—Mini Review—

Human preimplantation embryo culture media: past, present, and future

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Abstract: Systematic studies of mouse embryo culture beginning in 1949 led to an understanding of essential medium components for early mammalian embryos, and embryo culture from the zygote to the blastocyst stage was achieved in 1968. Since then, medium components that are either beneficial or detrimental for embryo culture have been identified. A variety of culture media that mimic the female reproductive tract, such as human tubal fluid medium and sequential media, were developed from the 1970s to the 1990s, and a single medium in which the concentrations of components were determined by a simplex optimization method was introduced for clinical use in 2002. While either sequential media or a single medium is currently used in most cases, no conclusion has yet been reached as to which of the two approaches is the best. That we are now easily able to culture embryos is the result of the work of pioneers. This review presents a chronological overview of media development from initial attempts at mouse embryo culture using synthetic media to the human embryo culture media used today. It also presents the characteristics of sequential media and a single medium. Finally, problems observed with current embryo culture media are discussed, along with future development in this area.

Key words: Preimplantation embryo culture, Sequential media, Single medium, Albumin, Growth factors

Introduction

In 1882, the first tissue culture medium was successfully used by Ringer [1, 2]. He formulated a balanced salt solution based on the inorganic salt compositions of blood serum to maintain a frog heart beating *in vitro*. His studies showed that animal tissues could survive *in vitro* if the osmotic pressure, pH, and inorganic ion concentrations were at physiological levels. Subsequently, Ringer's solution was modified, and a variety of balanced salt solutions were developed [3–9], including Tyrode's solution and Krebs–Ringer–bicarbonate (KRB) solution (Fig. 1). In 1907, Harrison *et al.* [10] were the first to successfully culture animal somatic cells using lymph as a medium, and they used it to observe the outgrowth of frog embryo nerve fibers *in vitro*. Their results accelerated animal cell culture study, including the culture of preimplantation embryos from various animal species (e.g., guinea pig and rabbit [11–14], mouse [15], monkey [16], and human [17, 18]), using natural media such as lymph [19] (Table 1). However, the use of natural media composed of unknown components presented problems in terms of experimental reproducibility, and it does not enable the identification of the components necessary for the developing embryo *in vitro*. Thus, there was a shift to synthetic media based on balanced salt solutions [20] (Table 1).

Whitten [21–23] developed mouse embryo culture media based on KRB solution, and was able to reproduce embryo development throughout the preimplantation period *in vitro*. Using Tyrode's solution, Yanagimachi and Chang [24] succeeded in inducing mammalian sperm capacitation *in vitro*. Their methods were applied by Edwards and Steptoe *et al.* [25] to human *in vitro* fertilization (IVF), and were linked to successes in assisted reproductive technology (ART) in 1978 [26]. According to the concept that the reproductive environment should be mimicked during embryo culture *in vitro*(the so-called "back to nature" approach [27]), human oviduct and uterine fluids were analyzed, and human tubal fluid (HTF) [28] and Gardner's G1/G2 media [29] were developed. Of these, media having similar characteristics to G1/G2 have become known as "sequential" or "two-step" media. Meanwhile, in another approach, there was no attempt to mimic *in vivo* environments, but rather the appropriate concentration of each medium component *in vitro*

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was determined by sequential simplex optimization using mouse embryo assays (the so-called “let the embryo choose” approach [27]). This approach led to the development of potassium simplex optimized medium (KSOM) for mouse embryo culture [30]. This medium and the $KSOM^{AA}$ medium [31], which is KSOM supplemented with amino acids, are effective not only for the culture of mouse embryos, but also for human embryo culture [32]. Under the trademark of Global, $KSOM^{AA}$ is widely used in human ART. A medium having characteristics similar to $KSOM^{AA}$ is currently called a “single medium” (or a “single-step medium,” or a “one-step medium”).

The following chapter presents a chronological overview of media development from initial attempts at mouse embryo culture using synthetic media to the human embryo culture media used today, to understand how pioneers cultured preimplantation embryos and what medium components are either essential or detrimental. The second chapter presents the characteristics of sequential media and single media to indicate which approach is best for each laboratory. The final chapter discusses problems observed with current embryo culture media to assist the development of further optimized embryo culture media.

The Development of Embryo Culture Media

The trajectory towards successful embryo culture

Mouse embryos

Systematic research using synthetic media began in 1949 with the use of mouse embryos. Hammond [33], using a simple medium of NaCl, KCl, $CaCl_2$, $MgCl_2$, and glucose supplemented with egg white and egg yolk, successfully developed 8-cell-stage mouse embryos, that were collected from a fallopian tube, to the blastocyst stage. However, the blastocyst formation rates were low, and it was also difficult to develop embryos before the 8-cell stage with this medium. In 1956, Whitten [21] showed that the pH of Hammond’s medium rose rapidly from 7.0 to 7.8, and confirmed that embryos do not undergo cleavage at pH 7.7 or above. He also found that bovine serum albumin (BSA) could be used instead of egg whites, and he used KRB solution with glucose, BSA, and antibiotics as an embryo culture medium because this solution had pH buffer capacity. This medium greatly improved embryo development from the 8-cell stage to the blastocyst stage, and at this time, it became clear that glucose was necessary for development from the 8-cell stage. McLaren and Biggers [34] transplanted blastocysts cultured from 8-cell-stage embryos using Whitten’s method to a surrogate mother and successfully obtained viable young. In 1957, Whitten [22] found that adding lactate to the medium enabled embryos to progress from the 2-cell stage to the blastocyst stage, but did not enable embryos to develop from the 1-cell stage.

Brinster made several major contributions to medium development which enabled culture from the 1-cell stage to the 2-cell stage in synthetic media. He determined appropriate pH levels and osmotic pressure for embryo culture and investigated the effects of the energy substrates, BSA, and amino acids that are used to supplement embryo culture media [35–37]. Brinster also discovered that the 2-cell-stage mouse embryo did not utilize glucose as an energy source, and that it required either pyruvate, lactate, oxaloacetate, or phosphoenolpyruvate [36]. Because the best results were obtained using a combination of pyruvate and lactate [38, 39], he developed Brinster’s medium for ovum culture 2 (BMOC2) containing pyruvate and lactate. This improved the development of embryos from the 2-cell stage to the blastocyst stage. Subsequently, Whittingham and Biggers [40] cultured embryos from the 1-cell to the 2-cell stage in a medium supplemented with pyruvate and lactate, and then obtained blastocysts within fallopian tubes undergoing organ culture. Furthermore, they showed that pyruvate, but not lactate, acted as an energy source for oocyte maturation and the first cleavage division, although lactate could act as an energy source for oocytes and fertilized eggs indirectly through cumulus cells [41]. Finally, in 1968 Whitten and Biggers [23] successfully reproduced all preimplantation development stages *in vitro* using a medium containing BSA, pyruvate, lactate, and glucose. The medium had lower osmolarity than previous media. However, Whitten and Biggers were only able to culture fertilized eggs from F1 hybrid mice, and most embryos from other strains of mice showed developmental arrest at the 2-cell stage in this medium. This phenomenon is known as the “2-cell block”.

Human embryo

In 1969, around the time of Whitten’s success (Fig. 1), the research group of Edwards and Steptoe [25, 42] reported the first indubitable evidence of fertilization of human ova using a medium based on a modification of Tyrode’s solution. This medium was selected based on the hamster IVF successes of Yanagimachi and Chang [24, 43]. Subsequently, in order to culture human fertilized eggs, Edwards *et al.* [44] screened five types of existing media, including Whitten’s medium, and showed that Ham’s F-10 medium with 20% fetal calf serum (FCS) was suitable for human embryo culture. In 1971, Steptoe *et al.* [45] succeeded in culturing human embryos from the zygote to the blastocyst stage. Although Seitz *et al.* [46],

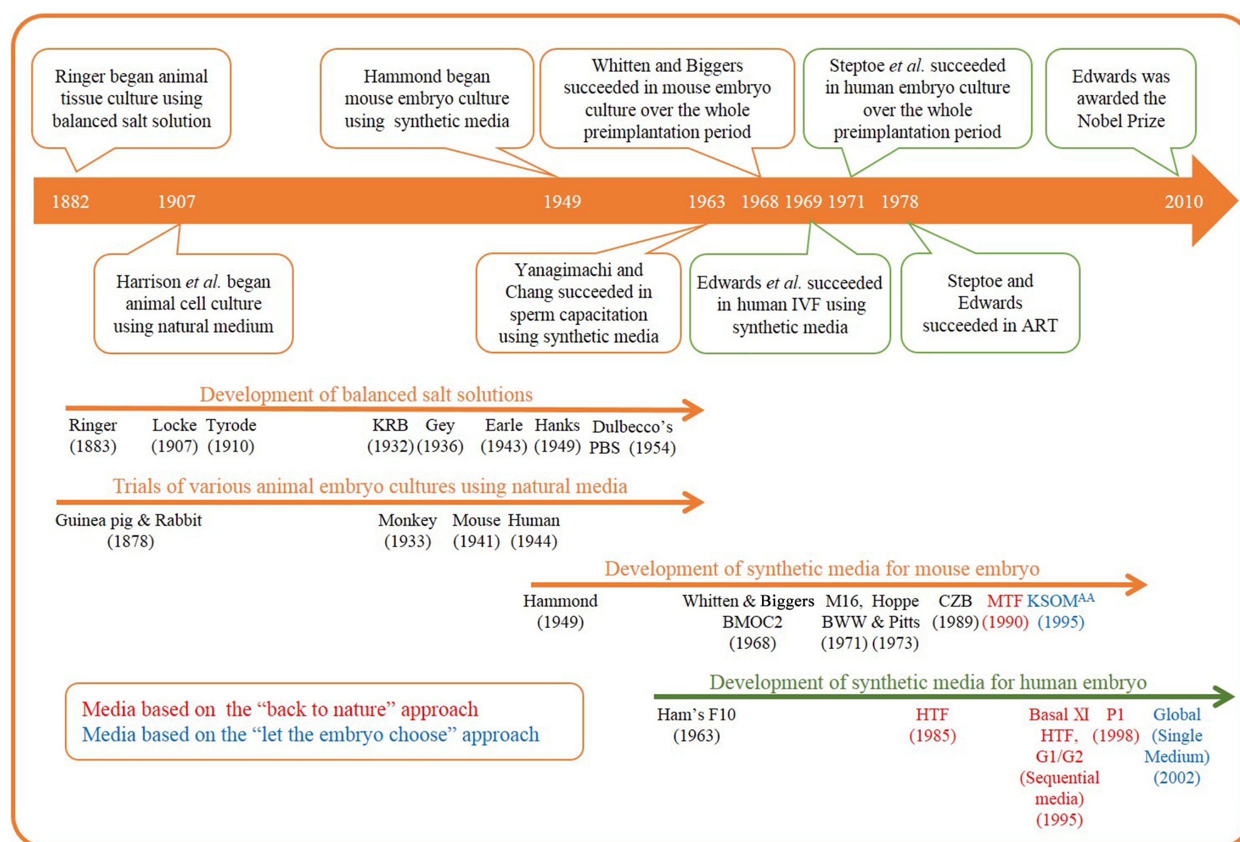

Fig. 1. Timeline from successful animal tissue culture to the appearance of a single medium. After the successful culture of animal tissues and cells, experiments were performed to culture a variety of animal embryos in natural media, while at the same time, balanced salt solutions were developed. Thereafter, progress was made on the study of mouse embryo culture using synthetic media, and based on successes in mouse embryo culture, a variety of human embryo culture media were developed.

Table 1. Categories of animal cell culture media

Categories	Definitions	Types	Examples
Natural media	These media consist of natural biological substances such as plasma, serum, and embryo extract.	Coagulant or clots	Plasma separated from heparinized blood, serum, and fibrinogen
		Biological fluids	Plasma, serum, lymph, amniotic fluid, and pleural fluid
		Tissue extracts	Extracts of chicken embryos, liver, spleen, and bone marrow extract
Synthetic media	These media contain partly or fully defined components, like a balanced salt solution, and organic compounds such as amino acids and vitamins.	Serum-containing media	Human, bovine, equine or other serum is used as a supplement
		Serum-free media	Crude protein fractions such as serum albumin, α, and β-globulin are used as supplements
		Protein-free media	Undefined components such as peptide fractions (protein hydrolysates) used as supplements
		Chemically defined media	Undefined components such as crude protein fractions, hydrolysates, and tissue extracts are not appropriate supplements. Highly purified components such as recombinant proteins are appropriate supplements.

Table 2. Human embryo culture medium composition

		1963	1985	1995		2002
		Ham's F-10	HTF	Sequential media		Single medium
				G1	G2	Global
Inorganic salts		○	○	○	○	○
Energy substrates* (mmol/L)	Glucose	6.1	2.8	0.5	3.2	0.2
	Pyruvate	1.0	0.3	0.3	0.1	0.2
	Lactate	–	21.4	10.5	5.9	10.0
Amino acids**	Essential	○	–	–***	○	○
	Nonessential	○	–	○	○	○
Vitamins		○	–	–	–***	–
Trace elements		○	–	–	–	–
Nucleic acid precursors		○	–	–	–	–
Chelators		–	–	○	–	○
Antibiotic agents		○	○	○	○	○
pH indicators****		○	○	○	○	○
Serum		○	–	–	–	–
Serum albumin		–	○	○	○	○

*Energy substrate concentrations in currently commercially available sequential media and single media are reported by Morbeck *et al.* [97]. **Amino acid concentrations in sequential media and single media are according to Eagle's MEM amino acids [87] (Table 3). ***Although not present in G1/G2 medium [216], these may be included in recent sequential media. ****Some manufacturers formulate embryo culture media without a pH indicator.

De Kretzer *et al*. [47], Kubo [48], and Lopata *et al*. [49, 50] made advances in the investigation of human IVF embryo transfer (ET) using Ham's F-10 medium, in 1978, Edwards and Steptoe [26] were the first to succeed.

The composition of Ham's F-10 medium is complex [51] (Table 2). It was originally developed in 1963, not for embryo culture, but for the culture of Chinese hamster ovary cells. Edwards *et al.* began studying human embryo culture using Ham's F-10 after the success of Daniel and Olson with rabbit embryos [52]. However, it was shown that hypoxanthine and trace elements included in Ham's F-10 medium had deleterious effects on embryos because they induce the production of reactive oxygen species (ROS) [53–56], and the use of this medium for human embryo culture was gradually discontinued. Around that time, in addition to Ham's F-10, a variety of media were used in attempts to culture human embryos, including Earle's solution with pyruvate [57], mouse embryo culture media, and a modification of Tyrode's solution known as T6 medium [58]. However, none of these media generated satisfactory results. Furthermore, although some positive results were obtained from the coculture of somatic cells of oviduct and uterine origin [59–61], this strategy did not become popular because of issues of complexity and reproducibility.

Overcoming the 2-cell block

After the successes of Whitten, the M16 medium [62], Biggers–Whitten–Whittingham (BWW) medium [63], and Hoppe and Pitts' medium [64] were developed (Fig. 1). However, with the exception of embryos from a few outbred or hybrid strains of mice, the 2-cell block could not be overcome. Phenomena resembling the 2-cell block were found at the 8–16-cell stage in bovine and sheep embryos, at the 4–8-cell stage in porcine and human embryos, and at the 2-cell stage in hamster embryos [65, 66]. Various attempts were made to identify ways of breaking through this block.

In 1977, Abramczuk *et al.* [67] found that the culture of fertilized mouse eggs on a single-layer sheet of somatic cells resolved the 2-cell block problem. In their search for causal agents, they found that the addition of ethylenediaminetetraacetic acid (EDTA), a divalent cation chelating agent, was effective at overcoming the 2-cell block in ICR and C57BL/6 mouse embryos. In 1988, Schini and Bavister [65] showed that the 2-cell block in hamster embryos was caused by glucose and phosphate. Based on these reports, Chatot *et al.* [68] developed Chatot–Ziomek–Bavister (CZB) medium with EDTA, substituted glutamine for glucose as a supplement for BMOC2, and eliminated phosphate. This medium enabled the culture of a variety of embryos from various strains of mice for

Table 3. Comparison of amino acid concentrations in Eagle's MEM and in embryo culture media (μmol/L)

		1959	1995		2002
		Eagle's MEM amino acids	Sequential media		Single medium
			G1	G2	Global
Essential amino acids	Arginine	600	0	600	300
	Cystine	100	0	100	50
	Glutamine	2000	1000	1000	1000*
	Histidine	200	0	200	100
	Isoleucine	400	0	400	200
	Leucine	400	0	400	200
	Lysine	400	0	400	200
	Methionine	100	0	100	50
	Phenylalanine	200	0	200	100
	Threonine	400	0	400	200
	Tryptophan	50	0	50	25
	Tyrosine	200	0	200	100
	Valine	400	0	400	200
Nonessential amino acids	Alanine	100	100	100	50
	Asparagine	100	100	100	50
	Aspartate	100	100	100	50
	Glycine	100	100	100	50
	Glutamate	100	100	100	50
	Proline	100	100	100	50
	Serine	100	100	100	50
Other amino acid	Taurine	0	100	0	0

Amino acid concentrations in currently commercially available sequential media and single medium are reported by Morbeck *et al.* [97]. *As a glycyl-glutamine supplement [217].

which the 2-cell block had been confirmed, but when developing "blocking strain" embryos from the morula to blastocyst stage, it was necessary to add glucose [69].

Development of media mimicking in vivo environments (the "back to nature" approach)

From about 1970, progress was made on compositional analyses of oviduct fluid and uterine fluid based on the "back to nature" approach. Synthetic oviduct fluid medium [70] based on sheep oviduct fluid, B2 medium [71] based on bovine oviduct/uterine fluid, HTF [28] based on human oviduct fluid, mouse tubal fluid medium [72] based on mouse oviduct fluid, and porcine zygote medium [73, 74] based on porcine oviduct fluid were developed, among others. Of these, B2 is also known as the "French medium" [75] because it was widely used in France in the early days of human ART.

The composition of HTF is simple (Table 2). It is composed of only inorganic salts, glucose, pyruvate, lactate, human serum albumin (HSA), and antibiotics. HTF was widely used in ART because of its ease of preparation and management. The composition of HTF was proposed by Quinn in reference to the analysis of data from Lippes *et al.* [76] and Borland *et al.* [77] for inorganic salts and glucose in human oviduct fluid. However, the composition of HTF was dissimilar to the published analyses of the composition of oviduct fluid [27].

After the introduction of HTF, glucose and phosphate were reported as potentially toxic, not only for hamster and mouse embryos, but also for human cleavage-stage embryos [78, 79]. Therefore, Basal XI HTF [79]—a modified HTF medium wherein, as with CZB, glutamine was substituted for glucose, phosphate was eliminated, and EDTA was added—was developed. Preimplantation stage-1 (P1) medium [80] was also introduced, in which citrate and taurine, respectively, were substituted for EDTA and glutamine in Basal XI HTF. Nevertheless, blastocyst formation rates with these media were not fully satisfactory, and during this time, cleavage-stage embryo transfer was the main method of ART. In subsequent studies, it was found that amino acids, vitamins, and EDTA supplementation ameliorated the toxicity of

Table 4. Characteristics of sequential media and single medium

	Sequential media	Single medium (renewed at Day 3)	Single medium (nonrenewable)
Environment changes at time of medium exchange (changes in medium composition, pH, temperature, gas phase, and exposure to light)	Large	Medium	None
Autocrine factors	Eliminated with medium exchange	Eliminated with medium exchange	Accumulated
Waste and toxic materials	Eliminated with medium exchange	Eliminated with medium exchange	Accumulated
Number of embryo culture media requiring management	2	1	1
Relative cost	High	Medium	Low
Labor of embryologists	High	Medium	Low
Continuous observation by time lapse from 1 cell to blastocyst	Complicated	Complicated	Simple
Changes in medium composition and culture environment during cultivation period	Simple	Complicated	Complicated

glucose for cleavage-stage embryos [81, 82], and the toxicity did not occur so long as the medium composition was appropriate [83, 84]. Thus, media developed from P1 once again included glucose.

Gardner's group [85] analyzed concentrations of glucose, pyruvate, and lactate in human oviduct and uterine fluids and found that the energy substrate concentrations differed, corresponding with changes in the nutritional demand of the cleavage- and blastocyst-stage embryos. Gardner and Lane [81, 86] also reported that adding amino acids to embryo culture media had positive effects, despite essential amino acids in Eagle's minimum essential medium (MEM) [87] inhibiting the development of cleavage-stage embryos, and EDTA inhibiting the development of blastocyst-stage embryos. Based on these results, Gardner's group [29] developed G1 and G2 as original sequential media in 1995 (Tables 2 and 3), in which energy substrates, amino acids, and EDTA compositions were sequentially modified from the cleavage stage (days 1–3) to the blastocyst stage (days 3–5/6). These media improved blastocyst formation rates, and blastocyst transfer became popular.

"Back to nature" approaches, including HTF and sequential media, are theoretically impressive. However, they have not been exhaustively analyzed and only a portion of the components in each medium reflects the concentrations *in vivo*, and analyses of human oviduct fluids have shown considerable differences [27]. Additionally, an embryo in culture generally experiences stresses unique to the culture environment that it would not experience in the reproductive tract (such stresses include exposure to light and high oxygen concentrations in the atmosphere, sudden changes in pH and temperature, and the accumulation of waste materials in the culture medium). Thus, it cannot be stated that mimicking, *per se*, *in vivo* compositions is necessarily the optimal approach.

Development of media using sequential simplex optimization (the "let the embryo choose" approach)

Within the National Cooperative Program on Non-Human *In Vitro* Fertilization and Preimplantation Development (called the "Culture Club"), in the United States, Biggers' group started to investigate optimized media via the use of a statistical approach, called the simplex optimization method. This method uses a computer-assisted algorithm to search the highest point of a concentration–response surface. Lawitts and Biggers [88] optimized the concentrations of 10 components (NaCl, KCl, KH_2PO_4, $MgSO_4$, lactate, pyruvate, glucose, BSA, EDTA, and glutamine) of CZB simultaneously using blastocyst formation rates *in vitro* for the response. In 1992, Lawitts and Biggers [89] reported simplex optimized medium (SOM) with reduced CZB concentrations of NaCl, KH_2PO_4, pyruvate, and glucose, and in 1993, KSOM [30] with increased potassium concentration. Ho *et al*. [31] then developed $KSOM^{AA}$ supplemented with 0.5×MEM essential and nonessential amino acids and reported improved blastocyst formation rates and cell numbers. KSOM and $KSOM^{AA}$ are effective not only for the culture of mouse embryos but also for bovine [90–92], rabbit [93, 94], pig [95], monkey [96], and human embryos [32]. $KSOM^{AA}$ is

widely used under the trademark Global (Tables 2 and 3) in human ART, and, other single media similar to Global have become commercially available [97]. Initially, medium exchange was necessary every 48 h with a single medium to eliminate waste produced by embryos and degradation products of medium components. However, more recently, media that do not require medium exchange from the fertilization to the blastocyst stage have become available [98].

MEM essential amino acids are not added to cleavage media of sequential media because they have deleterious effects on cleavage-stage embryos [86]. However, in studies using KSOM, such negative effects were not observed [31]. Furthermore, essential amino acids exist in the reproductive tract [92, 99–102], and are thought to be involved in imprinting and important reactions such as glutathione synthesis [103]; therefore, essential amino acids are added to some cleavage media that are currently being used [97]. EDTA—considered to have deleterious effects on blastocyst development [81] and removed from sequential media used for the blastocyst stage—does not present issues when used at appropriate concentrations [104]. Currently, about 10 *μ*M of EDTA is added to single media.

Comparison of Sequential Media with Single Medium

Comparative studies of sequential media and single media have been performed globally, but no conclusion has yet been reached as to which of the two is better [105, 106]. The best approach when selecting an embryo culture medium may be to understand the characteristics of the two types (Table 4) and choose the one best suited to each laboratory.

The advantage of a single medium is that there is no need to change the medium composition during the progression from the cleavage stage to the blastocyst stage, as is necessary with sequential media. Because there is only a single embryo culture medium that needs to be managed, this means reduced labor and costs and the avoidance of stresses on embryos that may occur when changing medium composition. A single medium, which requires no medium change, means that one can avoid the changes in pH, temperature, gas phase, and exposure to light that occur during the medium change. Moreover, the embryo does not lose the autocrine factors that it produces. The use of commercially available time-lapse devices in recent years has enabled continuous, seamless observations from the cleavage stage to the blastocyst stage. Thus, so long as the accumulation of waste and degradation products is tolerable, there are many advantages in using a single medium that does not require changing. Conversely, when aiming to deliberately change the medium composition and culture environment at the cleavage and blastocyst stage, respectively, sequential media offer benefits over a single medium. For example, the optimum pH of a medium may differ in the cleavage and blastocyst stages [107]. With a single medium, transfer of the medium to a separate incubator having a different CO_2 concentration, or change of incubator CO_2 concentrations, is needed for pH adjustment. However, with sequential media, if the manufacturer has prepared the medium so that the bicarbonate concentration is adjusted to provide the optimum pH at each stage, there is no need to change the incubator CO_2 concentration.

Issues with Current Embryo Culture Media

As distinct from somatic cells, mouse and human embryos can be developed from zygote to blastocyst in a simple medium composed of inorganic salts and energy substrates. In the early period, successful embryo culture was performed in media without amino acids or vitamins (for example, M16, BWW, and HTF). Later, the importance of amino acids was recognized, and they were added to embryo culture media (Table 2). Vitamins and/or growth factors and hormones are added to some embryo culture media, but we currently have insufficient knowledge of the appropriate concentrations and combinations of these substances or of the best time to add them to a culture. There are also cases where there is a variety of impurities in the medium in *in vitro* environments despite the best efforts of embryologists. Thus, impurity control is another key issue.

Amino acids and vitamins

Amino acids have a wide range of functions. They act as energy sources, precursors of biosynthesis, intracellular pH buffers, osmolytes, chelators, and antioxidants [108]. Adding amino acids to embryo culture media is generally known to improve embryo viability and enhance blastocyst formation rates [27, 108, 109]. Vitamins generally serve as precursors to coenzymes and are involved in carbohydrate, lipid, and amino acid metabolism, but these effects on embryo culture are unclear. O'Neill [110] reported that folate is necessary for the development of embryos from the zygote to the blastocyst stage, but folate is provided from the intracellular stock of the embryo itself and adding folate to a medium has been found to have no effect. The effects of inositol, nia-

cin, and vitamin B6 differ depending on the animal species [111–113], and as well as certain positive effects, their deleterious effects have also been demonstrated [111, 112]. Furthermore, the effects of vitamins within human embryo culture are still largely unknown [114], and some vitamins are unstable, making them difficult to use [115–117], meaning there are many examples of embryo culture media without vitamins.

The combinations and concentrations of amino acids and vitamins used for embryo culture media are, in most cases, based on the results of somatic cell experiments presented by Eagle in the 1950s [87, 118]. Eagle used human-derived HeLa cells and mouse-derived L cells to study the minimum substance requirements of somatic cells. He found that 13 amino acids and 8 vitamins were required, and developed MEM. The amino acids and vitamins included in this medium are called "MEM essential amino acids" and "MEM essential vitamins." These are packaged in the form of an easy-to-use concentrated solution and have been widely used to assess the effects of amino acids and vitamins on embryos [82]. Thus, the combinations and concentrations of amino acids used in current sequential and single media are, in most cases, based on MEM [97] (Table 3), yet they have never been optimized for embryos.

Optimizing 20 amino acid concentrations within an embryo culture medium using a statistical approach would require the use of huge numbers of embryos [27], which is not realistic. Specific amino acids such as glycine, alanine, glutamate, and taurine are found at higher concentrations in the oviduct fluid and uterine fluid of mice [101], rabbit [100], bovine [92], sheep [102], and pig species [99] than in serum. These higher concentrations have been reported to promote embryo development better than the amino acid concentrations set for somatic cells [119, 120]. In the future, media may be developed based on the amino acid concentrations from studies of human oviduct fluid and uterine fluid collected in their physiological state.

Growth factors and hormones

Growth factors and hormones are extremely important in the control of cell division, cell growth, and cell differentiation. In the case of somatic cells, the key to a successful cell culture without serum is based on setting growth factors and hormones in optimal combinations and concentrations [121, 122]. By contrast, most embryo culture media do not include these substances because embryos can develop to the blastocyst stage without any growth factors or hormones *in vitro*. While it is not clear to what extent the absence of growth factors and hormones negatively impacts cultured embryos, several deleterious effects have been indicated in comparison with embryos derived *in vivo*, including lower development rates, slower growth (bovine [123, 124] and hamster [125]), decrease in blastocyst cell numbers (mouse [126–129], pig [95, 130], and rat [131]), higher oxidation stress (mouse [132, 133]), metabolic changes (mouse [72, 134, 135] and rabbit [136]), changes in gene and protein expression (mouse [129, 137–140], bovine [141–143], and sheep [144]), changes in intracellular structure and increased lipid droplets (bovine [145–147]), decline in successful implantation and pregnancy rates (rat [131]), and changes in birth weights (human [148]). The absence of growth factors and hormones may account for some of these differences.

Within the female reproductive tract, an embryo develops interactions with the mother's body. This "embryo–mother communication" involves growth factors and their receptors [149, 150] (Table 5). For example, in the human reproductive tract, including the oviducts and uterus, various growth factors are expressed, such as epidermal growth factor (EGF) [151], granulocyte-macrophage colony stimulating factor (GM-CSF) [152], heparin-binding epidermal growth factor (HB-EGF) [153–155], insulin-like growth factor (IGF)-1 [156–160], and leukemia inhibitory factor (LIF) [161–165]. Human oocytes and embryos express receptors for these growth factors on their cell surfaces [158, 166–172]. Various positive effects have been reported resulting from the addition of such growth factors to human embryo culture media, including accelerated developmental speed, improved blastocyst formation rates, and increased numbers of blastocyst cells [159, 173–178].

In human ART, it has currently become common to use a medium supplemented with insulin or GM-CSF. In the mother's body, growth factors and hormones do not simply work alone; complex combinations of multiple growth factors and hormones affect an embryo at different development stages. Additionally, minute amounts of growth factors can have large impacts on cell growth and differentiation. Therefore, their concentrations must be set with careful consideration. Hopefully, more detailed studies will be performed to establish the optimal concentrations and combinations of growth factors and hormones, and the results will be reflected in media compositions. Following recent progress in analysis technologies [179], it may become possible to perform exhaustive analyses of physiological growth factors and hormones in the reproductive tract.

Table 5. Growth factors expressed within the human female reproductive tract and at the oocyte/embryo surface, and their effects on human embryos

	Expression of the growth factor within the human reproductive tract					Expression of the receptor by human oocytes/embryos					Supplementation of the human embryo culture media with growth factor	
	Follicular fluid	Cumulus/granulosa cells	Oviduct	Endometrium	Ref.	Oocyte	2–4-cell stage	6–8-cell stage	Blastocyst	Ref.	Beneficial effects	Ref.
EGF				○	[151]	○	○	○	○	[158, 166]	Increased plasminogen activator activity	[174]
GM-CSF			○	○	[152]		○	○	○	[171]	Increased developmental speed and number of blastocyst cells, improved blastocyst formation and hatching rates	[176]
HB-EGF			○	○	[153–155]	○	○	○	○	[169, 170]	Improved blastocyst formation and hatching rates	[175]
IGF-1	○	○		○	[156–160]	○	○	○	○	[158, 168]	Improved blastocyst formation rates, increased number of ICM cells, decreased apoptosis of blastocysts	[159, 178]
LIF		○	○	○	[161–165]	○	○	○	○	[167, 172]	Improved blastocyst formation rates	[173, 177]

Embryo culture medium contamination

While mouse and human embryos can develop into blastocysts in a protein-free medium [37, 180], the addition of HSA and other biogenic macromolecules is thought to improve blastocyst formation rates, hatching rates, blastocyst cell numbers, and implantation rates [181–184]. Thus, almost all human embryo culture media are supplemented with HSA or a serum substitute (HSA including α- and *β*-globulin) [185, 186].

HSA is the most abundant protein in the blood, accounting for 50%–70% of all serum proteins, and it binds with and transports a variety of molecules. For example, because long-chain fatty acids—reported to be used as an energy source and for the biosynthesis of lipids in an embryo [181, 187, 188]—have low aqueous solubility (typically <1 *μ*M), most are bound to HSA and transported within the blood [189]. Many other materials are bound to HSA and transported, including endogenous substances such as transition metals, amino acids, vitamins, and hormones, and exogenous compounds such as drugs and dyes [190]. In addition to these materials that bind to HSA, the commonly used HSA fraction V undergoes only partial purification using an ethanol fractionation procedure called Cohn's method, and thus includes a variety of serum-derived contaminants (impurities) [191]. For example, HSA contains at least 100 types of proteins (fragments) [192], of which some have been reported to have positive effects on an embryo, including growth factors and hormones such as vascular endothelial growth factor, IGF-2, and insulin [193]. Meanwhile, HSA may also contain substances that have negative effects on animal tissues such as endotoxins [194] and phthalates [195]. The problem is that there is no control of the types and concentrations of such albumin-binding substances and contaminants except endotoxins, with major variations among various product manufacturers and even among batches produced (fatty acids (Table 6), growth factors and hormones [193], endotoxins [194], and phthalate [195]). Thus, current embryo culture media cannot be considered as chemically defined media (Table 1), although they are serum-free media, and one can obtain scattered results from supplemented albumin [196–201]. A recent report suggested that depending on the HSA lot, the phenotype of the offspring may change [202]. Furthermore, although adequate viral testing is performed, one cannot exclude the possibility that unknown viruses may be present [203]. To increase the safety of ART and to obtain stable results, chemically defined media free of HSA or serum substitutes must be developed. To accomplish this, researchers must determine why HSA acts

Table 6. Fatty acid levels of HSA products (M/M albumin)

Manufacturer	A		B	C	D
Lot no.	I	II	I	I	I
Octanoate*	4.50	5.40	4.56	4.74	4.88
Laurate	0.00	0.00	0.00	0.00	0.00
Myristate	0.01	0.00	0.00	0.00	0.01
Palmitoleate	0.02	0.00	0.00	0.00	0.02
Palmitate	0.18	0.02	0.02	0.03	0.18
Linoleate	0.14	0.02	0.02	0.00	0.13
Oleate	0.19	0.01	0.01	0.00	0.18
Linoleate	0.01	0.00	0.00	0.00	0.03
Stearate	0.04	0.00	0.00	0.00	0.06
Arachidonate	0.01	0.00	0.00	0.00	0.01
Eicosapentaenoate	0.00	0.00	0.00	0.00	0.00
Docosahexaenoate	0.01	0.00	0.00	0.00	0.00

(Yao *et al.*, unpublished data) *Octanoate (synonym=caprylate) is used as a stabilizer [191, 218].

effectively on embryo development.

Potential risks remain for various medium contaminants, other than albumin, in culture environments, including toxic substances leached from filters used for sterilization, mineral oil [204–206], degradation products of components of the medium [207], and ROS generated by light irradiation [56, 208, 209]. To stably obtain high quality blastocysts *in vitro*, establishing chemically defined media will not be sufficient, as researchers will also need to strive to optimize the culture system as a whole. This will include establishing strict quality assessment methods for filters, mineral oil, and products that come into contact with embryo culture media, detailed investigations of the optimal numbers of embryos in the culture and the timing of medium exchanges, and the development of novel culture platforms [210].

Conclusions

Compared with the earliest days of human ART, there have been vast improvements in blastocyst formation and pregnancy rates [211, 212], and culture environments have generally reached a satisfactory level. However, differences in embryo culture media used in human ART result in differing offspring body weights [148], and it has been suggested that extended culture times for human embryos are associated with premature births and congenital anomalies [213]. Thus, it is wise to accept that culture environments remain suboptimal.

The embryo culture media that are currently used have not been optimized in terms of amino acid concentrations suited to the demands of an embryo, and the significance of adding vitamins remains unclear. Clinical use of human embryo culture media supplemented with growth factors and hormones is in its infancy. In the future, the types and concentrations of components to be added to the medium and their different combinations should be investigated. While only a limited number of embryos can be used for research, the number of possible medium composition types and combinations is nearly infinite. Thus, haphazard studies are unlikely to lead to the development of optimal media. It will be necessary to first establish methods of quantitative assessment that can clearly differentiate between normal and abnormal embryos (for example, using live-cell imaging technologies [214]). In addition, the accuracy of the “back to nature” and “let the embryo choose” approaches must increase. Combining these approaches will be efficacious for assessing which medium compositions are optimal for embryos.

Human ART has shown remarkable progress, and is now widespread. Globally, over one million cycles are performed in treatments every year [215]. Even a slight improvement in results would bring immense benefits. Therefore, progress must be made in studies aiming to establish optimal culture environments.

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