Thoughts on embryo culture conditions

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Abstract

This review discusses three topics: (i) the 'back to nature' and empirical optimization approaches to the design of chemically defined media for the culture of preimplantation embryos, (ii) the evolution of the simplex optimized family of media, and (iii) adaptation and stress in preimplantation embryos when placed in chemically defined media.

Keywords: *culture media, gene expression, osmolality, preimplantation embryo, stress*

Introduction

Two broad classes of media have been recognized since the pioneering phase of tissue culture that began with the work of Harrison (1907). These are biological media and chemically defined media. Biological media utilize natural body fluids, such as serum and chick embryo extract, and are therefore of unknown chemical composition. Chemically-defined media, in contrast, are made up of a set of known chemical constituents, not necessarily pure, dissolved in water. Nowadays, the preferred media are chemically defined. Their potential value was recognized by Lewis and Lewis (1911a,b), who argued that such media:

"(i) can be easily reproduced at different times in different laboratories, (ii) can be varied in a controlled manner, (iii) are free of unknown enzyme activities, *and hormones and growth factors*, which may interfere with the responses being studied". (italics are author's amendment).

These reasons are purely pragmatic in nature and are not founded on any physiological principles.

Two commercially available chemically defined media were recommended in the pioneering phases of human IVF and the culture of human preimplantation embryos. They were modified Earle's balanced salt solution and modified Ham's F10 (Edwards, 1981; Edwards *et al.*, 1981), both of which had been developed for other purposes. They were supplemented, however, with maternal serum thus converting them to biological media. An important contribution was made by Menezo *et al.* (1984), who showed that a chemically defined medium called B3 would support the development of human preimplantation embryos without the need of a serum supplement.

It is now recognized that chemically defined media used in human IVF needs to be improved. The design of media is complicated (reviews: Biggers, 1987, 1993, 1998; Gardner, 1994; Bavister, 1995; Barnett and Bavister, 1996; Gardner and Lane, 1999; Loutradis *et al.*, 2000; Lane, 2001). The components must be selected, and their concentrations determined in order to minimize the inevitable stress cultured embryos experience in a foreign environment. Some of the problems involved in the design of chemically defined media, based partially on the extensive studies on the culture of mouse zygotes in the SOM (Simplex optimized media) family of media, are discussed in this paper.

Theoretical model of preimplantation development

Biggers (1981) proposed a theoretical model summarizing the complex network of physiological processes that are involved in the initial stages of pregnancy (**Figure 1**). These processes involve changes in the patterns of energy metabolism and transport systems of the embryo itself (*a*), chemical fluxes between the embryo and its microenvironment (*b*), and chemical fluxes between the mother and the embryonic microenvironment (*c*). It was recognized that these processes may change as the embryo passes from the ampullary region of the oviduct to the uterus. It was also recognized that flows of fluid up or down the oviduct may also influence the compositions of the local environment of the embryo (*d*). Later, on the basis of this model, Biggers (1991) asked the questions: "Can a single culture medium be developed which supports development maximally throughout the preimplantation period? Should the composition of the medium be changed to correspond with the physiological changes which occur as development proceeds?" Two general decisions have to be made in the design of a chemically defined medium: (i) the compounds to include in the medium and (ii) the concentrations of each of the selected compounds.

The Venn diagram shown in **Figure 2** identifies three classes of compounds that could be included in a chemically defined medium for the culture of preimplantation embryos. These are (i) compounds found naturally only in the oviduct/uterus, (ii) compounds that occur naturally in the body, and (iii) non-natural compounds. Although specific oviductal factors have been postulated from time to time (Kane *et al.*, 1997; Buhi *et al.*, 2000), none of these has been routinely incorporated in media for preimplantation embryo culture. A few non-natural compounds have also been included, such as EDTA (Abramczuk *et al.*, 1977), anti-oxidants (review: Johnson and Nasr-Esfahani, 1993), and PVP or PVA (review: Biggers *et al.*, 1997). As indicated in **Figure 2**, the bulk of constituents in preimplantation embryo culture media are compounds found in many parts of the body and are not those specific to the oviductal fluid.

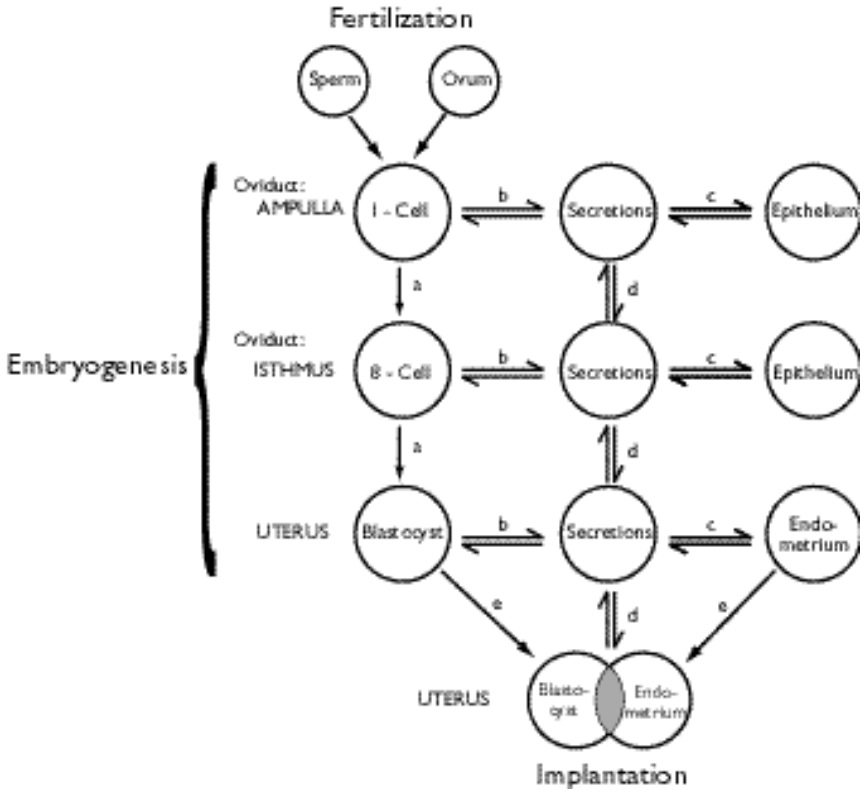

Figure 1. A theoretical model showing the physiological processes that influence the microenvironments of the preimplantation embryo. The composition of the secretions is determined by several transport mechanisms: between the embryo and the secretions in which it is bathed (*b*); between the oviduct or uterus and the bathing secretions (*c*). Further, mixing is produced by flows of secretions up and down the genital tract (*d*) (from Biggers, 1981).

In general, the media that have been used for the culture of preimplantation embryos can be arbitrarily classified into two groups, containing less than or greater than 12 components. The existence of these two groups is the result of historical quirks. Simple media, with less than 12 components, arose from the work of Whitten (1956), who showed that 8-cell mouse ova could be cultured to the blastocyst stage in Krebs–Ringer bicarbonate (Krebs and Henseleit, 1932) supplemented with glucose and bovine serum albumin. The need for complex media (>12 components) was signalled by Purshottam and Pincus (1961), who showed that rabbit zygotes would not develop in supplemented Krebs–Ringer solution but would develop in the more complex medium described by Eagle (1959). Since this early work, many simple and complex media have been described for the culture of preimplantation embryos. Some investigators use media sequentially: mouse (Lane and Gardner, 1997); human (Gardner, 1994; Gardner *et al.*, 1996); bovine (Gandhi *et al.*, 2000).

The choice of the concentrations of the compounds selected for inclusion in a chemically defined medium is not a simple issue. An intuitive approach is to use concentrations similar to those found in the genital tract. This approach was recently dubbed by Leese (1998) as the 'back to nature' principle. An alternative approach is to seek experimentally optimized concentrations (Biggers *et al.*, 1957, 1971; Lawitts and Biggers, 1991).

Back to nature

The 'back to nature' approach requires that we know the composition quantitatively of the microenvironments in which the mammalian preimplantation embryo develops. However, our knowledge about these environments, particularly the oviduct, is far from complete (review: Leese, 1987). A major experimental problem is the collection of fluid from the Fallopian tube for chemical analysis, as it produced very slowly in minute volumes. Restall (1966) listed the problems with the techniques that had been used previously. Ligation inhibits the secretion of tubal fluid by raising the intraluminal pressure; flushing of the tract precludes the determination of concentrations; the mechanical expression of fluid from dissected oviducts may contaminate the fluid with cellular components, and post mortem samples of oviductal fluid collected in abattoirs are likely to be altered by rapid ionic shifts. Accordingly, Restall adopted a cannulation technique which allowed the collection of fluid over several hours. Unfortunately this technique will obliterate any regional differences in the composition of the fluids. Only the development of microchemical analytical methods allows sampling of fluids by micropuncture and microsampling

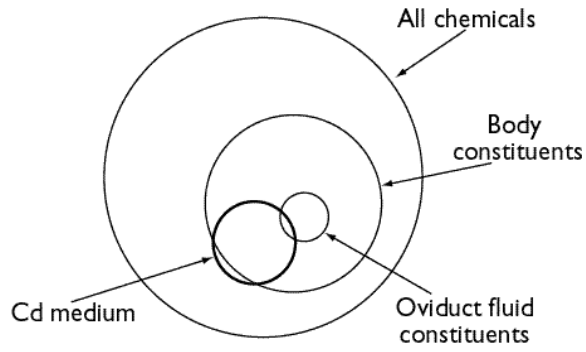

Figure 2. The classes of compounds included in media for the culture of preimplantation embryos.

techniques (mouse: Roblero *et al.*, 1976; Borland *et al.*, 1977; Gardner and Leese, 1990; human: Borland *et al.*, 1980; Gardner *et al.*, 1996). Micropuncture techniques allowed the real time sampling of the bursal, ampullary and isthmal fluids around mouse preimplantation embryos during the first 36 h post-coitum (Borland *et al.*, 1977). No significant changes in the concentrations of Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+} were observed. The real time sampling of the fluids surrounding human preimplantation embryos has limitations, since sampling from the oviduct of normal women when preimplantation embryos are present cannot be done for ethical reasons. Instead, a heuristic approach has been adopted by sampling fluids from patients undergoing normal menstrual cycles and assuming that the results obtained from individual patients at different stages of the cycle approximate the oviductal composition should embryos be present. Thus, Borland *et al.* (1980) used a microsampling technique to sample the ampullary fluid in the human oviduct from 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+} were found, relative to serum concentrations. The concentrations of Na^+ and Mg^{2+} in the oviduct fluid and serum were the same. Microsampling techniques were used by Gardner *et al.* (1996) to sample the ampullary fluid and uterine fluid from human patients undergoing laparoscopy for infertility. They found that the concentrations of pyruvate and lactate were higher in the ampullary region of the oviduct during the midcycle than in the uterus during the luteal phase. In contrast, the concentration of glucose was considerably lower in the ampullary region of the oviduct during the midcycle than in the uterus during the luteal phase.

Unfortunately, using the concentration of a substance found in the oviduct does not guarantee conditions in which preimplantation development can occur. This fact is well illustrated by the confusing results of studies on the effects of K^+ on the development of the mouse preimplantation embryo *in vitro*. The mouse oviductal fluid, collected by micropuncture, has a very high concentration of K^+, ~25 mmol/l (Roblero *et al.*, 1976; Borland *et al.*, 1977). Earlier, Wales (1970) had reported that 2-cell mouse embryos would develop into blastocysts in potassium concentrations in the range 1–48 mmol/l. Subsequently, Roblero and Riffo (1986) reported that 25 mmol/l potassium supported the preimplantation development of the mouse. In contrast, Whittingham (1975) found that mouse zygotes did not develop in concentrations of potassium in the range 12–48 mmol/l. Further, Wiley *et al.* (1986) observed that mouse embryos developed best in a medium containing <6 mmol/l potassium.

Three media for the culture of preimplantation embryos have been claimed to mimic the concentrations of the components found in oviductal fluid. These media are medium sheep oviduct fluid (SOF) for the sheep (Tervit *et al.*, 1972), medium human tubal fluid (HTF) for the human (Quinn *et al.* 1985), and medium mouse tubal fluid (MTF) for the mouse (Gardner and Leese, 1990). Unfortunately, the names given to three of these media, synthetic oviduct fluid, human tubal fluid and mouse tubal fluid, are potentially misleading, since the media only marginally mimic the natural oviduct environments. Quinn *et al.* based their medium on analyses of several components of human oviduct fluid made by Lippes *et al.* (1972) and Lopata *et al.* (1976). These analyses are compared with the composition of HTF in **Table 1**. Only in three out of eight of the components is there a close correspondence between their concentrations in the oviduct fluid and HTF. Tervit *et al.* (1972) based their medium on the analyses of sheep oviduct fluid reported by Restall and Wales (1966). Again there is only a rough correspondence between the figures published by Restall and Wales and the concentrations of the compounds used in SOF. Medium MTF was produced by Gardner and Leese by replacing the concentrations of pyruvate, glucose and lactate in medium M16 (Whittingham, 1971) by the concentrations of these compounds measured on microsamples of mouse oviduct fluid. The concentrations of K^+ and Cl^- in M16 do not closely correspond with the concentrations observed in mouse ampullary fluid obtained by micropuncture (Borland *et al.*, 1977).

The differences in the concentrations of pyruvate, lactate and glucose found in the oviduct and uterus of infertile patients with normal menstrual cycles led Gardner *et al.* (1996) to conclude that as the human preimplantation embryo passes from the ampullary region of the oviduct to the uterus, it encounters decreases in the concentrations of pyruvate and

Table 1. Comparison of the composition of medium HTF with published analyses of the composition of human oviductal fluid

Compound (mmol/l)	Lippes *et al.* (1972)[a]	Lopata *et al.* (1976)[b]	Medium HTF (Quinn *et al.*, 1985)	David *et al.* (1973)[c]	Borland *et al.* (1980)[d]
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+}	3.80–4.80	1.38	2.04	–	1.13
Mg^{2+}	–	0.19	0.20	–	1.42
Glucose	2.39–3.04	–	2.78	–	–
Pyruvate	–	0.18	0.33	–	–
Lactate	–	2.52	21.40	–	–

[a]Collected over 24 h by cannulation (16 specimens).
[b]Collected at laparoscopy (two specimens).
[c]Collected at laparotomy (33 specimens).
[d]Collected by microsampling (seven specimens).

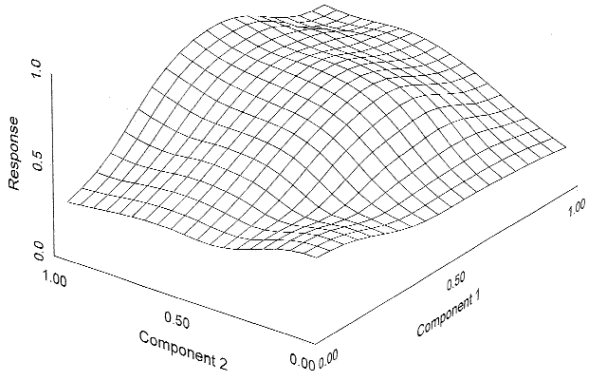

Figure 3. Two-dimensional concentration response surface.

lactate and an increase in the concentration of glucose. This can only be a tentative conclusion, since the patients were not pregnant. Nevertheless, these observations provided the basis for the introduction of two media (G1 and G2) which were used sequentially for the culture of human preimplantation embryos from the zygote to the blastocyst stage. Later, in the justification of the sequential use of two culture media, Gardner (1998) stressed that "in order to support development of a competent zygote to the viable 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". Many of these changes are well documented in the mammalian preimplantation embryo, such as the increase and decrease in the utilization of pyruvate and glucose, respectively, at about the morula stage (review: Leese, 1995). Such physiological changes, however, provide no rigorous proof that the microenvironments of the developing preimplantation embryo alters even if the changes in the embryo correlate in time with possible changes in the environments in the female genital tract.

Empirical optimization

An alternative approach to media design is to optimize the concentrations of the components by bioassay (Biggers *et al.*, 1957). The problem of optimizing a mixture, however, is not simple, and cannot be done by using the intuitive approach of varying the composition of each component one at a time, keeping the concentrations of the other components constant. An understanding of the problems involved needs consideration of a concentration response surface, a mathematical model introduced by Box (1957) in the field of chemical engineering, to describe the processes involved in the large scale manufacture of chemical products. For simplicity, consider the case of two components in which the concentrations of each are simultaneously varied. The responses to the different concentrations can be represented in a three-dimensional graph by a concentration response surface (**Figure 3**). It is clear that the properties of the concentration response line for compound A is dependent on the concentration of compound B. Thus the common practice of varying the concentration of a compound while holding constant the concentration of the other compound gives only partial knowledge of their joint actions. This partial knowledge can be misleading if there are interactions between the effects of the different components. These interactions can only be detected when the concentrations of both components are varied in combination to explore the concentration response surface. These surfaces can be explored using a variety of experimental designs, such as factorial, fractional factorial, rotatable and shell designs, provided the number of levels of each component is >2.

When more than two components are involved, the regression model is a surface in $(n + 1)$ – dimensional space where n is the number of components. An example is a 3^3 factorial design used by Lawitts and Biggers (1992) to study the joint effects of glucose, glutamine and NaCl in medium SOM, which can be represented in four-dimensional space. The exploration of such a surface by the designs listed above rapidly becomes impossible as n increases. Thus a factorial experiment involving 12 components each at three concentrations would require the comparison of $3^{12} = 531{,}441$ media! An alternative to exploring the surface in detail is to seek the coordinates of a useful point on the surface. An obvious choice is the maximum response, which is commonly identified as the optimal response. It must be emphasized, however, that the maximum response is not necessarily the natural response.

There are several procedures for finding the maximum of a regression surface (Everitt, 1987). One is sequential simplex optimization (Spendley *et al.*, 1962; Walters *et al.*, 1991). This experimental strategy was used by Lawitts and Biggers (1991) to produce medium SOM for the culture of mouse zygotes through the so-called 'two-cell block', and by Karlsson *et al.* (1996) to optimize procedures for the cryopreservation of mouse oocytes. A bonus from this work was that SOM also favoured the subsequent development of the embryos into blastocysts, although the response used to optimize the medium was passage through the two-cell block.

The Simplex optimized family of media

Medium SOM has been modified in several ways so that there is now a family of these media (**Figure 4**). The first modification arose from studies on the intracellular concentrations of Na^+ and K^+ in 2-cell mouse embryos exposed SOM, which showed a very low K^+/Na^+ ratio (Biggers *et al.*, 1993). This led to the formulation of KSOM (Lawitts and Biggers, 1993) (**Table 2**). Subsequently, KSOM was modified in two ways. Ho *et al.* (1995) showed that the addition of amino acids (AA) improved the development of mouse zygotes to the blastocyst stage. Further properties of this medium, called $KSOM^{AA}$, have been recently described by Biggers *et al.* (2000). Summers *et al.* (1995) showed that KSOM would support IVF in the mouse provided the concentration of glucose was raised to the normal concentration found in blood. This medium was denoted mKSOM. Very recently, it has been shown that the results of IVF could be improved by adding AA to mKSOM (denoted $mKSOM^{AA}$) to the fertilization medium (Summers *et al.*, 2000). The concentrations of AA used in our current formulation is half strength Eagle's amino acids (Biggers *et al.*, 2000) (**Table 3**).

The most spectacular effect of supplementing KSOM with AA is the improved development of the inner cell mass in the

blastocyst (**Figure 5**). Biggers *et al.* (2000) found that the addition of AA significantly increased the number of cells that develop in the inner cell mass of embryos cultured from the zygote stage for 144 h post-human chorionic gonadotrophin (HCG) from a median of 11 to a median of 20. There was also an increase in the number of cells in the trophectoderm, although less spectacular, from a median of 63 to a median of 85. Further the extracellular matrix that separates the primitive endoderm and primitive ectoderm, as revealed by staining for collagen IV, was better organized (**Figure 5**).

KSOM will not support the fertilization of mouse ova *in vitro*. Fertilization does occur in KSOM, however, if the glucose concentration is raised from 0.2 mmol/l to 5.56 mmol/l (Summers *et al.*, 1995, 2000). Mouse zygotes produced by IVF in KSOM also develop into blastocysts in the presence of 5.56 mmol/l glucose. The result was surprising, since there was considerable previous evidence that glucose inhibited the early development of mammalian zygotes *in vitro* (review: Biggers and McGinnis, 2000). Glucose can, however, inhibit the development of mouse zygotes in other culture media for reasons that are still not clear.

The work on the effects of supplementing KSOM independently with AA and glucose for different purposes has now converged (**Figure 4**). Summers *et al.* (2000) have shown that the addition of AA to mKSOM (mKSOM^{AA}), to provide a fertilization medium, increases the percentage of blastocysts that hatch, increases the number of cells in the blastocysts particularly in the inner cell mass and supports in the blastocysts a more organized extracellular matrix.

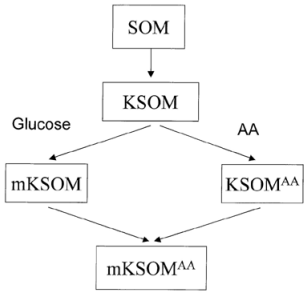

Figure 4. The SOM family of media.

The concentrations of the AA added to KSOM are half of those used by Eagle (1959). These concentrations give almost the same responses as the full strength concentrations. These concentrations of AAs have not been optimized. To do so would involve a prodigious amount of testing which is logistically unattainable at the present time.

Pathophysiology of preimplantation development *in vitro*

Placing the embryos in a chemically defined medium exposes them to stress, as it is inevitable that a chemically defined medium provides only a partial representation of the natural environment the preimplantation embryo requires to develop. In order for the embryo to survive and develop under such conditions it must fulfil two functions: contain or be able to synthesize essential compounds not provided by the medium, and adapt to the abnormal conditions which are imposed by the artificial environment (Biggers, 1993, 1998; Leese, 1995; Lane, 2001).

Abnormal gene expression in preimplantation mouse embryos *in vitro*

It has now been established that the expression of some genes in preimplantation mouse embryos are affected by the medium used to support preimplantation development *in vitro*. Ho *et al.* (1995) examined the expression of nine genes in blastocysts cultured from the 2-cell stage in a medium described by Whitten (1971) and KSOM^{AA}. The abundance of mRNA from five of these genes (actin, G3PDH, Na^+/K^+-ATPase, Sp1, TATA box binding protein) were the same in blastocysts that developed in both media. In contrast, the mRNA from the other four genes (IGF-I, IGF-IR, IGF-II, IGF-IIR) was more abundant in blastocysts produced using the AA supplemented

Table 2. Composition of KSOM

Component	*Concentration (mmol/l)*
NaCl	95.0
KCl	2.5
KH_2PO_4	0.35
$MgSO_4.7H_2O$	0.2
DL-Lactate (Na salt)	10.0
Pyruvate (Na salt)	0.2
DL(+)-Glucose	0.2
$NaHCO_3$	25.0
$CaCl_2.2H_2O$	1.7
L-Glutamine	1.0
EDTA (Na salt)	0.01
Bovine serum albumin	1 mg/ml
Penicillin G	100 IU/ml
Streptomycin sulphate	5 µg/ml

Table 3. Concentrations of amino acids added to KSOM to give KSOM^{AA} (half strength AA)

Amino acid	*Concentration (mmol/l)*
L-Alanine-HCl	0.05
L-Arginine-HCl	0.30
L-Asparagine-H_2O	0.05
L-Aspartic acid	0.05
L-Cystine	0.05
L-Glutamic acid	0.05
Glycine	0.05
L-Histidine-HCl-H_2O	0.10
L-Isoleucine	0.20
L-Leucine	0.20
L-Lysine-HCl	0.20
L-Methionine	0.05
L-Phenylalanine	0.10
L-Proline	0.05
L-Serine	0.05
L-Threonine	0.20
L-Tryptophan	0.025
L-Tyrosine	0.10
L-Valine	0.20

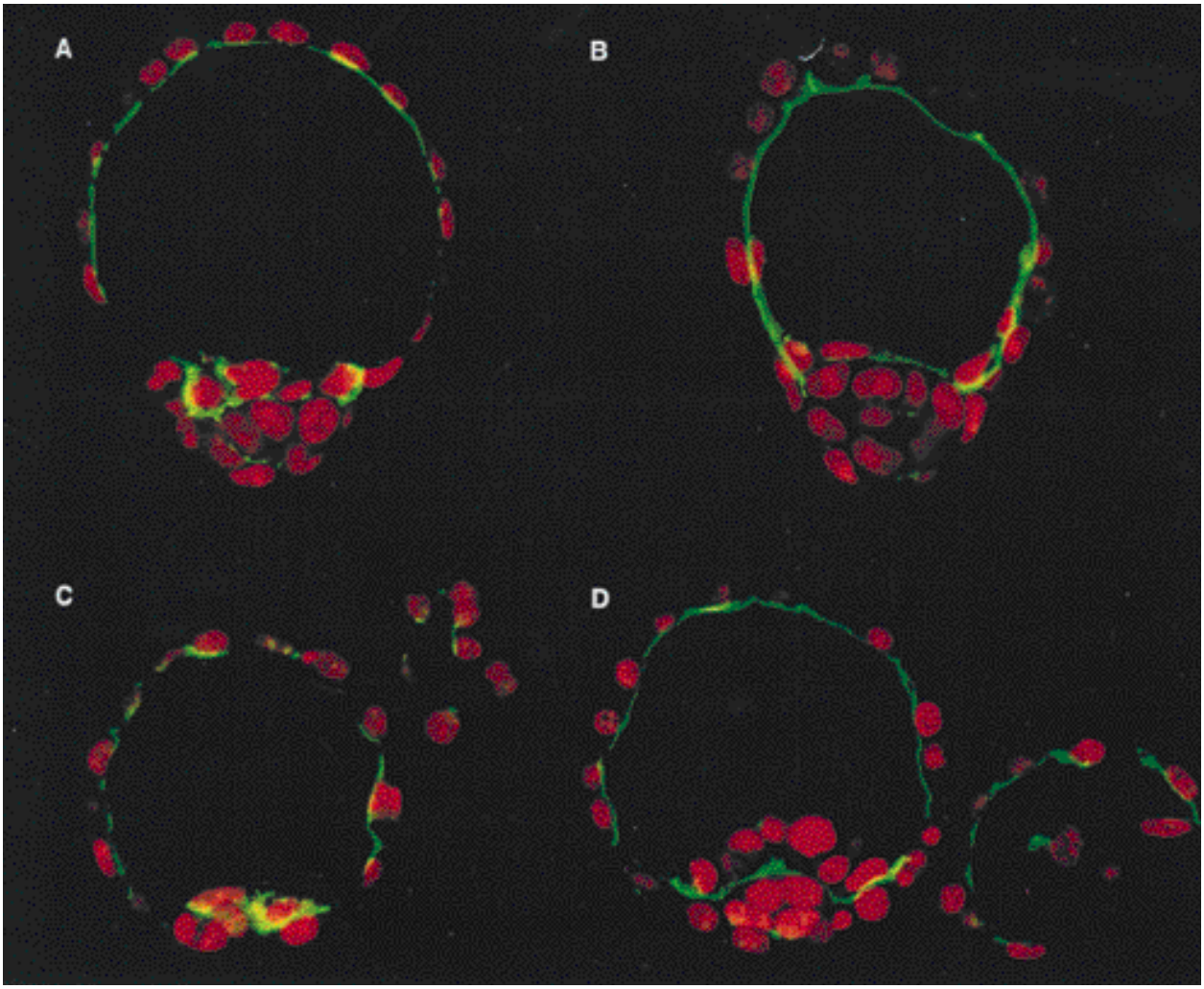

Figure 5. Optical sections obtained by confocal microscopy of mouse blastocysts stained for collagen IV blastocysts, flushed from the uterus (A, B), and cultured in either KSOM (C) or KSOM^{AA} (D). Original magnification ×500 (from Summers *et al.*, 2000).

KSOM. In a further study, Doherty *et al.* (2000) have compared the expression of the H19 gene, which is preferentially expressed in the maternal allele, in blastocysts produced from the 2-cell stage in Whitten's medium and KSOM^{AA}. The gene was abnormally expressed in both the maternal and paternal alleles in embryos that developed in Whitten's medium, and was normally expressed in the maternal allele only in blastocysts produced in KSOM^{AA}. It is possible that the expression of other genes may be affected by the media used for in-vitro studies. Sensitive molecular biological methods have now established the occurrence of gene expression at several stages between the zygote and blastocyst of the mouse preimplantation development. (Ko *et al.*, 2000; Latham *et al.*, 2000). Ko *et al.* mapped 798 new genes on the mouse genome and reached the following conclusions: "(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...". Thus the long held view that early preimplantation development merely involved a sequence of cell divisions is clearly not the case. The potential seriousness of genetic effects produced by culture conditions on later development will depend on the redundancies in the genetic programme, a topic on which we are largely ignorant.

Adaptation to osmotic stress

To adapt, an embryo must make use of innate physiological mechanisms. As an example we consider the response of preimplantation embryos to changes in tonicity. The normal osmolality found in the mouse oviduct falls in the range of 290–300 mOs/kg (Collins and Baltz, 1999). Traditionally, the effects of changing the tonicity of the environment of cells has been explained solely in terms of the properties of semi-permeable membranes. It is now known that all cells have more complex, delicately balanced mechanisms which continually function to maintain the normal cell volume (reviews: Lang *et al.*, 1998; Lange, 2000). These mechanisms provide a buffer against the fluctuations in cell volume due to the dynamic state of the cell cytoplasm in which the production and loss of small molecules continually occurs. Three responses may occur in sequence when a cell is placed in a hypertonic solution depending on the duration of the osmotic stress (**Figure 6**). The first response is a rapid

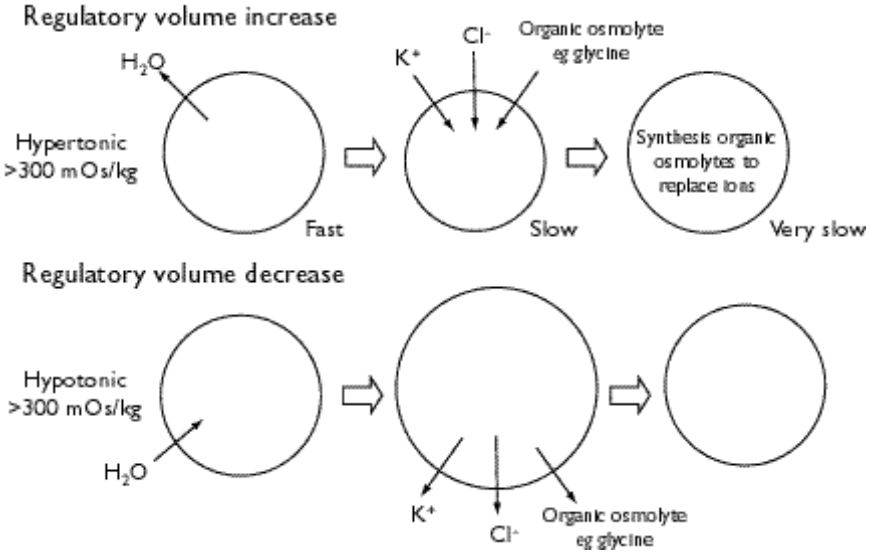

Figure 6. The control of regulatory volume increase and regulatory volume decrease in response to hypertonicity and hypotonicity.

movement of water out of the cell causing it to shrink. This shrinkage is followed by the uptake of ions and organic osmolytes from the environment, which if sufficient will restore the cell volume. If the volume is not restored, a slow response occurs in which genes are activated in the cell leading to the synthesis of organic osmolytes. If a cell is placed in a hypotonic solution the reverse of these processes occur. This new understanding of the importance of maintaining normal cell volume was triggered by the discovery of organic osmolytes (Yancey *et al.*, 1982).

The results summarized in **Figure 7** show that the development of preimplantation mouse embryos is particularly sensitive to hyperosmolality (Dawson and Baltz, 1997).

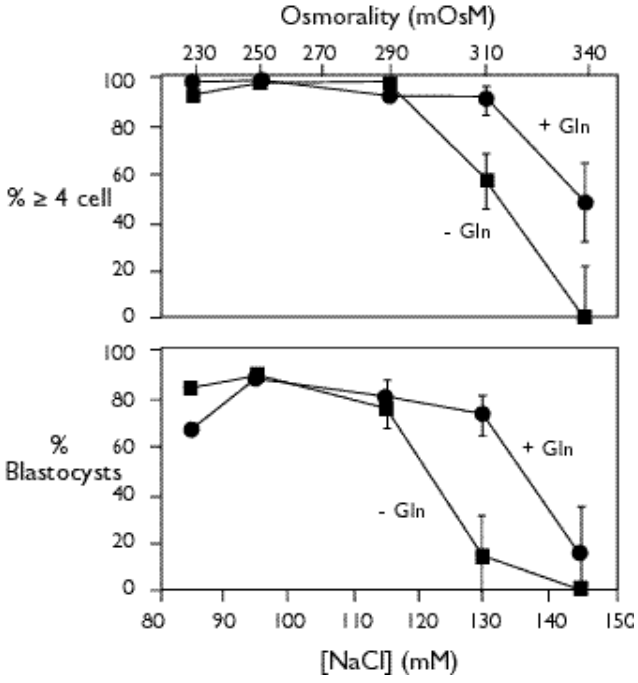

Figure 7. The development of mouse zygotes in KSOM in media of different osmolality produced by varying the concentration of NaCl (from Dawson and Baltz, 1997).

Further, glutamine, a weak organic osmolyte, protects the preimplantation embryo developing *in vitro* in KSOM against the deleterious effects this hypertonicity. Several other organic osmolytes are now known to protect mouse preimplantation embryos against hypertonic solutions: glycine, betaine, proline, alanine, and hypotaurine (Van Winkle *et al.*, 1990; Biggers *et al.*, 1993; Dawson and Baltz, 1997). Thus, the beneficial effects of adding AA to culture media may be due in part to the fact that some are organic osmolytes. At present it is not known whether the prolonged exposure of preimplanation embryos to hypertonic conditions in the absence of external organic osmolytes activates the expression of genes to stimulate the production of endogeneous organic osmolytes.

Hsp70 and preimplantation development

The response of preimplantation embryos to stress has received little study, with the exception of hsp70, one of the heat shock proteins (reviews: Luft and Dix, 1999). The protein is produced constitutively in the mouse from the zygote to the 8-cell stage, and can be induced in response to stress, such as heat or arsenite, only after the 4-cell stage is reached. Dix *et al.* (1998) showed that the transfection of antisense oligonucleotides to hsp70 into 4-cell mouse embryos inhibited development into blastocysts cultured in KSOM, establishing that hsp70 subserves a normal function for part of preimplantation in the mouse. Hsp70 acts as a molecular chaperone ensuring the normal tertiary structure of new proteins as they are synthesized. It seems likely that hsp70 would be particularly important at the time of the zygotic transition when the genes contributed by the fertilizing sperm begin to be translated. Dix *et al.* (1998) have also shown that inhibition of hsp70 by its complimentary antisense oligonucleotide sensitizes the 4-cell embryo developing *in vitro* in KSOM to the toxicity of arsenite. It was not demonstrated whether this effect is due to the inhibition of the hsp70 produced constitutively or inhibition of inducible hsp70.

Other aspects of stress

The study of the molecular mechanisms involved in the effects of stress on cells is a particularly active field at the present

time (reviews: Mathias *et al.*, 1998; Ronai, 1999; Hannun and Luberto, 2000). For example, several types of stress can cause the accumulation of ceramide, a compound involved in sphingolipid metabolism. Ceramide has been shown to be involved in cell-cycle arrest and apoptosis. Could this type of mechanism, still not studied in preimplantation embryos, be induced by adverse culture conditions? If so, could cells be differentially eliminated, say in the blastocysts, with resultant effects manifested later after transfer for development in surrogate mothers?

Conclusion

Two approaches have been used to determine the composition of chemically defined media for the culture of preimplantation embryos: the 'back to nature' approach and empirical optimization. Both methods provide only a subset of the compounds found in the natural environments in which the embryo develop. As a result cultured embryos are subjected to continual stresses. They will only be able to develop if they can adapt to these stresses. Our knowledge of the responses of preimplantation embryos to stress is still very limited. It is suggested that the empirical optimization approach to media design will compensate for these putative stresses, thereby providing a favourable environment in which embryos can adapt and develop.

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