A brief history of the development of the KSOM family of media

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Mouse embryo development in vitro

Hammond (1949) using a physiologic saline solution supplemented with hen egg white and yolk observed that eight cell mouse embryos developed into blastocysts, whereas two-cell embryos typically arrested and did not undergo further development [1]. Whitten (1956) using modified Krebs-Ringer bicarbonate as the physiologic saline supplemented with glucose, penicillin G, streptomycin and egg white confirmed good development of eight-cell embryos into blastocysts [2]. Later, egg white was replaced by bovine serum albumin (BSA). In 1958, McLaren and Biggers showed that mouse blastocysts produced by Whitten's technique developed into normal adults following transfer to the uteri of surrogate females [3]. The results were unequivocal since genetic markers were used to distinguish mice derived from cultured embryos of one strain and transferring then into surrogates of a different strain. These pioneering studies paved the way for the experimental manipulation of the mammalian preimplantation embryo in vitro. However, the early days of preimplantation mouse embryo culture were complicated by the two cell block to development (reviews [4, 5]). Several unrelated ways were subsequently developed to overcome the two-cell block to development and led to the view that the media being used were not optimal.

The concentrations of the compounds targeted for inclusion in a chemically-defined medium is a very complicated matter.

One approach uses the '*back to nature*' principle [6]. Such an approach appears intuitively attractive since the concentrations used would be similar to those found in the female reproductive tract. There are however drawbacks with this approach (reviews: [7, 8]). Importantly, the approach will fail to detect any interactions between other compounds in the medium. An alternative approach is to determine experimentally optimized concentrations. The subject was treated theoretically by Biggers et al. (1957 [9]). The second approach has been called the '*let the embryo choose*' principle.

Sequential simplex optimization

The measured total response of embryos to a mixture of compounds in a culture medium can be represented by a concentration-response surface [10]. To study the interactions between several components is possible using a factorial design to construct a concentration-response surface. The approach becomes impossible with greater numbers of compounds, for example ten compounds, and other strategies are needed. One approach is called sequential simplex optimisation. Lawitts and Biggers [11, 12] found that sequential simplex optimisation represented a practical strategy to develop media for the culture of mouse zygotes which overcame the two-cell block to development. The measured response was the proportion of embryos that developed beyond the two-cell stage as the concentrations of compounds in the initial medium (START) were simultaneously varied, according to the rules determined by the optimisation protocol. After twenty cycles of optimisation a medium called simplex optimised medium (SOM) was developed from the START medium which overcame the two-cell block. The concentrations of NaCl and KCl were subsequently increased based on measurements of the intracellular

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Capsule In honoring Professor Biggers on the occasion of his 90th birthday, this article reviews his seminal contributions towards the development of KSOM media.

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ionic composition of K^+ and Na^+ determined by electron probe microanalysis in blastomeres of two-cell stage embryos that developed from zygotes. This medium was called KSOM, potassium-supplemented SOM [12]. It was first shown that KSOM supported a high yield of mouse blastocysts from zygotes that were capable of developing into fetuses after transfer to the uteri of surrogate mothers [13]. KSOM has been further modified in several ways to produce a family of KSOM-derived media.

Amino acids

KSOM was greatly improved when Ho et al. [14] supplemented the medium with 19 natural amino acids (Eagle's essential and non-essential amino acids, AA) noting that L-glutamine was already present in the START medium. The AAs added to KSOM are at half the concentration described by Eagle [15]. Dose response studies showed there was little difference in response between full strength and half strength concentrations of the AAs [16]. The addition of AAs resulted in very high yields of expanded blastocysts with markedly increased numbers of inner cell mass (ICM) and trophoblast (TE) cells compared to KSOM alone [16]. The extracellular matrix separating the primitive endoderm and ectoderm was also better organised. The medium is called KSOM^{AA}. The concentrations of the different AAs have not been optimised at this time.

L-glutamine has traditionally been used in the majority of media for the culture of mouse embryos, but is chemically unstable resulting in the accumulation of ammonium in the culture medium. Ammonium may be detrimental to preimplantation embryo development, but the experimental data are equivocal [8, 17]. The accumulation of ammonium can be largely avoided by replacing L-glutamine with a dipeptide containing L-glutamine. Dose response studies of KSOM^{AA} supplemented with two dipeptides, L-alanyl-L-glutamine and glycyl-L-glutamine, have been described [18]. Glycyl-L-glutamine may be preferable since there is evidence that it favours the development of the ICM and is therefore included in all currently used variants of KSOM^{AA}.

In vitro fertilisation (IVF) of mouse ova

KSOM will not support the fertilisation of mouse ova in vitro. Modified KSOM (mKSOM) was introduced to support IVF in the mouse. The original version of KSOM contains a low concentration of D-glucose (0.2 mmol/L) and BSA (1 mg/mL). The concentration of both constituents was increased (D-glucose, 5.56 mmol/L and BSA, 4 mg/mL) to permit high rates of in vitro fertilisation of mouse ova from a variety of

different strains [19]. Mouse zygotes derived from IVF also showed high rates of blastocyst development when cultured in KSOM supplemented with 5.56 mmol/L D-glucose. The result may have been surprising since the higher concentration of D-glucose did not prevent the subsequent cleavage divisions, contrary to the widespread dogma asserting that glucose inhibits the early development of the preimplantation embryo [8]. Earlier studies with SOM had also suggested that high concentrations of D-glucose did not inhibit early embryo development in the mouse [20]. Biggers and McGinnis [21] showed, for example, that the response of mouse zygotes to joint changes in the concentrations of D-glucose and potassium dihydrogen phosphate (KH_2PO_4) in KSOM is a flat two-dimensional concentration-response surface indicating no significant interactions between the effects of glucose and KH_2PO_4 . Moreover, no significant effects of D-glucose were noted on the development of zygotes into blastocysts. When the concentration-response surface is flat it is not feasible to define an optimal concentration for D-glucose. In this setting it is reasonable to apply the 'back to nature' principle and use the measured concentration of D-glucose in the oviduct. A detailed discussion of this matter is also found in Biggers and McGinnis [21]. D-Glucose-supplemented KSOM is now called KSOM_g.

Summers et al. (2000 [22]) showed that the addition of AAs to KSOM_g to provide a medium for IVF increased the percentage of blastocysts that hatched, increased the number of cells in the ICM and supported a more organised extracellular matrix based on the pattern of staining of Collagen IV. Further improvements in IVF were also made when L-glutamine was replaced with glycyl-L-glutamine [23]. The replacement of L-glutamine with glycyl-L-glutamine favoured the development of relatively more ICM cells than TE cells and reduced the number of pyknotic and fragmented nuclei in the blastocysts that developed in vitro.

One-step versus two-step culture of preimplantation mammalian embryos

Two-step protocols have been widely used to culture human zygotes to the blastocyst stage; this followed the recommendations of Gardner [24]. The protocol involves the sequential culture of the embryos in media of different chemical composition. The sequential media are purportedly designed to mimic the changing microenvironment of the female reproductive tract as the developing embryo passes along the oviduct into the uterus. A second justification for changing the medium is to remove putative toxic substances that may have accumulated during prolonged culture. This has been critically reviewed [7, 8]. There are several pairs of such media available commercially (review: [25]). Earlier studies described by Biggers and Racowsky [26] using KSOM^{AA} and Macklon

et al. [27] using the so-called “Rotterdam” medium, failed to show an advantage of the two-step procedure for the culture of human preimplantation embryos, suggesting that a more thorough examination of the practical advantage of the two-step procedures should be undertaken [8, 28].

Biggers et al. [29] compared the development of mouse zygotes in several sequential formulations and a single medium, KSOM^{AA} and found no significant differences in the proportions of blastocysts, rates of hatching, numbers of cells in the ICM and TE. No significant differences were noted in the body weights of the fetuses cultured from zygotes in a single medium and a sequential formulation, G1.2/G2.2. It was concluded that two-step protocols are sufficient to support preimplantation mouse development in vitro, but are not necessary. Recent studies comparing several commercial sequential media formulations and a single medium derived from KSOM^{AA} (Global® medium; LifeGlobal) for the culture of human preimplantation embryos have also noted no significant differences in the development of human zygotes to the blastocyst stage or pregnancy rates following intrauterine embryo transfer [30–33]. A comprehensive review of this topic is beyond the scope of this article.

It is now apparent that different culture media are able to produce human embryos with similar morphologic scores, rates of development in vitro and pregnancy rates following embryo transfer, suggesting that preimplantation embryos are able to adapt to different culture conditions [5, 7, 8, 33]. The next challenge is to define in more detail the effects of different media on cell stress, epigenetics and the control of gene regulatory networks in the developing embryo. Does the embryo pay a long term price for adapting to different culture conditions? The answer will require a multi-disciplinary systems biology approach to media development [25].

It is well established that different culture media can change the pattern of gene expression in preimplantation mouse embryos. Doherty et al. [34] showed there were differences in the pattern of expression of the imprinted gene, *H19*, in mouse embryos when cultured in Whitten’s medium and KSOM^{AA}. *H19* expression and DNA methylation were adversely affected by culture in Whitten’s medium due to abnormal methylation of the upstream imprinting control region, resulting in a high level of biallelic *H19* expression. The response of *H19* following embryo culture in KSOM^{AA} followed more closely the pattern of expression observed with in vivo generated blastocysts. Subsequently, Rinaudo and Schultz [35] showed differences in the global expression of genes in mouse blastocysts cultured from the zygote to the blastocyst stage: 114 genes were abnormally expressed in Whitten’s medium and only 29 genes in KSOM^{AA}. Differences were noted in the level of specific genes involved in protein synthesis, cell proliferation and transporter function.

Human IVF and culture media

The ultimate aim of human IVF is the successful term delivery of a healthy singleton foetus. There has always been a concern of the potential deleterious effects of human embryo culture on the health of the offspring [7, 8, 36]. A higher incidence of low birth weight (LBW) is seen in singleton pregnancies following IVF [37, 38]. There is also the suggestion that human IVF is associated with an increased incidence of imprinting gene disorders [39–41]. A sense of urgency occurred when Dumoulin et al., [42] reported different distributions in the birth weight of newborns when embryos were cultured in two different commercially available sequential media. A follow up study confirmed their earlier observations [43]. However, three recent retrospective studies have not shown an effect of culture medium on newborn birth weights [44–46]. All these studies compared different commercial media and the retrospective nature of the studies weakens the findings. One cannot preclude the possibility that some commercial media used for the culture of human embryos have a greater or lesser deleterious effect on pre- and post-implantation embryo development. This is a complicated topic that requires careful deliberation, and will not be further discussed in this short article. One problem that needs to be addressed is the lack of information on the constituents and their concentrations in all the commercial media used for the culture of human embryos. A serious scientific debate on the impact of different culture media on the long term health of offspring conceived through the use of IVF cannot be held unless this issue is resolved. This matter has previously been discussed at some length [8, 47].

It is worth noting that under the conditions used for mouse IVF that even brief exposure (4 h) to different medium used for IVF can impact the subsequent development of the zygote into a blastocyst [22]. Moreover, changing one compound (replacing L-glutamine with glycyl-L-glutamine) in KSOM^{AA} changes the ICM/TE cell ratio [23]. These changes are subtle, but significant.

Concluding comments

The initial work on the development and design of SOM and KSOM was done as part of the National Cooperative Programme on Non-Human In-Vitro Fertilisation and Preimplantation Development, sponsored by the National Institute of Child Health and Development (NICHD), between 1986 and 1996. The impetus for much of the early work was to overcome the two-cell block to development in the mouse. Many other researchers who were part of the cooperative programme made equally important contributions in the field of early mammalian embryo development, including the development of other culture media, such as CZB [48] patterns of gene

expression in cultured embryos and the effects of culture media on genomic imprinting, and so on. A similar type of effort will likely be required to study effectively the impact of culture media on pre and post-implantation human embryo development.

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