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Reflections on the culture of the preimplantation embryo

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ABSTRACT There has been a considerable improvement in the media available for the culture of preimplantation mouse embryos during the 40 years since mouse embryos were first cultured and successfully transferred to uterine foster mothers. Two new media, KSOM and mMTF, are becoming more commonly used. The history of the development of these media, including recent work on KSOM and mMTF, is reviewed. A major artefact in the earlier work was the two-cell block. The causes of the two-cell block and the methods by which it has been overcome are reviewed. It is concluded that even the best available media inevitably cause imbalances in the environment in which the embryos are forced to develop, because they consist of only a small subset of the compounds present in the natural environments. As a result, the embryos must adapt to these abnormal conditions if they are to survive. The implications of these conclusions on the choice of media for specific purposes are discussed.

KEY WORDS: *media, KSOM, embryo culture, blastocyst, concentration-response surfaces*

The possibility of experimentally studying the mammalian preimplantation embryo *in vitro* became a reality between 1956 and 1959 with the publication of three papers. Whitten (1956) reported that eight-cell mouse embryos would develop into blastocysts when cultured in a simple chemically defined medium containing only nine components, including water. McLaren and Biggers (1958) then showed that blastocysts produced in this way could develop into outwardly normal young after being transferred into the uterus of surrogate mothers. Finally, Chang (1959) successfully fertilized rabbit ova *in vitro* and produced apparently normal young. By the use of genetic markers, both McLaren and Biggers, and Chang unequivocally ruled out the possibility that the surrogate mothers produced young from their own oocytes.

This pioneer work was done at a time when chemically defined media were finally being perfected to replace biological media for the culture of cell lines. Biological media contain biological fluids of unknown chemical composition, such as serum, while chemically-defined media are solutions in water of a set of compounds of known composition. The idea of using chemically defined media for the culture of cells and tissues was not new. Only four years elapsed between the first tissue culture experiments by Ross Harrison (1907), who used a biological medium, and the recognition of the potential usefulness of chemically defined media. Two papers were published in 1911 by Warren and Margaret Lewis entitled:

(1) The growth of embryonic chick tissues in artificial media, agar and bouillon.

(2) The cultivation of tissues from chick embryos in solutions of NaCl, $CaCl_2$, KCl and $NaHCO_3$.

They extended the earlier work begun by Ringer (1883) on the study of tissues removed from the body that resulted to the design of physiological salines for the study of the beating heart. Lewis and Lewis 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 reasons for utilizing chemically defined media instead of biological 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 enzyme, hormonal and other activities which may interfere with the responses being studied. Thus, the reasons are not dependent on any fundamental physiological principles.

Generally, a chemically defined medium is an artificial mixture of known chemical components that can substitute for the natural microenvironment of cells after they, or aggregates of them, are explanted. A conditional definition of a chemically defined medium, which applies specifically to the culture of preimplantation embryos, is an artificial mixture of known chemical components that can substitute for the natural microenvironments encountered by an embryo as it develops from the one cell zygote to the blastocyst,

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while traveling from the ampullary region of the oviduct to the uterus. There are two steps in the design of a chemically defined medium, the choice of compounds to include, and the concentrations of selected compounds.

A surprise from Whitten's work was that the eight-cell mouse embryo would develop into a blastocyst in a very simple solution based on a physiological saline, described earlier by Krebs and Henseleit (1932), supplemented only with a carbon source (glucose) and bovine plasma albumin (BSA) (Table 1). The first three

TABLE 1

A COMPARISON OF THE CHEMICAL COMPOSITIONS OF WHITTEN'S ORIGINAL MEDIUM FOR THE CULTURE OF 8-CELL MOUSE EMBRYOS AND KREB'S RINGER BICARBONATE

Component	Whitten's original medium	Kreb's Ringer Bicarbonate
NaCl	118.50	118.50
KCl	4.74	4.74
KH2PO3	1.18	1.18
CaCl2	2.54	2.54
MgSO4	1.18	1.18
NaHCO3	24.90	24.90
Glucose	5.56	-
BSA (mg ml-1)	1,00	-

cleavage divisions would not develop in this medium. It was even more surprising when Whitten (1957) reported that the two-cell stage mouse embryo, but not the zygote, would develop into a blastocyst if lactate was added to the medium. These observations raised the possibility that the oviduct provides a unique microenvironment for the preimplantation embryo. This idea was supported in part when Biggers, Gwatkin and Brinster (1962) showed that the preimplantation mouse embryo would develop from the zygote to the blastocyst in explants of the fallopian tube. The possibility that the oviduct might provide different environments which are species dependent was also suggested by the work of Purshottam and Pincus (1961), who found that a medium similar to Whitten's did not support the development of rabbit preimplantation embryos. Instead, they found that the much more complex Eagle's medium, developed earlier for the culture of human cell lines, supported cleavage to the morula stage (Eagle, 1959). Thus, in the early 1960s, it appeared that some species of preimplantation embryos could develop in very simple chemically defined media, while others could not. Nevertheless the notion that the oviduct provided a unique microenvironment for the preimplantation embryo was not generally accepted, particularly by clinicians treating human infertility due to occluded oviducts. At this time these patients were treated by using the Estes' operation in which the ovary, with its pedicle intact, was inserted into the uterine cavity where it was hoped that ovulation would occur. Although the results were equivocal, the oviduct was believed by some investigators to be dispensable (reviews: Adams, 1979; Biggers, 1984). It was from this background that Ralph 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) . A singular result from this work was that only four members of the glycolytic pathway and the citric acid cycle would support the development of the two-cell mouse embryo to the eight-cell stage (pyruvate, lactate, oxaloacetate, phosphoenolphosphate), while many additional compounds, including glucose, would support the development of the eight-cell stage to the blastocyst. This work led to the formulation of a well-known medium called BMOC2 (Table 2) (Brinster, 1972). When parallel studies showed that only two compounds (pyruvate and oxaloacetate) would support the final maturation of the oocyte and the first cleavage division (Biggers *et al.*, 1967), a working hypothesis was developed that the energy pathways became restricted during oogenesis and were restored during cleavage after fertilization (review: Biggers, 1971). It is now generally accepted that pyruvate is an essential component of media to support preimplantation development in all species.

Several other chemically defined media were proposed for the culture of preimplantation mouse embryos in the ensuing 20 years after Brinster's work. The most widely used medium was M16 (Table 2), designed by Whittingham (1971), and a variant of it, M2 (Quinn *et al.*, 1982) in which part of the bicarbonate buffer in M1 was replaced by the Hepes buffer. These are the media recommended in the most widely used manual on techniques for the experimental study of the development of mouse embryos (Hogan *et al.*, 1994). Other media were produced in an effort to overcome the phenomenon known as the two-cell block (review: Biggers, 1987).

The two cell block was recognized as the result of four observations. Whitten (1957) had shown that two-cell mouse embryos would develop into blastocysts *in vitro* but not zygotes, and Biggers *et al.* (1962) had shown that zygotes would develop in organ cultures of the oviduct. When Cole and Paul (1965) reported that mouse zygotes would cleavage to the two-cell stage *in vitro* and then degenerate, it was suggested that the oviduct protects the embryo over this apparently vulnerable stage. This hypothesis was confirmed when Whittingham and Biggers (1967) and Whittingham (1968) demonstrated that two-cell stage embryos that had arrested *in vitro* could be rescued by transferring them into the lumen of organ cultures of the oviduct, specifically the ampullary region. A simple explanation for these results was that the oviduct secretes some substance that is necessary for the second cleavage division to occur. Subsequent work by several investigators has now

TABLE 2

THE COMPOSITIONS OF SEVERAL MEDIA THAT HAVE BEEN USED TO CULTURE MOUSE ZYGOTES SENSITIVE TO THE TWO-CELL BLOCK

Compound	BMOC2*	M16*	CZB	START*	SOM	KSOM	MTF
NaCl	88.98	94.7	81.62	80	85	95	94.7
KCl	4.83	4.78	4.8	5	0.25	2.5	4.78
KH2PO4	1.18	1.19	1.18	1	0.35	0.35	1.19
MgSO4	1.18	1.19	1.18	1	0.2	0.2	1.19
CaCl2	1.7	1.71	1.7	1.71	1.71	1.71	1.71
NaH2CO3	25.12	25	25.12	25	25	25	25
Lactate	29.9	23.3	31.3	1.5	10	10	4.79
Pyruvate	0.27	0.33	0.27	0.3	0.2	0.2	0.37
Glucose	5.55	5.56	0	0.1	0.2	0.2	3.4
Glutamine	0	0	1	1	1	1	0
EDTA	0	0	0.11	0.01	0.01	0.01	0
BSA (mg ml-1)	5	4	5	2.5	1	1	4

* Two-cell block occurs.

resulted in media that has overcome the two-cell block, but not through the finding of a specific oviductal factor.

The notion that the two-cell block was caused by the absence of a specific oviductal factor came into question when Whitten and Biggers (1968) showed that it was strain dependent. They demonstrated that, while the block occurred in inbred strains, it did not occur in F_1 hybrids between inbred strains, suggesting that the block was caused by some defect within the ovum itself. Further doubt of the hypothesis that the two-cell block was caused by the absence of an oviductal factor was later provided by the observation that the block could be overcome by adding EDTA to the medium (Abramczuk *et al.*, 1977). This finding suggested that a chelating agent was needed to remove any deleterious trace elements from the medium used for cultivation. Thus proximate causes, arising within the embryo, or ultimate causes, present in the environment provided by the medium, could cause the two-cell block (Biggers, 1993).

Proximate causes of the two-cell block may be regarded as a deficiency in some essential molecule(s) produced intrinsically by the embryo that is necessary for development to proceed. Indirect evidence for this suggestion was provided by Muggleton-Harris *et al.* (1982), who showed that the two-cell block in a blocking strain could be overcome by the injection of cytoplasm from a zygote of a non-blocking strain. Later it was shown that the production of the putative cytoplasmic factor reaches a maximum between the G_2 and M phases of the second cleavage division (Pratt and Muggleton-Harris, 1988). The factor(s) could be small regulatory molecules or transcription factors since protein synthesis is retarded in culture conditions that cause the two-cell block (Poueymirou *et al.*, 1989).

Ultimate causes of the two-cell block arise in the microenvironment provided by the culture conditions. They presumably act by causing a proximal defect. Known ultimate causes can be classified into three types: simple toxicity, simple deficiency and environmental imbalance (Biggers, 1993). Simple toxicity may arise from too high concentrations of trace metals, as suggested by the effectiveness of adding EDTA. Another example is the two-cell block produced by oxygen-free radicals, which can be overcome by the addition of apoferritin, transferrin and diethylenetriaminepentaacetic acid (Nasr-Esfahani *et al.*, 1990; Legge and Sellens, 1991; Noda *et al.*, 1991). An example of simple deficiency as a cause of the two-cell block was provided by Chatot *et al.* (1989) who showed that the replacement of glucose with glutamine in medium BMOC-2 would allow zygotes of a blocking strain to develop into blastocysts. In these experiments the absence of glutamine is a simple deficiency.

A modification of BMOC-2, called CZB, was proposed by Chatot *et al.* (1989), which contained EDTA, an increased lactate/pyruvate ratio, and glutamine as a replacement for glucose (Table 2). This modification was introduced partly because Schini and Bavister (1988) had reported that glucose and phosphate caused a two-cell block in the hamster, and partly because Carney and Bavister (1987) had demonstrated the beneficial effect of glutamine, also in the hamster. CZB allowed the development of the zygote to the blastocyst of several strains of mice that normally blocked at the two-cell stage (Chatot *et al.*, 1989,1990). Only when the embryos had developed to the morula stage was it necessary to add glucose to ensure a high yield of blastocysts. Although the medium contained EDTA and glutamine, both of which had been shown to independently overcome the two-cell block using other media, the two-cell block occurred using CZB if the glucose was raised to normal blood concentration.

To understand the third ultimate cause of the two-cell block, imbalances in the environment, we need to discuss the relationship between the responses of the embryos in culture to different concentrations of the substances that make up the medium. In designing a medium for any type of culture, microbial, cell, organ or embryo culture, it is a common practice to examine the effect of a series of concentrations of a compound on a response of interest to determine a concentration-response line. The concentration selected to include in the medium is usually near the maximum of this line. However, this approach, which examines the effects of one compound at a time, is limited since it will not detect any interactions with the effects of other compounds in the medium. The subject was treated theoretically by Biggers *et al.* (1957) who proposed that the totality of responses to the mixture of compounds in a medium be represented by a concentration-response surface. This model was again suggested for the development of media for the culture of preimplantation embryos by Biggers *et al.* (1971). If there are n compounds in the medium, then the concentration-response surface can be modeled in $(n+1)$-dimensional space. Thus the problem of determining the concentrations of the components of a medium involves finding the co-ordinates of an acceptable point on the concentration-response surface. Such concentration-response surfaces may be complex having both a global maximum and other local maxima. These surfaces were first proposed in a technique for increasing industrial productivity, called evolutionary operation (Box, 1957). They have now found application in many areas, including the optimization of microbial processes (Bull *et al.*, 1990). A recent monograph on their exploration has been written by Drain (1997).

The first study of the joint action of two compounds in a medium for the culture of the preimplantation embryo was done by Brinster (1965d), who studied the effect of simultaneously varying the concentrations of lactate and pyruvate on the development of the two-cell mouse embryo. A 2x2 factorial design was used. Unfortunately 2^n factorial experiments cannot detect curvatures in a concentration-response surface and therefore they cannot detect global and local maxima in a concentration-response surface. At least a 3^n factorial design is required. These designs are logistically impossible to execute because of the large number of media that need to be prepared and their effects compared. Thus a medium containing 10 compounds would require the preparation of 59,049 media. Other experimental strategies are required, such as the method of steepest ascent or sequential simplex optimization (Everitt, 1987; Walters *et al.*, 1991; Drain, 1997). Lawitts and Biggers (1991,1992) found that sequential simplex optimization was a logistically practical strategy to optimize a medium for the culture of the mouse zygote which overcame the two-cell block. The composition of the initial medium (START) is shown in Table 2. Although START contained glucose, glutamine and EDTA it nevertheless caused a two-cell block in zygotes from the outbred CF-1 strain of mouse which is notorious for its susceptibility to the block. After 20 cycles of optimization a medium called simplex optimized medium (SOM) was produced whose composition is shown in Table 2. The concentrations of NaCl and KCl were later increased because analyzes of the intracellular K^+/Na^+ ratio in the two-cell stages that developed from zygotes in SOM were only about 3 instead of the normal value of about 10 (Biggers *et al.*, 1993). The new medium was called KSOM (Table 2) (Lawitts and Biggers, 1993; Erbach *et al.*, 1995).

The effects of simultaneously varying the concentrations of NaCl, glucose and glutamine in SOM, using a 3^3 factorial experi-

ment, yielded some surprising results (Lawitts and Biggers, 1992). Raising glucose to 5 mM (~blood concentration) was not inhibitory, contrary to the earlier work of Chatot *et al.* (1989). Increasing the concentration of NaCl to 125 mM inhibited development when glutamine was omitted, whereas the presence of glutamine protected the embryos from the damaging effects of high concentrations of NaCl. Possibly the glutamine exerts its protective action by acting as an organic osmolyte, as does glycine (Van Winkle *et al.*, 1990) and betaine (Biggers *et al.*, 1993). The non-inhibitory effect of glucose on the development of CF-1 zygotes to blastocysts has been repeatedly shown using KSOM (Summers *et al.*, 1995; Biggers *et al.*, 1997). Furthermore, phosphate is only marginally inhibitory to these zygotes (Biggers and McGinnis, unpublished), contrary to other observations on the hamster (Schini and Bavister, 1988) and mouse (Brown and Whittingham, 1992; Quinn, 1995). These examples stress the importance of environmental imbalances caused by interactions between the effects of the different components of a medium.

Brinster (1965c) reported that the development of the outbred two-cell mouse embryo into a blastocyst has no absolute requirement for exogenous amino acids. This observation was later confirmed by Cholewa and Whitten (1970). Other studies of explanted mouse blastocysts suggested that the need for amino acids develops around the time of implantation (Gwatkin, 1966; Spindle and Pedersen, 1973). Recently there has been a resurgence of interest in the need for amino acids in media for the culture of preimplantation embryos (Chatot *et al.*, 1989; Mehta and Kiessling, 1990). Gardner and Lane (1993) cultured F_1 hybrid zygotes in a modified tubal fluid medium (mMTF) supplemented with amino acids. The percentage of embryos that developed at least to the blastocyst stage were the same as obtained in the control unsupplemented medium. However, the embryos that developed in the amino acid-supplemented medium developed at a faster rate. Gardner and Lane (1993) also found that the addition of amino acids to mMTF increased the percentage of blastocysts that partially hatched, and significantly increased, albeit small, the total number of cells in the blastocyst. Ho *et al.* (1995) cultured C57BL/6J zygotes in KSOM supplemented with amino acids. The percentage zygotes that developed were the same in supplemented and unsupplemented media. Like Gardner and Lane (1993) they also found that the rate of development of the blastocysts was accelerated in the supplemented KSOM. Ho *et al.* (1995) also observed that added amino acids also caused a significant increase in partial hatching and in total cell count of the blastocysts. Similar observations have been reported on the effect of adding amino acids to KSOM for the culture of mouse zygotes by Biggers *et al.* (1997). Amino acid-supplemented KSOM has now been shown to support the development of cow and rabbit zygotes to blastocysts (Liu and Foote, 1995; Liu *et al.*, 1996). The role of amino acids in culture media is now under intense study, and the results obtained are likely to require an update of the composition of currently used media (review: Gardner and Lane, 1997).

The composition of media which have been described so far in this paper have been designed without reference to the composition of the naturally occurring microenvironments they experience in the female genital tract. Heuristically, it seems reasonable to assume that media should mimic the natural environments as closely as possible. This point of view has been taken by Leese (1988) and Gardner and Leese (1990). Unfortunately we are largely ignorant of the compositions of the oviductal and uterine fluids during the preimplantation stages of pregnancy, with the exception of the common elements (Borland *et al.*, 1977), carbon sources (Gardner and Leese, 1990) and a few macromolecules (review: Leese, 1988). Medium MTF (Mouse Tubal Fluid; Table 2) was designed by changing M1 to include the concentrations of glucose, pyruvate and lactate in the mouse oviduct (Gardner and Leese, 1990). Development of zygotes from F_1 hybrids was the same in both M1 and MTH. The required concentration of K^+ in media for the culture of mouse preimplantation embryos seems paradoxical. The concentration of K^+ in the mouse oviduct is high (29 mM). This concentration of K^+ in a medium supports the development of two-cell embryos to blastocysts (Wales, 1970; Roblero and Riffo, 1986). In contrast, Whittingham (1975) found concentrations above 6 mM K^+ to be toxic to zygotes of outbred mice. A striking feature of KSOM is the low concentration of K^+ (2.85 mM).

It must be stressed that currently used media that support preimplantation development contain only a small subset of the set of compounds found in the natural microenvironments. Such media could easily produce environmental imbalances because of the absence of compounds needed to balance the effects of other compounds included in the medium. It is likely that embryos are able to develop in these artificial media only because they are able to adapt to the conditions (Biggers, 1993; Leese 1995). The physiological analyses of such adaptations may well be a fruitful area of research, such as the study of the regulation of intracellular ion concentrations (Baltz *et al.*, 1966) and osmolarity and cell volume regulation (Dawson and Baltz, 1997; Seguin and Baltz, 1997).

The choice of a medium depends on the purpose for which it is to be used. The uses fall into three categories: (a) biotechnical, including assisted reproduction, (b) experimental for the study of development, and (c) experimental for physiological studies. In all of these applications it is important to be aware of the artefacts that can be produced by the culture media. In general the biotechnical applications require the use of a medium that is as simple and economic as possible, but at the same time ensures that the young eventually produced after development in a surrogate uterus are normal or at least viable. It is for this reason that the final evaluation of a medium will be made by the determination of the rate of normal embryos born (review: Gardner and Lane, 1997). Recently Gardner and Lane (1997) have proposed that a two-step culture system be adopted; a medium for the culture of the pre-compaction stages and a more elaborate medium for the culture of the post-compaction stages. Whether this procedure yields more robust young than those produced by a one-step culture method involving only one medium needs confirmation. Developmental research often involves the study of the action of some gene product or a growth factor. Our interpretation of negative results requires caution, for if a strain of mouse is used which is susceptible to the two cell block completely misleading results will be obtained. However, there may be more subtle artefacts. Biggers (1993) wrote:

... if a growth factor has no effect on an embryo in culture, it would be rash to conclude that the embryo has no receptor for the factor. There may be many other possible explanations. For example, the conformation of the cellular proteins involved in the response to the growth factor may have been altered by the composition of the medium, Just as the effect of a gene often depends on

the genetic background of an individual, the action of a growth factor under in vitro conditions may depend on the background provided by a culture medium.

If a negative result is obtained it may be necessary to repeat the experiment with other media. Physiological research may be particularly prone to artefacts produced by culture media since the experiments are often of short duration and may inadvertently be done during the adaptations to the initial shock of explanation. Even if the embryos are given time to adapt the experiments may be studying systems that have changed due to the non-normal environments. These changes may become more serious the longer the cultures are maintained.

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