.*,1991; Williams *et al*., 1991; Kallen *et al*., 2003). Several recent articles have reviewed the genetic control of trophoblast differentiation and placental function (Cross *et al*., 2002; Pardi *et al*., 2002; Regnault *et al*., 2002; Edwards, 2003; Handwerger and Aronow, 2003). There seems little doubt that most cases of IUGR are the result of placental dysfunction either as a result of maternal factors or from abnormalities in placental development and placentation. In the more extreme cases of placental insufficiency, such as asymmetric or 'head-sparing' IUGR, loss of umbilical artery end-diastolic blood flow or even end-diastolic reverse flow with resultant dynamic shunting of blood to the brain (carotid artery) away from the gastrointestinal (superior mesenteric artery) and renal systems (renal artery) can be demonstrated using Doppler velocimetry (Wladimiroff *et al*., 1986; Arduini *et al.*, 1987; Kempley *et al*, 1991; Akinbi *et al*., 1994; Pardi *et al*., 2002). Consequently, the physiological adaptation of the fetus to a suboptimal intrauterine environment as a result of chronic under-perfusion may permanently change the structure and function of a specific terminal organ that may not be apparent until later in life (for a review, see Desai and Hales *et al*., 1997). One example is the possible relationship between delayed fetal growth, congenital oligonephropathy and adult-onset disease (Mackenzie and Brenner, 1995; Mackenzie *et al*., 1996; for a recent review, see Moritz *et al*., 2003). It has been proposed (Brenner *et al*., 1988) that essential hypertension is inversely related to nephron endowment at birth. Recently, direct experimental evidence of an inverse relationship between nephron number and essential hypertension in humans has been presented (Keller *et al*., 2003).

Issues raised by the commercialization of embryo culture media

The increasing use of extended culture of human preimplantation embryos has raised some concerns given the findings from animal studies (e.g. Sinclair *et al*., 2000; Khosla *et al*., 2001b; Rycke *et al*., 2002). A second issue related to the use of extended culture methods is that many of the companies which now advertise media apparently designed for the culture of human preimplantation embryos do not provide a full listing of the constituents in the media, or their concentrations. Is it scientifically or ethically correct to expose human embryos to chemical solutions of unknown concentration and composition? Although attention was first drawn to these issues by Biggers (Biggers, 2000), others have more recently raised similar concerns (Schatten, 2002; Winston and Hardy, 2002; Powell, 2003). One of the principal themes of this review is that many of the components used in chemically defined media for the culture of preimplantation embryos probably act in a combinatorial fashion, and it is thus imperative that the concentrations of the constituents be determined when they act in concert. Since knowledge of these concentrations is so important, it is particularly disturbing that the quantitative concentrations of the majority of commercially available media is unavailable for protective commercial reasons. The claims by the manufacturers of the superior quality of their products can only be ascertained by comparing their effects one with another. Many of the companies advertise that the composition of their media is optimized for production of blastocysts from zygotes *in vitro*, but it is not possible to determine precisely what is actually being optimized. Unless the concentrations of the components are known, the media cannot be subject to critical analysis. In 1987, Erwin Chargaff, the late professor emeritus at Columbia University, wrote an interesting and provocative article titled, ''Engineering a Molecular Nightmare'' (Chargaff, 1987). Chargaff was neither an embryologist nor a reproductive endocrinologist by profession, but as a biochemist and refugee from Europe possessed a profound understanding of the forces that shape scientific progress and social change. As commented by Chargaff (1987): ''Is it completely out of the question that during normal conception factors play a role that are not duplicated in the synthetic milieu in which *in vitro* fertilization takes place? (I am not thinking of mystical influences but of good solid chemistry.) In manipulating processes worked out by nature in the wisdom of millions of years one must be aware of the danger that our shortcuts may carry a bleeding edge.''

Given the uncertainties with the extended culture of human preimplantation embryos to the blastocyst stage, perhaps some restraint needs to be exercised on its widespread use. Clearly, there are regional differences in the use of extended culture of human embryos from IVF, for example, North America compared with Europe. This might reflect more aggressive marketing strategies by the various companies themselves. Perhaps there is a more ready acceptance by some human IVF clinics to promote the latest technologies for promotional and marketing purposes in a competitive environment, or possibly as one approach to reduce the multiple pregnancy rates seen with human IVF. There are published studies reporting on comparable success rates between Day 3 and Day 5 embryo transfers in selected groups of patients (e.g. Coskum *et al*., 2000; Plachot *et al*., 2000; Rienzi *et al*., 2002; Bungum *et al*., 2003). A recent review (Kolibianakis and Devroey, 2002) critically examined the published clinical studies on human blastocyst transfer and the use of sequential media, and concluded that there are insufficient data from properly designed studies to support the widespread use of blastocyst transfer. Recently,

Houghton and coworkers have described non-invasive methods using amino acid turnover to assess single human embryo viability on day 2–3 of culture (Houghton *et al*., 2002). These authors showed that the net turnover of several amino acids in cleavage-stage embryos was predictive of subsequent blastocyst development. These data suggest that it is possible to select embryos for transfer prior to cavitation and obviate the need for extended culture with its attendant uncertainties. Preliminary clinical data have been presented to support this idea (Brison, 2003). Moreover, the preliminary findings also support the inclusion of all 20 amino acids in the culture medium during the cleavage stages of preimplantation development (Houghton *et al*., 2002). This is a position we have always held. For example, the amino acid L-leucine is actively metabolized during the cleavage stages of human preimplantation embryos cultured *in vitro*, but is not included in most commercially available sequential media (Houghton *et al*., 2002; Brison, 2003). As wisely noted by Chargaff (1987): ''The life of man is an unrepeatable experiment. No placebos, no controls''.

Concluding remarks

It has been commented previously that the limiting factor controlling the efficiency of human IVF is the health of the zygote, and that research should be focused on the problem of improving the quality of the zygote produced by IVF, rather than on media development (Hardy *et al*., 2001). The justification of improving media, however, involves not only improving the yield of embryos for transfer but also minimizing the effects of stress which may impair later fetal development. Over the years, two basic approaches have been used to develop chemically defined media: 'back-to-nature', and 'let the embryo choose'. These two approaches, however, are not mutually exclusive and have in combination resulted in a marked improvement in the media available for the culture of mammalian preimplantation embryos. These improvements include elimination of the need to supplement media with complex sera, and the addition of amino acids and vitamins. Much work still needs to be done, however. Leese, who over the years has done much to promote the 'back-to-nature' approach to media development, recently advocated a 'let the embryo choose' principle with regard to the amino acid requirements of human embryos in culture (Houghton *et al*., 2002). There was a time, not so long ago, when it was sufficient to obtain cleavage-stage embryos which, after embryo transfer into the uteri of foster mothers, produced outwardly normal offspring. It is now apparent that there are many more subtle molecular and gene regulation issues to consider and take account of when designing culture media for preimplantation embryos. These include development of the zygote to the blastocyst stage with adequate numbers of inner mass and trophectoderm cells together with their supporting extracellular matrix that best approximates to the situation *in vivo*, along with a normal pattern of temporal and differentiated gene expression, including imprinted genes and genes related to stress, such as the heat shock genes. Improvements will require not only the inclusion of key constituents but also the choice of the concentrations of each of the constituents that support growth and development. Most importantly, this will involve the design of media that minimize the inherent effects of stress that inevitably are imposed on preimplantation embryos when placed in a chemically defined environment.

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