Connections between preimplantation embryo physiology and culture

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Abstract

Purpose To review the history of experimental embryo culture and how culture media that permitted complete preimplantation development in vitro were first discovered, and the physiological insights gained.

Methods This article reviews the history of in vitro mammalian embryo culture, in particular the efforts that led to the current generation of successful culture media and how these reflect embryo physiology, highlighting the contributions of Dr. John D. Biggers and his colleagues and students.

Results The culture of mammalian embryos began about a century ago. However, defined media without biological fluids were only developed in the late 1950s, and the first live young born from cultured embryos, using these media, were produced by McLaren and Biggers in 1958. It wasn't until the late 1980s, however, that preimplantation mammalian embryos could generally be cultured in vitro from fertilized eggs to blastocysts. These new media led to insights into embryo physiology, including the importance of cell volume homeostasis to early embryo viability.

Conclusions The development of successful preimplantation embryo culture media has had a profound effect on assisted reproduction technologies and on research into early embryo physiology.

Keywords Cell volume · Culture · Embryo · Preimplantation · Metabolism · Osmolarity

It has been known for more than a century that mammalian embryos can be transferred from a donor into a recipient to produce live young. The first recorded successful embryo transfer was performed by Heape in 1890 [44], when live young were born after 4-cell Angora rabbit embryos were transferred to a Belgian hare recipient, an achievement described in detail by Biggers [12]. Over the subsequent decades, successful embryo transfers were accomplished in a number of other species including rodents and large domestic animals [41]. Similarly, it has been about a century since mammalian preimplantation embryos were first successfully cultured, beginning with Brachet's demonstration in 1912 that rabbit blastocysts could continue to grow for up to 2 days in culture [17], which Pincus and coworkers later extended to culture through the cleavage stages [41]. The ability of primate embryos to also develop in vitro was confirmed by Lewis and Hartmann, who not only cultured rhesus embryos from the 2-cell to 8-cell stage, but filmed them for the duration of the culture period in 1933 [59]. Human embryos were not successfully cultured until this was accomplished by Edwards nearly four decades later [28]. Culture of preimplantation embryos of the mouse, a commonly used laboratory species for research in reproduction, was first achieved by Hammond, who cultured their cleavage stage embryos to blastocysts in 1949 [42]. However, it was not until the late 1950s that embryo culture and embryo transfer were combined to produce live offspring, when a landmark paper was published by McLaren and Biggers in which they reported that 8-cell mouse embryos cultured to the blastocyst stage produced normal fetuses and live young after transfer into recipients [60]. This was extended the following year by Chang to the production of live young after in vitro fertilization of rabbit ova, which were cultured to the 4-cell stage before transfer [22]. About 20 years later, Steptoe and Edwards reported the first successful human embryo IVF, culture and transfer that resulted in a live birth [71].

Capsule A historical perspective of the development of embryo culture media is reviewed in the context of advances made in our understanding of the physiology of preimplantation mammalian embryos.

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The 1958 Nature paper by McLaren and Biggers and the subsequent paper by Chang were milestones in our ability to perform experiments on clearly viable preimplantation mammalian embryos and represented a major advance in the development of assisted reproduction technologies for animals and humans. The McLaren and Biggers paper was not only the first demonstration that cultured embryos were completely viable, but they also transferred embryos that had been cultured in an entirely defined culture medium. Most previous embryo culture had been carried out in complex biological fluids such as serum or plasma, or in synthetic culture media supplemented with biological fluids including serum and chicken egg extracts and was of limited success in most species, notably including poor development of rodent embryos [3, 41, 63]. However, during 1956 and 1957, Whitten formulated a completely defined medium based on Krebs Ringer Bicarbonate (KRB) that supported the development of 2-cell mouse embryos to blastocysts [85, 86]. It was Whitten's medium in which the mouse embryos were cultured by McLaren and Biggers to obtain live offspring [60].

Development of mouse embryos from fertilized eggs in a defined medium was not achieved, however, until Brinster found that pyruvate was the major energy source for early preimplantation embryos [18] and carried out his extensive series of studies on the optimization of metabolites and physicochemical parameters for mouse embryo culture (reviewed in detail by Hammer [41]), following which Whittingham and Biggers demonstrated that medium to which pyruvate had been added supported development through preimplantation embryogenesis [89]. These findings led directly to the development of classic mouse embryo culture media that included Whittingham's M16 [88], which was the standard for mouse embryo culture for decades [13, 46], as well as related widely-used human embryo culture media such as HTF [8, 64].

Complete development of mammalian embryos from fertilized eggs to blastocysts in vitro was still not generally possible in these media, however. In Whittingham and Biggers's study [89], fertilized eggs cleaved to the 2-cell stage, but became arrested at that point unless they were reinserted into oviducts for a period before they could be cultured further from the 2-cell to blastocyst stages. This "2-cell block" to in vitro mouse development afflicted embryos derived from many genotypes of females, particularly those of outbred and many inbred strains, with F1 hybrids between inbred strains being most resistant, as first shown by Whitten and Biggers in 1968 [87]. Embryos of other species also suffered similar developmental blocks at characteristic points of development, such as the 4- to 8-cell stages in human [16], 2- to 4-cell in hamster [65], 8-cell in cow [20], and 2-cell in rat [49]. The physiological cause of these developmental blocks is still not really clear, although, at least in the mouse, embryos become arrested at the G2/M border, pointing to a defect in cell cycle control induced by culture conditions [7, 34, 83]. Overcoming

these developmental blocks became an intense focus of research over several decades through the 1980s.

Early attempts to overcome the 2-cell block in mouse yielded interesting clues. The chelator EDTA allowed development through the 2-cell block in some mouse strains that otherwise blocked in vitro [1]. High phosphate and glucose were found by Bavister's group to induce a similar 2-cell block in hamster embryos [65] which was also confirmed in mouse [37]. Phosphate and glucose are not universally inhibitory, however, since Biggers and colleagues showed they can have little or no detrimental effect when the base medium has a different composition [13, 73]. In addition, glutamine, which markedly improved development of hamster embryos from the 8-cell stage [21] was later shown to be able to relieve the 2-cell block in mouse [23, 54]. Perhaps most intriguingly, the 2-cell block was able to be overcome by a factor in the cytoplasm of non-blocking embryos, since Muggleton-Harris, Whittingham and Wilson showed that cytoplasmic transfer allowed development of mouse embryos from fertilized eggs past the 2-cell block to blastocysts [61]. Since such disparate factors could modulate in vitro developmental blocks to embryo development, this implied that these blocks were most likely an artifact of culture caused by stress acting at particularly vulnerable points of development. Such an interpretation is also consistent with the observation that the embryonic stage where developmental blocks occurred in different species (above) generally corresponded to the stage where their zygotic genomes become globally activated [76].

By the 1980s, it was evident that there were scattered leads but no clear path forward. The inability to culture fertilized eggs through complete preimplantation development in vitro was hampering the ability to treat human infertility by in vitro fertilization and assisted reproductive technologies, making experimental work difficult with most animal models, as well as making the use of these types of technologies in animal husbandry difficult. There was also concern that the clearly stressful conditions in culture could cause detrimental effects on embryos that resulted in lower success rates and the need to transfer a large number of embryos into women with resultant multiple births. As well, there was concern that fetal and offspring health was being compromised, a possibility since made real by demonstrations that the early origins of health and disease extend even to the preimplantation embryo period [31]. The US National Institute of Child Health and Human Development, NIH, responded to this by launching a National Cooperative Program on Non-Human In Vitro Fertilization and Preimplantation Development under the leadership of NICHD's Richard Tasca.

The first iteration of this group grant program, more commonly known as the "Culture Club" (a reference to the contemporary English New Wave band headed by Boy George, apparently a favorite of the researchers involved), was largely aimed at overcoming in vitro developmental blocks to

preimplantation embryo development. Remarkably, it led to the formulation of defined culture media that supported development of fertilized mouse eggs to blastocysts, eliminating developmental blocks within just a few years. The successful media were developed primarily by two groups within the Program, those of Bavister and Biggers (although, as a cooperative program, it is difficult to separate out the contributions of individual groups from the efforts of the program as a whole). Bavister's group had worked primarily with hamster, and developed media that improved in vitro preimplantation development in that species. Key findings from Bavister's work with hamsters were that glutamine was beneficial [21] and that glucose and phosphate influenced whether embryos became blocked in vitro [65]. His group applied these findings to mouse in collaboration with Ziomek, leading to the development of the first successful medium that supported mouse embryo development from the fertilized egg to blastocyst, CZB [23]. CZB was generally employed as a two-stage medium, with glucose omitted for the first 48 h of culture and then introduced for subsequent culture to blastocysts.

Lawitts and Biggers pursued a unique strategy, which was to adapt an unbiased optimization process called simplex optimization that had been previously used in realms such as chemical engineering. Biggers adapted the simplex algorithms to the optimization of culture media, accounting for the inherent variability of biological systems [53]. The initial medium at the start of the optimization was similar to Whittingham's M16 [88] and included the same eight components as M16 plus two additional components: EDTA, previously identified by Abramczuk, Solter, and Koprowski as alleviating the 2-cell block in some mouse strains [1], and glutamine, adopted from the work by Bavister's group that led to CZB. Thus, the optimization was carried out on $n=10$ components of the medium [53]. For simplex optimization, each round required $n+1$ or 11 different media. The starting set of media still largely caused the 2-cell block, but after the first few cycles of optimization, media formulations arose in which the 2-cell block was only partial and some embryos had escaped [53]. After running 20 such optimization cycles, a final medium was produced that was designated Simplex Optimized Medium (SOM), in which the 2-cell block did not occur [54]. SOM was later modified further by increasing the potassium concentration, after measurements by Biggers, Lawitts, and Lechene had shown that intracellular potassium levels were abnormally low in embryos cultured in SOM [14]. This final medium was designated KSOM [29, 55] and resulted in a further improvement of blastocyst development over SOM and CZB under the same conditions [29].

In addition to the major advance of producing a medium, KSOM, in which most strains of mouse embryo will develop from fertilized eggs to blastocysts, the process of simplex optimization yielded some very interesting insights. Because the simplex procedure does not rely on any assumptions about

the relative importance or function of the optimized variables, the embryos essentially were able to "choose" conditions that were most favorable for their development in vitro. A key feature of the optimized media that permitted development past the 2-cell block was their markedly low osmolarity [8, 9]. While mouse oviductal fluid has an osmolarity of about 290–310 mOsM [24, 30], which is close to that of mouse serum [84, 90], SOM and KSOM have osmolarities of only approximately 230 and 255 mOsM, respectively [54, 55], and the osmolarity of CZB is about 275 mOsM [27]. In contrast, M16 medium, in which the 2-cell block occurs, has a "physiological" osmolarity of around 290 mOsM [46]. We subsequently showed that osmolarity itself was a key factor, since a 2-cell block could be made to occur even in embryos from a "non-blocking" F1 hybrid female by specifically increasing the osmolarity of KSOM, although a higher osmolarity was required to block development of embryos from F1 hybrids than for embryos from "blocking" random bred females [37].

Another feature of the new media was the inclusion of glutamine. Biggers's group then showed that glutamine levels interacted strongly with NaCl levels in determining whether embryos would become blocked, with higher glutamine levels protecting against the deleterious effects of increased NaCl [54]. This was reminiscent of Van Winkle's earlier observation that glycine protected development of mouse embryos from the 2-cell stage against increased NaCl [79], where he had proposed that glycine may play a role as an organic osmolyte in embryos. Organic osmolytes are compounds used by cells to provide benign intracellular osmotic support that maintains cell volume while avoiding the deleterious effects of increased ionic strength in the cytoplasm [45, 93, 94]. When it initially became apparent that the optimization of media to allow development past the 2-cell block resulted in low osmolarity, Biggers also speculated on the possibility that organic osmolytes may be required [53] and that glutamine may exert a beneficial effect by acting as an organic osmolyte [54]. This hypothesis was strengthened by his group's subsequent demonstration that a major organic osmolyte in the kidney, betaine (N,N,N-trimethylglycine), which unlike glutamine is not metabolized as an energy source, was also highly protective against increased NaCl for mouse embryos [14]. Furthermore, Schultz's group, also part of the Culture Club, showed that protein synthesis in embryos was disrupted in when they were cultured at increased NaCl, and that this deleterious effect was reversed by betaine [6], also consistent with a role for organic osmolytes in embryos.

The low osmolarity of the optimized media, abilities of glutamine, glycine and betaine to protect against increased NaCl and osmolarity, and perturbation of metabolism at increased osmolarity all pointed to a central role for cell volume regulation in the etiology of the 2-cell block. In mammalian somatic cells, volume is controlled acutely by the accumulation and release of inorganic ions [45]. When cell volume

becomes too large, the swelling is relieved by the release of anions (and organic osmolytes) through volume-activated anion channels [45, 72]. We have shown that mouse and human embryos also possess such volume activated channels that allow them to recover from cell swelling [40, 50, 51, 67].

The major acute mechanism for correcting cell volume decreases is a functionally-coupled set of two transporters, the Na^+/H^+ exchanger and $\text{HCO}_3^-/\text{Cl}^-$ exchanger [45]. Decreased cell volume activates the NHE1 isoform of Na^+/H^+ exchanger, which both imports Na^+ and slightly raises intracellular pH [2]. The increase in intracellular pH, in turn, activates the AE2 isoform of $\text{HCO}_3^-/\text{Cl}^-$ exchanger, importing Cl^- and neutralizing the pH increase through the export of bicarbonate [47]. Thus, pH remains unchanged, but Na^+ and Cl^- are accumulated to raise intracellular osmotic pressure and quickly correct the cell volume decrease. NHE1 is activated by decreased cell volume through a mechanism that involves the tyrosine kinase Janus Kinase 2 (JAK2)-mediated activation of calmodulin, which directly activates NHE1 [33]. We have recently shown that this mechanism operates in preimplantation mouse embryos at all stages and mediates their recovery from unwanted cell volume decreases [95]. A more detailed discussion of these mechanisms in preimplantation embryos can be found in a recent review [10].

The accumulation of excessive levels of NaCl to maintain cell volume is likely a major cause of the deleterious effects of older media that resulted in the 2-cell block. Such a model explains why lowering osmolarity eliminated the block, since a reduced level of intracellular inorganic ions would then be required to maintain normal embryo volume. It also provides a mechanism for why glutamine, betaine, or glycine protected against increased osmolarity and allowed development past the block, since their role as organic osmolytes is to replace a portion of the intracellular inorganic ions (reviewed in [10]).

We followed up on the seminal work by Biggers to investigate whether cell volume dysregulation and lack of appropriate organic osmolytes was a factor in the 2-cell block to embryo development in vitro. Once media in which complete development of embryos from the fertilized egg to blastocyst were available, it became possible to ask what conditions would cause the blocks to reappear, which opened new avenues of inquiry. A systematic search for putative effective organic osmolytes that could displace deleterious levels of intracellular inorganic ions in mouse embryos was done using KSOM medium without glutamine and with the osmolarity increased so that nearly all embryos blocked at the 2-cell stage. Adding candidate organic osmolytes to this medium confirmed the efficacy of glutamine, glycine and betaine, and additionally identified proline, β -alanine and hypotaurine [25]. The first clear indication that several of these compounds actually functioned as organic osmolytes in mouse embryos was achieved when it was shown that glycine was accumulated to higher levels in 2-cell mouse embryos when the

external osmolarity was increased [26], that betaine was transported by 1-cell mouse embryos [38] and its accumulation is stimulated by increased osmolarity [5], and that β -alanine accumulation was similarly stimulated at increased osmolarity [39].

In mammalian somatic cells, there are four known transporters of organic osmolytes: the $\text{Na}^+/\text{myo-inositol}$ transporter (SMIT), betaine transporter (BGT1), taurine/ β -amino acid transporter (TAUT) that transports β -amino acids such as taurine and β -alanine, and the System A amino acid transporter (ATA2) that transports small, neutral amino acids [32, 52, 62]. β -alanine and hypotaurine are both transported by TAUT, which is the route they almost certainly take in preimplantation mouse embryos [39, 81]. However, these β -amino acids are unlikely to have a significant function in preimplantation embryos as organic osmolytes [10, 39]. Surprisingly, the other mammalian organic osmolyte transporters did not participate in transport and accumulation of effective organic osmolytes in mouse embryos, since BGT1 is absent [38], System A only first appears in blastocysts [48], and SMIT transports only myo-inositol, which is ineffective in early embryos [25].

To investigate the mechanisms by which early mammalian embryos use organic osmolytes, we focused on glycine. Glycine is highly effective at protecting early mouse embryos against increased osmolarity [10, 37, 79]. It is present in oviductal fluid of many species [36, 43] at concentrations shown to be maximally protective for embryo development in vitro [25], and endogenous intracellular levels of soluble glycine in embryos are very high [66, 75, 78]. Furthermore, the single route of glycine transport in mouse embryos had previously been identified by Van Winkle as the classical amino acid transport system designated GLY [80], which is also present and active in human embryos [40].

The GLY transport system corresponds to a single transport protein, GLYT1, encoded by the *Slc6a9* gene [19, 35]. Inhibition of GLYT1 eliminates glycine transport and also prevents increased glycine accumulation in response to higher osmolarity in mouse embryos [70]. Most importantly, GLYT1 activity is required for mouse eggs and early embryos to maintain normal volume and to develop in vitro past the 2-cell block at the osmolarity of oviductal fluid [69, 70, 75]. Cell volume regulation using GLYT1 transport of glycine is apparently unique to early preimplantation embryos, as this mechanism has not been found in somatic cells. Furthermore, the mechanism by which GLYT1 regulates volume is different from that of the somatic organic osmolyte transporters, since the latter require transcription and translation of transporter proteins, while glycine accumulation as an organic osmolyte in embryos is independent of protein synthesis [70].

What about the other organic osmolytes effective for embryos? Glutamine, which had been fortuitously included in their culture media by Bavister and Biggers, is also

transported by GLYT1 in mouse embryos [58]. This likely explains its ability to protect embryos when glycine is absent (as in CZB and KSOM), although glycine predominates when it is present. Initially, it was proposed that betaine, a derivative of glycine, was also transported by GLYT1 and that there was a single organic osmolyte transporter in early embryos for all but the β -amino acids [25]. However, further investigations revealed that betaine was, unexpectedly, not a GLYT1 substrate [38]. Instead, we found that a related transporter, SIT1, encoded by the *Slc6a20* gene [19, 74] was the sole betaine transporter in early preimplantation mouse embryos [4] and that it also transports proline [5]. Like GLYT1, this role for SIT1 in volume regulation is apparently unique to early embryos and has not been reported in somatic cells. Thus, there are several transporters that can function in organic osmolyte accumulation and cell volume regulation in early preimplantation embryos: GLYT1, SIT1, which appear unique to early embryos, and to a lesser extent, TAUT, which shares the same function in somatic cells.

Both GLYT1 and SIT1 are active only within fairly restricted windows during oocyte maturation and preimplantation embryo development. GLYT1 is activated at ovulation, and becomes fully active during meiotic maturation, during which period glycine is initially accumulated to high intracellular levels [75]. GLYT1 becomes inactive after about the 8-cell stage, at which time embryos lose the ability to accumulate glycine at increased levels in response to increased osmolarity [40, 75, 77]. SIT1 is active during an even shorter period, being first activated after fertilization and persisting only through the 2-cell stage [4]. Thus, the organic osmolyte transporters unique to early embryos are present in mouse embryos at precisely the period when they are most vulnerable to osmotic stress, when they must avoid the 2-cell block. The current model is thus that the earliest stages of embryos possess the same mechanisms for acute cell volume regulation utilizing inorganic ion transport that are employed by somatic cells. However, early preimplantation embryos are, for unknown reasons, particularly sensitive to even modest increases in intracellular ionic strength. To overcome this, they replace inorganic ions with organic osmolytes using mechanisms unique to preimplantation embryos, principally relying on glycine. A more extensive discussion can be found in recent reviews [9, 10].

From the earliest attempts to manipulate preimplantation mammalian embryos in vitro there has been a close two-way connection to discoveries about their physiology. The work on culture media and embryo transfer in the late 1950s led to extensive investigations, initially carried out mainly by Biggers and his colleagues and students [57], that elucidated the unique metabolism of each stage of preimplantation embryos. The development of successful culture media for complete preimplantation development, particularly Biggers's work within the Culture Club in formulating KSOM, led to

the identification of osmolarity and cell volume control as key factors in determining whether embryos will become arrested in culture [8–10]. This in turn led to the discovery of mechanisms of cell volume regulation unique to early embryos [69, 70, 75]. Furthermore, this spurred investigations into how preimplantation embryos detect osmotic stress and stress in general, and how they adapt, including our work [95] and that of Watson's [11] and Rappolee's [91, 92] groups. It seems likely that further improvements in embryo culture and assisted reproductive technologies will continue to be intimately connected with improvements in our understanding of preimplantation embryo physiology, with insights continuing to flow both ways. This connection is currently particularly evident in the recent discovery that culture conditions profoundly affect epigenetic gene regulation in embryos and can have major effects on the health of offspring into adulthood [31, 68, 82]. As predicted by Biggers and Summers, "[f]uture research on the design of media for the culture of preimplantation embryos will focus mainly on the avoidance of epigenetic effects...Advances in this area will require replacing the classical way in which we look at embryos in terms of morphology and biochemistry, and focusing more on the effects of culture media on gene regulatory networks, the chronicity of gene expression patterns during early embryonic development and the fluxes they control through networks of metabolic reactions" [15]. Appropriately, betaine, one of the compounds originally used by Biggers's and Schultz's groups to show that embryos likely require organic osmolytes [6, 14], has recently been found to also have a function as a major source of the methyl groups used in the mouse blastocyst for epigenetic marking by methylation ([56] and our unpublished results), thus making yet another connection from the development of culture media to embryo physiology.

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