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REVIEW ARTICLE

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Animal-cell culture media: History, characteristics, and current issues

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Abstract

Background: Cell culture technology has spread prolifically within a century, a variety of culture media has been designed. This review goes through the history, characteristics and current issues of animal-cell culture media.

Methods: A literature search was performed on PubMed and Google Scholar between 1880 and May 2016 using appropriate keywords.

Results: At the dawn of cell culture technology, the major components of media were naturally derived products such as serum. The field then gradually shifted to the use of chemical-based synthetic media because naturally derived ingredients have their disadvantages such as large batch-to-batch variation. Today, industrially important cells can be cultured in synthetic media. Nevertheless, the combinations and concentrations of the components in these media remain to be optimized. In addition, serum-containing media are still in general use in the field of basic research. In the fields of assisted reproductive technologies and regenerative medicine, some of the medium components are naturally derived in nearly all instances.

Conclusions: Further improvements of culture media are desirable, which will certainly contribute to a reduction in the experimental variation, enhance productivity among biopharmaceuticals, improve treatment outcomes of assisted reproductive technologies, and facilitate implementation and popularization of regenerative medicine.

KEYWORDS

cell culture technique, cell proliferation, culture media, cultured cells, serum

1 | INTRODUCTION

The influence of cell culture technology on human society has been immeasurable. Progress in biology in recent years, for example, has depended heavily on cell culture technology.[1] In addition, cell culture-based practical technologies have been developed in various areas, including the assessment of the efficacy and toxicity of new drugs, manufacture of vaccines and biopharmaceuticals, and assisted reproductive technology. As the reprogramming of somatic cells became technically feasible recently, researchers around the world are fiercely competing for leadership in the advances of regenerative medicine. In this area likewise, cell culture technology is regarded as a foundation for further development and popularization.

No one probably would argue against the claim that a culture medium is the most important factor in cell culture technology. A medium supports cell survival and proliferation, as well as cellular functions, meaning that the quality of the medium directly affects the research results, the biopharmaceutical production rate, and treatment outcomes of assisted reproductive technology. It is essential, therefore, for investigators who are working with cell cultures to select an appropriate medium that is suitable for their aims. In some

TABLE 1 Categories of animal-cell culture media

Category	Definition	Type	Example
Natural media	Consisting of natural biological substances, such as plasma, serum, and embryo extract	Coagulant or clots	Plasma separated from heparinized blood, serum, and fibrinogen
		Tissue extracts	Extracts of chicken embryos, liver, and spleen and bone marrow extract
		Biological fluids	Plasma, serum, lymph, amniotic fluid, and pleural fluid
Synthetic media	Composed of a basal medium and supplements, such as serum, growth factors, and hormones	Serum-containing media	Human, bovine, equine, or other serum is used as a supplement
		Serum-free media	Crude protein fractions, such as bovine serum albumin or α- or β-globulin, are used as supplements
		Xeno-free media	Human-source components, such as human serum albumin, are used as supplements but animal components are not allowed as supplements
		Protein-free media	Undefined components, such as peptide fractions (protein hydrolysates) are used as supplements
		Chemically defined media	Undefined components, such as crude protein fractions, hydrolysates, and tissue extracts, are not appropriate as supplements, but highly purified components, such as recombinant proteins are appropriate supplements

cases, researchers should modify a medium themselves. In addition, when facing problems, researchers have to know the properties of the medium in order to identify the cause of any problem with their experiments.

At present, synthetic media can be classified into several groups, based on the type of supplements added; for example, serum-containing media, serum-free media, protein-free media, and chemically defined media (Tables 1 and 2). Serum-containing media naturally contain various serum-derived substances, which make the medium composition unclear and whose concentrations can fluctuate from batch to batch. This situation makes the culture results less reproducible and poses a risk of microbial contamination. Serum-containing media, however, can be designed easily and be used effectively for a variety of cell types because serum includes a lot of active substances that are necessary for the survival and growth of animal cells.[2] Serum-free media, in contrast, have a defined composition, resulting in a high reproducibility of results, and the cultivation process can be validated. In addition, target cells can be grown selectively in an intermingled cell population if the culture conditions are configured to benefit them. Among the serum-free media, subgroups of protein-free media (which do not contain any protein at all) and chemically defined media (which do not contain any undefined ingredient) provide additional stability and reproducibility for culture systems, facilitating the identification of the cellular secretions and reducing the risk of microbial contamination. However, the serum-free media are difficult to design: only specific cell types have been cultivated this way to date.[3]

At present, formulations of popular media for cell culture are available commercially. Thus, there are investigators who are using culture media without understanding their details and background, particularly regarding the rationale for their development, the exact ingredients, as well as the cell types that these media are suitable for. This review article briefly describes the history of the development of animal-cell culture media, with comments on the types of media in general use today regarding their characteristics, roles of the medium components, and pitfalls or problems with their use.

2 | HISTORY OF CELL CULTURE MEDIA

2.1 | Dawn of cultivation experiments (1882–1907)

In 1882, Sydney Ringer developed Ringer's solution, a balanced salt solution of a composition that is close to that of bodily fluids, and successfully kept frog hearts beating after dissection and removal from the body.[4,5] This is said to be the first instance of in vitro cultivation of animal tissue. Balanced salt solutions were developed one after another in the wake of Ringer's report, including Locke's solution,[6] Tyrode's solution,[7] the Krebs–Ringer bicarbonate solution,[8] Gey's solution,[9] Earle's solution,[10] and Hanks' solution.[11] The composition of these balanced salt solutions is simple and includes only inorganic salts, sometimes with glucose added as a nutrient. Nonetheless, their pH, osmotic pressure, and inorganic salt concentrations were calibrated to physiological conditions and these solutions can be used successfully to keep tissues and cells outside the body alive for short periods, generally up to a few days.

After the success of Ringer's solution, researchers began to pay attention to cells in culture devices and tried to maintain the cells. Nonetheless, the cells usually did not survive and rarely showed mitotic figures.[12–14] In 1907, however, Ross G. Harrison successfully monitored an apparent outgrowth of nerve fibers of a frog for several weeks in lymph fluid that had been freshly drawn from the lymph sacs of an adult frog.[15] This experiment is considered to be the beginning of animal cell cultivation.

TABLE 2 Types and characteristics of basal media

Category	Name (author, year)	Features
Connaught Medical Research Laboratories (CMRL) media	Medium 199 (Morgan et al. 1950)	Developed in order to cultivate chicken embryonic cells under protein-free conditions, it is prepared by the sequential addition of amino acids, vitamins (including fat-soluble vitamins), and nucleic-acid precursors. Its composition is extremely complex because the components that are thought to be necessary on theoretical grounds, including inactive components, are added to the medium. Often used for organ culture
	CMRL1066 (Parker et al. 1957)	Created by amending Medium 199 in order to culture mouse L cells under protein-free conditions, the modifications included increased levels of reducing substances (cysteine, glutathione, and ascorbic acid), the elimination of fat-soluble vitamins, changes in the types of nucleic-acid precursors, and the addition of coenzymes
Eagle media	Basal medium Eagle (BME) (Eagle 1955)	Supplemented with the minimal components that are necessary for mouse L cells and human HeLa cells to reach the index of proliferative capacity and including 13 amino acids and eight vitamins, it is unsuitable for cells whose cultures require many components because of its simple composition
	Minimum essential medium (MEM) (Eagle 1959)	BME modified according to the cellular need for amino acids, so the concentrations of the majority of amino acids are twofold, as compared to BME. Non-essential amino acids, which cells can biosynthesize, were not included in the original MEM formulation; however, researchers can add non-essential amino acids to reduce the biosynthetic load
	Dulbecco's modified MEM (DMEM) (Dulbecco and Freeman 1959)	Modified to have fourfold the concentrations of amino acids and vitamins that are present in BME and developed to study the plaque-forming ability of the polyoma virus in mouse embryonic cells. Various modifications have been made since, with supplementation, for example, of the non-essential amino acids, glycine and serine, iron, and pyruvate. The glucose concentration also can be increased to 25 mmol L^{-1} in order to accommodate cells with high nutritional requirements. In the event that pH changes are suspected due to metabolites, sodium bicarbonate is doubled in concentration, equilibrated, and then used at 10% CO_2
	α-MEM (Stanners et al. 1971)	MEM modified for hybrid-cell-line research on mice and hamsters, its composition is MEM supplemented with non-essential amino acids and vitamins (ascorbic acid, biotin, and cyanocobalamin), pyruvate, lipoic acid, and nucleosides
	Iscove's modified DMEM (IMDM) (Iscove and Melchers 1978)	Supplemented with several non-essential amino acids and vitamins that are not present in DMEM (cyanocobalamin and biotin) and additional selenite, pyruvate, and HEPES. Transferrin, bovine serum albumin, and soybean lipids are added as serum substitutes. With its high concentrations of amino acids and vitamins, IMDM is suitable for high-density cultures and cultures of rapidly proliferating cells
Tissue Culture Section of the National Cancer Institute (NCTC) media	NCTC109 (McQuilkin et al. 1957)	Developed to culture L cells under protein-free conditions, its amino acid composition is based on the results of a componential analysis of the compounds that have been ultrafiltered from horse serum and chicken embryonic-tissue extract. Its composition is quite complex, with not only coenzymes, nucleobases, and reducing agents, but a wealth of vitamins added, as well (ie, A, C, D, E, and K, in addition to the B-group vitamins). Cysteine was included in the original composition, but after it was found to negatively affect cells, a version was developed from which cysteine was removed (NCTC135)
Ham media	Ham's F-10 (Ham 1963)	Enables colony formation by a single Chinese hamster ovary (CHO) cell under serum-free conditions, developed by adding two kinds of purified serum proteins (serum albumin and fetuin), instead of serum, and by examining in detail the types and concentrations of amino acids and trace elements This medium was the first to contain the trace elements, copper and zinc (iron having been included in other media already). The CHO cells have an inferior proliferative capacity in this medium alone, compared to the one with serum added. Furthermore, culture of the cell lines other than CHO necessitates the addition of serum
	Ham's F-12 (Ham 1965)	The serum albumin and fetuin (used in Ham's F-10) are replaced by two compounds with definite chemical composition: linoleic acid and putrescine, which enables colony formation by a single CHO cell under protein-free conditions. Often cited as the world's first chemically defined medium, the levels of several amino acids are higher than in Ham's F-10, while those of the vitamins (except choline and inositol) and potassium phosphate are reduced. Its composition must be modified (eg, by reducing the zinc concentration) for a protein-free culture of cells other than CHO cells. MCDB301, in which 20 trace elements were added, was later developed after it became obvious that trace elements contaminating water or raw materials are necessary for a protein-free culture of CHO cells in Ham's F-12.
	Kaighn's modified Ham's F-12 (Ham's F-12K) (Kaighn 1974)	The concentrations of the amino acids, pyruvate, biotin, calcium, magnesium, putrescine, and phenol red are increased with respect to those in Ham's F-12, among other compositional modifications, in order to support the proliferation and differentiation of primary cultured cells

(Continues)

TABLE 2 (Continued)

Category	Name (author, year)	Features
Roswell Park Memorial Institute (RPMI) media	RPMI 1640 (Moore et al. 1966)	Based on the 5A Medium (developed by McCoy et al. 1959) and modified for the long-term culture of peripheral blood lymphocytes, it is characterized by low levels of calcium and magnesium and high levels of phosphate. Multiple media were developed on the path to this medium (eg, RPMI 1629, 1630, and 1634). It is widely used as a medium for suspension cultures; for example, of white blood cells, lymphocytes, and hybridomas
Molecular, Cellular, and Development Biology (MCDB) media	For example, MCDB202 (McKeehan et al. 1976) MCDB301 (Hamilton et al. 1977) MCDB153 (Peehl et al. 1980) MCDB110 (Bettger et al. 1981) MCDB402 (Shipley et al. 1983) MCDB170 (Hammond et al. 1984) MCDB131 (Knedler et al. 1987)	A series of Ham's F-12-based media that was developed in order to grow specific cell types in serum-free culture. The composition of each MCDB medium is optimized to promote the growth of a specific cell type. In particular, MCDB202 is a medium for the serum-free culture of chicken embryo fibroblasts; MCDB301 is for the serum-free culture of CHO cells; MCDB153 is for human keratinocytes; MCDB110 is for human fibroblasts; MCDB402 is for mouse fibroblasts; MCDB170 is for the mammary epithelium; and MCDB131 is a medium for the serum-free culture of human vascular endothelial cells
Mixed media	DMEM/F-12 (Barnes and Sato 1979)	Constitutes a 50:50 mixture of component-rich Ham's F12 medium and the nutrient-rich DMEM medium and compatible with the requirements of a variety of cell types, it is most often used as a basal medium for serum-free culture
	RPMI 1640/DMEM/F-12 (RDF) (Murakami 1984)	Developed for the serum-free culture of hybridomas, it is a mixture of RPMI 1640, DMEM, and Ham's F-12 at 2:1:1 and is typically used as a serum-free medium with added insulin, transferrin, ethanolamine, and selenite
Other media	Waymouth's MB752/1 (Waymouth 1959)	Developed with as simple a composition as feasible, so that mouse L929 cells could be cultured without the addition of serum and other proteins, it is composed of a total of 40 components, including glucose, inorganic salts, amino acids, vitamins, purine bases, and hypoxanthine. It is characterized by high concentrations of glucose, histidine, lysine, glutamine, choline, and thiamine
	Trowell's T-8 (Trowell 1959)	Designed for the long-term culture of adult rat liver epithelial cells. With its comparatively simple composition, it contains no non-essential amino acid and hardly any vitamins, but is characterized by high glucose and insulin concentrations. It is used for short-term organ culture
	Leibovitz's L-15 (Leibovitz 1963)	The buffering capacity is mediated by phosphates and free basic amino acids instead of sodium bicarbonate, so that a culture's pH is maintained in ambient air without the use of a CO_2 incubator. Instead of glucose, pyruvate (and galactose) is added at a high concentration in order to control pH drops due to the lactic acid that is produced during glucose metabolism and to promote the release of CO_2 from the respiratory chain. Another major characteristic is that each of its constituent amino acids is added at approximately its respective maximal concentration. Once used for cell and tissue transport and primary cultures, its popularity has declined as researchers began to use HEPES as a buffering agent and as they realized, moreover, that a certain amount of sodium bicarbonate is necessary for optimal cell proliferation
	Fischer's Medium (Fischer and Sartorelli 1964)	Contains a high concentration of folate because this medium was developed by using folate-dependent L5178Y lymphoma cells

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2.2 | Use of natural media (1907-)

Alexis Carrel was a French surgeon and biologist who received the Nobel Prize in Physiology and Medicine in 1912 for his research on the vascular suture and transplantation of blood vessels and organs. He contributed greatly to tissue culture technology by devising a prototype of the cell culture flask that is used widely today and by establishing the aseptic manipulation technique.[16]

The first success of animal cell culture by Harrison inspired Carrel to send Montrose T. Burrows to work under Harrison's supervision in 1909. There, Burrows found that lymph is unsuitable for the cultivation of cells from warm-blooded animals and used plasma instead. Thereafter, blood plasma had become a major culture medium for a variety of animal cells. He successfully cultivated chicken embryonic cells by using chicken blood plasma, which is readily available,[17] and later successfully cultivated mammalian cells as well.[18] In 1912, Carrel demonstrated that the long-term cultivation of the cells that have been obtained from the connective tissues of chick fetuses is possible (for several months) with a periodic exchange of the medium.[19] In 1913, he discovered that adding embryonic extract to blood plasma can dramatically increase cellular proliferation and extend the culture period of fibroblasts from the chick embryo heart.[20,21] Meanwhile, because the composition of the lymph, plasma, and embryonic extract was unknown, it became a new scientific inquiry regarding which of their components affected the survival and growth of animal tissues and cells. This situation led to a period when researchers attempted to identify the growth-promoting substances within these ingredients of natural origin and to replace them with ingredients of definite composition.

Carrel is widely believed to be the first person in the world to successfully culture mammalian somatic cells, but the Biographical Memoirs of the National Academy of Sciences contends that the cultivation of guinea pig bone marrow specimens in 1908 by Margaret Reed, the future Mrs. Warren H. Lewis, precedes his success.[22]

2.3 | Endeavors toward synthetic media (1911-)

Margaret R. Lewis and Warren H. Lewis (1911) demonstrated that the Locke–Lewis solution—which is modified Locke's solution that additionally contains amino acids, bouillon, and glucose (or maltose)—is more effective for chick embryo cell cultivation than simple balanced salt solutions.[23,24] They reported that glucose is especially important: if the concentration of glucose is not sufficient in the medium, the chick embryo cells show vacuolar degeneration and die within a few days.[25] In contrast, in their search for the active ingredients in embryonic extract, it was ascertained that the active substance is in the protein fraction[26] and that the partially hydrolyzed proteins effectively promote the cell growth of chick embryo fibroblasts.[27] Those researchers also confirmed the growth-promoting activity of amino acids[28] and glutathione for chick embryo fibroblasts.[29] They hypothesized that glutathione is required for the control of the redox environment during cell cultivation. Carrel's medium was supplemented with several natural products, such as casein digests (thymus-derived) nucleic acids, liver ash, and hemoglobin. Thus, their research did not lead to the molecular identification of any substance that is essential for cultured cells. Joilannes P. M. Vogelaar and Eleanor Erlichman, in contrast, confirmed the effectiveness of two hormones (insulin and thyroxine) by successfully cultivating human fibroblasts for >3 months in a medium that consisted of Ringer's solution with these hormones, along with glucose, cysteine, hemin, peptone, and blood plasma.[30] Researchers continued the efforts to specify the composition of media thereafter, including the development of Baker's medium, which contains vitamin A, ascorbic acid, vitamin B_1, vitamin B_2, glutathione, and blood plasma.[31] Nevertheless, this approach did not lead to culture media that did not include natural products, such as blood serum or plasma.

2.4 | Birth of established cell lines (1940-)

It is rare for healthy somatic cells that are derived from animals to acquire unlimited proliferative capacity during cultivation. They typically stop growing after a certain number of divisions (ie, the Hayflick limit).[32] As a result, researchers would conduct studies by using cells that had been sampled from animals in each experiment. In 1940, Wilton R. Earle et al. used carcinogens to successfully create immortal mouse fibroblasts (L cells),[10] revealing that proliferation from a single cell is possible.[33] In 1951, George O. Gey and his coworkers created an infinitely proliferating human cell line from a tissue of a patient with uterine cervical cancer (HeLa cells).[34] Due to the emergence of these established cell lines, the sampling of cells from the tissues of animals in each experiment became unnecessary, enabling researchers worldwide to perform assays by using the same homogenous population of cells. This state of affairs made it easier to examine and to precisely quantify the subtle differences in the effects of culture media on cells. Thus, the development of culture media advanced rapidly as a result.

2.5 | Establishment of basal media and research into protein-free media (1946-)

Baker's medium and the other media that had been developed up to this point contained naturally derived components of unknown composition, including plasma, serum, bouillon, peptone, and tissue extracts. In order to find the crucial components in those natural materials and to develop defined media that are comparably efficient in the cultivation of cells, relative to the media containing natural ingredients, two main strategies were undertaken. The first strategy was to use dialyzed serum for the support of cells at minimum levels and to add defined components to maximize the proliferation of cells. The second strategy was not dependent on serum, or even proteins at all, and involved the formulation of media exclusively from definitive components.

Fischer was a pioneer of the first strategy. He dialyzed blood plasma to remove the low-molecular-weight fraction. Culture media that were supplemented with dialyzed blood plasma could sustain cells only for a short period, indicating that the low-molecular-weight fraction was essential for the survival of cells. Then, he discovered that the amino acids are the key substance in the low-molecular-weight fraction.[35,36] This creation of dialyzed media, along with the established cell lines,

made it possible to determine, in a systemic way, whether the cells require low-molecular-weight substances under culture conditions. For example, in 1955, Harry Eagle studied the minimum necessary amounts of low-molecular-weight components that are required by mouse L cells and HeLa cells by using a balanced salt solution with the addition of dialyzed serum, on the basis of Fischer's method.[37,38] He found that 13 amino acids and eight vitamins are necessary.[39] Going on to study the amino acid requirements of a variety of cells, Eagle then developed the minimum essential medium (MEM), which is composed of the minimal essential components that he identified (glucose, six inorganic salts, 13 amino acids, eight water-soluble vitamins, and dialyzed serum).[40] Renato Dulbecco and Marguerite Vogt,[41] Clifford P. Stanners et al.,[42] and Norman N. Iscove and Fritz Melchers[43] made improvements to MEM, according to the desired cell type and culture purpose (Table 2). Later, using dialyzed serum that had been prepared in accordance with Fischer's method, Thomas A. McCoy et al. confirmed that carcinosarcoma cells require pyruvate.[44] At this time, the Roswell Park Memorial Institute (RPMI) modified the 5A medium that had been developed by McCoy,[45] in terms of its calcium and magnesium concentrations. The result then was reported as the RPMI 1640 medium for use in lymphocyte cultivation.[46,47]

The other strategy—the total omission of serum and proteins—is thought to have begun in 1946, with full-scale research by Philip R. White. He developed a chemically defined medium that was composed of glucose, inorganic salts, amino acids, iron, vitamins, and glutathione, with no protein at all. Using this medium, he successfully cultivated chick embryo-derived fibroblasts and cardiac muscle cells for ~2 months.[48] Other researchers were unable to repeat his findings and argued that White's Medium still needed 10%-20% serum for the reported results.[49] In 1950, Connaught Medical Research Laboratories (CMRL), led by Raymond C. Parker, developed Medium 199, which includes fat-soluble vitamins, cholesterol, and nucleic acid precursors, in addition to the ingredients of White's Medium (Table 2). Instead of adding serum or proteins, Parker's team used the strategy of adding as many low-molecular-weight substances as possible, according to what was considered theoretically necessary for cell culture.[50] Using this medium, they cultured chick embryo-derived cells for 3-4 weeks. Serum and embryonic extract were still necessary for the primary culture. Other researchers also reported the necessity of a small amount of serum in Medium 199 for cell proliferation.[37] The team at CMRL thereafter developed a chemically defined medium, CMRL1066, using mouse L cells and their proliferation as an indicator of the effectiveness of the medium. This CMRL1066 medium, consisting of 58 components, was developed through several modifications, such as increasing the levels of reducing substances (cysteine, glutathione, and ascorbic acid), removing fat-soluble vitamins, changing the nucleic acid precursors, and adding coenzymes (Table 2).[51,52] In contrast, during the same period, the National Cancer Institute's Tissue Culture Section (NCTC), led by Wilton R. Earle, developed a chemically defined medium that was composed of 68 ingredients, named NCTC109 (Table 2).[53] These two media were difficult to prepare because of their complex composition. Thereafter, Charity Waymouth developed the MB 752/1 medium (Table 2), which is composed of 40 ingredients (glucose, inorganic salts, amino acids, vitamins, purine bases, hypoxanthine, and glutathione). This was the simplest-possible chemically defined medium at the time.[54] CMRL1066, NCTC109, and MB 752/1, however, all were designed for the best growth of mouse L cells. Therefore, they were not necessarily suitable for the serum-free culture of other cell types. In addition, cell cloning was not possible in those protein-free media. Waymouth hypothesized that the proteins that are produced by cells are necessary for proliferation under protein-free conditions: the so-called "auto/paracrine effect." It was in this context that Richard G. Ham developed Ham's F-10 medium in 1963 (Table 2). With two kinds of serum protein fractions (albumin and fetuin) instead of serum, he successfully made a single Chinese hamster ovary (CHO) cell form a colony under serum-free conditions.[53] The composition of this medium, however, was undefined because it contained serum protein fractions and the proliferative capacity it conferred fell short of that induced by serum-containing media. There was also a challenge: serum supplementation remained necessary for the cultivation of cell types other than CHO cells. In order to solve these issues, Ham replaced the albumin and fetuin of biological origin with low-molecular-weight substances—linoleic acid and putrescine[55,56]—and developed Ham's F-12, a completely synthetic medium of definite composition. During this refinement of the medium, the concentration of each ingredient in Ham's F-10 was reviewed and modified (Table 2).[57] With the reduced amount of zinc, this medium supported colony formation by mouse L cells, but protein-free cultivation of other cells remained difficult.[58,59] It became clear in later studies that trace elements contaminating the water or raw materials they used are necessary for the protein-free cultivation of CHO cells in Ham's F-12 medium, leading to the development of MCDB301, a medium that is supplemented with 20 trace elements (Table 2).[60] Toshiko Takaoka and Hajim Katsuta successfully cultured various kinds of cells long-term in a simple medium that did not contain proteins or lipids;[61] however, only a few cell types can adapt to protein- and lipid-free conditions and the fatty-acid composition of the cells thus cultured differs greatly from the cells cultured in serum-containing media.[62]

In the field of life sciences, the "reductive approach" and the "synthetic approach" are often used for elucidating a complicated biological process. The reductive approach is used to analyze and identify the essential parts in the complex biological process. The synthetic approach is used to reconstruct the biological process by putting the known parts together. Both approaches were instrumental in the development of synthetic culture media because these approaches had disadvantages relative to each other. First, the reductive approach was undertaken strenuously by Carrel and others because the synthetic approach was almost impossible without any information about the active substances for the cultivation of cells; accordingly, the substances that were important for cell culture in natural media were lined up. In contrast, the reductive approach could not be entirely successful at elucidating the complexities of the natural media because of the quantitative and qualitative limitations of the analytical method, complicated components of the natural media, and their variation from batch to batch. Accordingly, the candidate substances based on the knowledge of the reductive approach were tested by means of the synthetic approach by Eagle and others and the substances that are essential for cell culture were eventually identified. It seems to be important when and how these approaches are used for the successful development of culture media.

2.6 | Identification of serum substitutes and the development of serum-free media tailored to a cell type (1970-)

Insulin was discovered earlier by Frederick Banting and Charles Best (1921), but full-scale research into this peptide as a supplement for culture media began in the 1960s.[63,64] Initially, the effectiveness of insulin alone was found to be inferior to that of serum,[65,66] but the use of insulin in combination with low-concentration serum yielded a higher level of efficacy of baby hamster kidney (BHK)21 cell growth. This finding led researchers to conclude that insulin acts in a coordinated manner with serum components. Growth factors were discovered one after another during this era: nerve growth factor,[67] epidermal growth factor,[68,69] insulin-like growth factor,[70–73] fibroblast growth factor (FGF),[74,75] platelet-derived growth factor,[76–78] and transforming growth factor (TGF).[79,80] The addition of these growth factors to a culture medium increased cellular proliferation. Nevertheless, their effect on cell proliferation, as with insulin, was found to be almost always inferior to the effect of serum.[81–85]

Under these circumstances, in 1976, three key reports were published that accelerated the development of serum-free media. Ham's group discovered that a trace element of selenite is necessary for the serum-free cultivation of human diploid cells[86] and Larry J. Guilbert and Iscove showed that, besides selenite, a combination of transferrin and albumin is a good serum substitute.[87] Izumi Hayashi and Gordon H. Sato discovered that a combination of several hormones and growth factors is an effective serum substitute.[58] Prompted by their discoveries, attempts at serum-free culture by using serum substitutes (eg, several hormones and growth factors, transferrin, and selenite; Table 3) grew in number and a variety of serum-free media was developed, with each medium tailored to researchers' cell type of interest.

When developing serum-free culture media, researchers typically test combinations of individual serum substitutes. As many cells require insulin, transferrin, and selenite,[88] ITS, a supplement that contains a mixture of these three substances, has become commercially available. In addition, Hiroki Murakami et al. found that ethanolamine is essential for the serum-free cultivation of hybridomas and developed the supplement, ITES, which consists of ITS plus ethanolamine.[89] Various other supplements were designed for different cell types and cultivation purposes, with the aim of the addition of various trace elements to protein-free culture media. These include Synthetic Serum Replacement, in which various trace elements are stabilized by ethylenediaminetetraacetic acid, citric acid, and aurintricarboxylic acid;[90] the B-27 supplement that was created for use with nerve cells (it contains progesterone, putrescine, triiodothyronine, fatty acids, vitamin E, bovine serum albumin [BSA], and glutathione, in addition to ITS);[91,92] and Knockout Serum Replacement (KSR), a formulation of various active ingredients of serum (eg, BSA, amino acids, antioxidants, ITS, and trace elements).[93]

2.7 | Improvements to basal media (1970-)

In addition to being a source of hormones, growth factors, carrier proteins, and lipids, serum increases the levels of various low-molecular-weight compounds in basal media. As a result, traditional basal media from which serum is excluded were sometimes unable to adequately support cell growth.[94] For example, Barnes et al. and Murakami et al. reported that the performance of DMEM, MEM, and Ham's F-12 as basal media in serum-free cultures is inadequate, but that the DMEM/F-12 medium, in which Ham's F-12 and DMEM are combined in a 1:1 ratio, shows better performance occasionally when used for certain types of cells.[95,96] The reason seems to be the large number of constituents in Ham's F-12 and the high concentration of several nutritional constituents in DMEM: mixing the two allows each to complement the weaknesses of the other. Moreover, Murakami et al. reported that the RDF medium—a 2:1:1 mixture of RPMI 1640, DMEM, and Ham's F-12—yields more effective cell growth of hybridomas than does the DMEM/F12 medium.[97] Mixed media, however, do not always show a level of performance that is better than that of a single medium. For example, the ferrous sulfate that is contained in Ham's F-12 is toxic to nerve cells and nerve cells proliferate more readily at a reduced osmolarity. Thus, DMEM alone is more effective than DMEM/F-12 in this case.[91] Naturally, the composition of a basal medium that is used for serum-free culture should be optimized for each cell type. In addition, it seems that the optimization also depends on the scale of the culture and its method.[98]

2.8 | Medical and industrial applications of animal-cell culture technology (1978-)

2.8.1 | Culture media for the production of recombinant pharmaceuticals

Inspired by the 1982 clinical application of recombinant human insulin expressed in *Escherichia coli*, researchers actively proceeded to produce growth hormones, interferon α, and other substances by using *E. coli* or yeast as a host. With *E. coli* and yeast, however, it was impossible to produce proteins with glycosylation. Animal cells thus started to be used for the production of recombinant proteins, like tissue plasminogen activator, erythropoietin, interferon β, and monoclonal antibodies. The host cells that have been used in the manufacture of biopharmaceutical products include CHO cells, mouse myeloma NS0 cells, BHK cells, human embryonic kidney 293 cells, and human retinal cells. Among these, the CHO and NS0 cells have become especially popular in the field of biopharmaceutical manufacturing for the following reasons: (1) technological advances in mass-culture methods for these two cell lines; (2) sufficient knowledge about the safety of viruses that these two cell lines contain; and (3) remarkable advances in high-expression sublines that were derived from these two cell lines.[99]

In order to enhance the efficiency of the production of biopharmaceuticals, one must increase the production rate of the target protein in a culture medium that contains none or a minimal amount of ingredients of biological origin, like serum, because they significantly hamper the process of product purification. Research in this direction has been conducted to efficiently optimize the medium's composition, for example, by means of approaches that are based on the monitoring of changes in the concentration of the medium components and by-products in the culture,[100] as well as genomics- and proteomics-based

TABLE 3 Characteristics and limitations of serum substitutes

Category	Name	Characteristic	Limitations
Serum, tissue extracts	For example, fetal bovine serum protein,bovine pituitary extract	Contain various components, including proteins and lipids of serum or tissue origin and contribute to improved cellular proliferation and survival	Composition is undefined and therefore there is large lot-to-lot variation and a high risk of contamination; for example, by viruses
Hydrolysates	For instance, animal-derived (animal tissues, milk), microorganism-derived (yeast), plant-derived (soy, wheat, rice)	Supply cells with vitamins, lipids, inorganic salts, low-molecular-weight peptides, and amino acids. Confirmed efficacy for culturing Chinese hamster ovary, hybridoma, baby hamster kidney, Vero, and lymph cells. Purification of antibodies and recombinant proteins is simplified because the components in question contain only low-molecular-weight substances, owing to ultrafiltration. Very low-cost, as compared with serum	Composition is undefined; thus, there is a large lot-to-lot variation. A risk of contamination, for example, by viruses when the origin is from animals. The risk is non-zero even when the origin is plants: for instance, when in contact with animals or animal-source products during cultivation or the manufacturing process. Caution is necessary because the raw materials could have been exposed to high concentrations of pesticides or herbicides
Growth factors	For example, EGF, FGF, IGF, NGF, PDGF, TGF	Act in small quantities on cells; for example, to induce proliferation, differentiation, migration, secretion, or import. Many cells require supplementation of the medium with growth factors under serum-free conditions. Acidic FGF readily degrades if heparin sulfate is not present on the surface of the target cells; therefore, heparin (or synthetic dextran as a substitute) is added to the medium in some situations	Animal-derived growth factors pose a risk of contamination; for example, by viruses. The use of recombinant proteins reduces the risk of contamination; however, the risk is non-zero because the proteins could have been produced by means of animal-derived enzymes in the manufacturing process and for other reasons. Released from platelets, TGF-β acts as a growth inhibitor on many epithelial cells
Hormones	For example, growth hormone, insulin, hydrocortisone, triiodothyronine, estrogen, androgens, progesterone, prolactin, follicle-stimulating hormone, gastrin-releasing peptide	Growth hormone and insulin enhance the proliferation of a variety of cells. Hydrocortisone improves the cloning efficiency of the glial cells and fibroblasts and is necessary for the maintenance of the epidermal keratinocytes and several other endothelial cell types. Triiodothyronine is necessary for MDCK epithelial cells and is used for the pulmonary epithelium. Along with hydrocortisone and prolactin, various combinations of estrogen, androgens, and progesterone are necessary for the maintenance of the mammary epithelium	Insulin is unstable at 37°C (especially in the presence of a high concentration of cysteine) and therefore must be added to a medium at a comparatively high concentration. In addition, zinc is necessary for insulin to exert its biological action and researchers ideally should use a zinc-supplemented medium. The hydrocortisone that is present in the fetal bovine serum acts as a growth inhibitor in high-density cultures (many cells that are closely packed; eg, glial cells, pulmonary epithelial cells). Conversely, it sometimes promotes growth in low-density cultures
Carrier proteins	Albumin, transferrin, lactoferrin, and others	Albumin is used as a carrier of a variety of substances, including lipids (eg, fatty acids, cholesterol), trace elements (eg, copper, nickel), amino acids (cysteine, tryptophan), and vitamins (pyridoxal phosphate: ie, the active form of vitamin B_6). As lipids cannot dissolve in an aqueous solution alone, they are more effectively supplied to cells after the formation of complexes with albumin. In addition, albumin has toxin-neutralizing, antioxidant, and shear stress-reducing effects. Transferrin is used as a carrier of iron. Lactoferrin can serve as a substitute for transferrin	If these agents are serum-derived, there is a risk of contamination; for example, by viruses. Most of the serum-derived albumin in distribution today is purified from corn by using the cold ethanol fractionation method: the products that are prepared this way contain lower proportions of other proteins. Moreover, the levels of lipids and trace elements that are bound to albumin vary from lot to lot. Sometimes, differences between lots are observed as a result: researchers should perform batch screening before using these products. Serum-derived transferrins include compounds of porcine, bovine, and human origin. As bovine transferrin typically has low activity, researchers must in some cases work around this issue; for example, by raising the concentration

(Continues)

TABLE 3 (Continued)

Category	Name	Characteristic	Limitations
Lipids and related components	Cholesterol, steroids, fatty acids (eg, palmitate, stearate, oleate, linoleate), ethanolamine, choline, inositol, and others	Serve various roles: as membrane components, in nutrient storage and transport, and in signal transduction. Many established cell lines can biosynthesize the lipids that are necessary for metabolism from acetyl coenzyme A, but adding the lipids to the medium lessens the biosynthetic load. In addition, some cells lack the enzymes that are necessary for the cholesterol-biosynthetic pathway: a source of sterols must be added to the medium in order to culture such cells. The use of lipoproteins and albumin is the physiologically closest and most effective way to solubilize proteins and deliver them to the cells	When building a culture medium under protein-free conditions, researchers must use ethanol, surfactants (eg, Pluronic F-68, Tween 80), or cyclodextrin to solubilize the lipids. If ethanol is used for solubilization, one must typically add a quantity of ≤1 mL/L (v/v) in view of its negative effects on cells. Caution is advised when using surfactants and cyclodextrin: they are toxic to cells at high concentrations, result in poor lipid solubility at low concentrations, and can be removed by filtration. Many commercially available lipids are animal-derived and their performance varies
Transition metals	For example, Fe, Zn, Cu, Cr, I, Co, Se, Mn, Mo	These are transition elements because they readily undergo electron transfer, so they function in the active centers of enzymes and physiologically active substances inside the cell. Se, Fe, Cu, and Zn, in particular, generally are used in cell culture. Se has an antioxidant activity in the form of selenoproteins, such as glutathione peroxidase and thioredoxin reductase	Chelating agents can serve as a substitute for the Fe carrier, transferring, in cases where it must be removed from the medium. Caution is still necessary: depending on the species and concentration of the chelating agent, not only could it be ineffective as a carrier, it might also promote the production of reactive oxygen species
Vitamins	Fat-soluble vitamins (A, D, E, K), water-soluble vitamins (eg, B_1, B_2, B_6, B_{12}, C, folate)	Necessary for cell division and growth as precursors of various cofactors. Vitamins C and E additionally have antioxidant effects. Vitamins are present in most of the basal media, but their types and amounts (especially of the fat-soluble vitamins) are limited in some situations; therefore, they are added according to the needs of the cell type	Vitamins A, C, D, and E are readily degraded by air oxidation. Vitamin C, moreover, reacts with trace elements and oxidatively decomposes, sometimes generating reactive oxygen species. Vitamins A, B_1, B_2, B_{12}, C, and K are readily degraded by light; vitamins B_1 and B_5 are easily degraded by heat. Folate has poor solubility and is partially removed during filtration sterilization in some cases. Hydroxocobalamin and vitamin C interact, promoting mutual degradation
Polyamines	Putrescine, spermidine, spermine	Low-molecular-weight, basic, physiologically active amines that exist ubiquitously in cells and promote protein or nucleic-acid synthesis. Intracellular concentrations of polyamines are regulated and maintained both by biosynthesis or decomposition inside the cell and by transport from outside the cell	Cell growth halts if the intracellular polyamine concentrations drop too low due to a disrupted balance among polyamine biosynthesis, decomposition, and transport. Moreover, apoptosis is induced if the polyamine concentration rises too much
Reductants	2-mercaptoethanol, α-thioglycerol, reduced glutathione	Import of cystine or cysteine into cells is necessary to maintain the intracellular redox environment and the addition of reducing agents to the culture medium of cells that lack cystine transporters (eg, lymphocytes and embryonic stem cells) converts cystine into cysteine, which the cells then are able to import	Caution is necessary when adding reducing agents in the absence of albumin: this approach can damage cells
Protective additives, detergents	Carboxymethyl cellulose, polyvinyl pyrrolidone, Pluronic F-68, Tween 80, and others	Reduce the shear stress generated in stirred cultures and by pipette manipulation. Pluronic F-68 and Tween 80 also are used as solubilizers of lipophilic substances (eg, lipids, fat-soluble vitamins)	Surfactants sometimes show cytotoxicity, depending on their concentration
Adhesion factors	For example, fibronectin, laminin	Promote the adhesion of anchorage-dependent cells to vessels	Pose a risk of viral contamination if components of biological origin are used

EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MDCK, Madin-Darby canine kidney; NGF, nerve growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

approaches.[101] Through such efforts, as well as host cell modifications,[102] the per-cell production yield has increased nearly 10-fold from 1986 to 2004.[103] The composition of the various culture media that are used in biopharmaceutical manufacturing today has not been disclosed for commercial reasons, but the composition of a previously reported serum-free culture medium that is used for CHO cells is detailed in Table 4 for reference.

2.8.2 | Culture media for use with pluripotent stem cells

Since the establishment of human ES cells by James A. Thomson et al. in 1998 and human iPS cells by Shinya Yamanaka et al. in 2007, the demand for these cells has increased rapidly due to their usefulness in basic and clinical studies for regenerative medicine, as well as in a variety of possible applications, such as disease modeling, drug discovery, and cytotoxicity studies. Thus, culturing these cells in a simple, low-cost, and highly productive way became an important issue. These cells initially were cultivated on a layer of feeder cells in a serum- or KSR-supplemented medium,[104] but in anticipation of clinical applications, the efforts shifted to the development of culture conditions that are feeder-free and xeno-free (Table 5).[105–111] Among these, the E8 medium that was developed by Guokai Chen et al. has become popular. With its simple composition—DMEM/F-12 supplemented with insulin, sodium selenite, transferrin, ascorbic acid (stable form), FGF2, TGF-β1 (or Nodal), and sodium bicarbonate—this medium allows for the long-term growth of human iPS cells.[109] Moreover, in recent years, developments have continued in the field of culture media that contain low-molecular-weight compounds instead of expensive growth factors (eg, FGF2 and TGF-β1; Table 5).[112]

2.8.3 | Culture media for use in assisted reproductive technology

The development of in vitro fertilization, embryo culture, and embryo transfer technologies has progressed on the basis of animal experiments, primarily on rabbits and mice.[113–116] In 1978, the first clinical application was successful, as implemented by Patrick C. Steptoe and Robert G. Edwards.[117] The medium they preferred to use for human zygote cultures was Ham's F-10, supplemented with serum.[118] It was later revealed, however, that hypoxanthine and the trace elements in Ham's F-10 negatively affected the embryos via the production of reactive oxygen species.[119–122] The metabolism of a pre-implantation embryo differs greatly from that of somatic cells; therefore, the embryo can be cultured up to the blastocyst stage by using a simple medium that consists of a balanced salt solution that is supplemented with glucose, pyruvate, lactate, and albumin.[123] The concentration of glucose that was used usually for somatic cells in those days, which was as high as 5.5 mmol L^{-1}, turned out to be unfavorable to zygote cleavage. Therefore, glucose is often added to the culture medium for use with zygotes at the low concentration of 0.2-0.5 mmol L^{-1}.[124] The detailed composition of the embryo culture media that is used clinically has not been published to date for commercial reasons; however, according to the analysis by Morbeck et al.,[125] it resembles either the G1/G2 medium that was developed by Gardner and Lane[126] or the $KSOM^{AA}$ medium that was developed by Biggers et al.[127] More information on the culture media for assisted reproductive technology is available elsewhere.[124]

3 | SELECTION OF A BASAL MEDIUM

Each basal medium has been designed in each case on the basis of the cell type, the origin (animal species), and the purpose of the culturing. In fact, the medium composition can differ greatly depending on such background factors. Whether supplementation with natural products is allowed is another important presupposition for the choice of a basal medium. For example, MEM (developed by Eagle) was designed under the assumption of serum supplementation and accordingly includes only the minimum necessary components (inorganic salts, sugar, essential amino acids, and water-soluble vitamins). In contrast, Medium 199 and Ham's F-12, intended for serum-free culture, contain various other components. Regarding the selection of basal media, readers can be referred to the literature and suppliers'

TABLE 4 Serum-free culture media for Chinese hamster ovary cells

Name (author[s], year)	Basal media	Supplements	Remarks
MCDB 301 (Hamilton and Ham 1977)	Ham's F-12	Trace elements (Al, Ag, Ba, Br, Cd, Co, Cr, F, Ge, I, Mn, Mo, Ni, Rb, Se, Si, Sn, Ti, V, and Zr)	A medium with 20 trace elements that are not present in Ham's F-12
GC_3 (Gasser et al. 1985)	Modified MEM/F-12	Insulin, transferrin, and selenite	Developed because Chinese hamster ovary cells could not be cultured in the MCDB301 medium
WCM5 (Keen and Rapson 1995)	IMDM	Amino acids, vitamins, transition metals (Cu and Zn), ferric citrate, insulin, ethanolamine, putrescine, Pluronic F-68, and soy peptone	Lacking high-molecular-weight proteins, it was developed for use with large-scale cultures (≥8000 L). Ferric citrate is used instead of transferrin
Name unspecified (Sung and Lee 2009)	IMDM	Amino acids, ascorbate, transition metals (Cu and Zn), ferric citrate, selenite, insulin, ethanolamine, phosphatidylcholine, hydrocortisone, putrescine, pyruvate, ascorbate, Pluronic F-68, dextran sulfate, and a hydrolysate mixture (yeast, soy, and wheat)	The combination and concentrations of the added hydrolysates were determined by using an experimental design method. It was developed to increase antibody productivity

TABLE 5 Serum-free culture media for embryonic stem/induced pluripotent stem cells

Name (author[s], year)	Basal media	Supplements	Remarks
Knockout DMEM (Amit et al. 2000)	DMEM	Amino acids, bFGF, 2-mercaptoethanol, and Knockout Serum Replacement (KSR)	A medium with lower osmotic pressure than DMEM and an added serum substitute containing animal-source components (KSR), it is for use with mouse embryonic stem cells. The cultures require feeder cells
TeSR (Ludwig et al. 2006)	DMEM/F-12	Vitamins, trace elements (V, Mn, Ni, Si, Sn, Mo, Cd, Cr, Ag, Al, Ba, Co, Ge, Br, I, F, Rb, Zr), selenite, LiCl, insulin, transferrin, human serum albumin (HSA), bFGF, transforming growth factor (TGF)-β1, γ-aminobutyric acid, pipecolic acid, glutathione, 2-mercaptoethanol, lipids (fatty acids, cholesterol), Pluronic F-68, and Tween 80	A xeno-free medium that does not require feeder cells
E8 (Chen et al. 2011)	DMEM/F-12	Ascorbate-2-phosphate, selenite, insulin, transferrin, bFGF, TGF-β1 or NODAL, and $NaHCO_3$	A TeSR-based medium. HSA (which results in large between-lot variation) and 2-mercaptoethanol (which negatively affects cells) were removed and supplements were refined down to the necessary minimum
(Name undefined) (Hasegawa et al. 2012)	DMEM/F-12	Amino acids, ascorbate, selenite, insulin, transferrin, Wnt3a, and indole derivative (ID)-8 (DYRK inhibitor)	The expensive bFGF and TGF-β are replaced with Wnt3a and the low-molecular-weight compound ID-8. Growth is slow, compared to conventional media
(Name undefined) (Hasegawa et al. 2015)	DMEM/F-12	Ascorbate, selenite, insulin, transferrin, ID-8, GSK3β inhibitor (eg, 1-azakenpaullone), and NFAT inhibitor (eg, tacrolimus)	Wnt is replaced with a GSK3β inhibitor and NFAT inhibitor (low-molecular-weight compounds), it can be manufactured cheaply, and quality management is simple

bFGF, basic fibroblast growth factor; DYRK, dual-specificity tyrosine-phosphorylation-regulated kinase; GSK, glycogen synthase kinase; NFAT, nuclear factor of activated T cells.

information; for instance, cell banks' sites (eg, www.atcc.org, www.phe-culturecollections.org.uk, cellbank.nibiohn.go.jp, and cell.brc.riken.jp). Table 2 shows the basic characteristics of each medium and researchers can select several candidates after comparing them experimentally. α-MEM, DMEM, or Ham's F-12 often are selected for adherent cell culture; the RPMI 1640 medium for suspension cell culture; and a mixed medium, like DMEM/F-12, for serum-free culture.

3.1 | Selection of a basal medium: the roles of the medium components

3.1.1 | Serum

Serum serves as a source of amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, inorganic salts, trace elements, and other compounds. It also improves the pH-buffering capacity of the medium and helps to reduce shear stress (ie, physical damage that is caused by pipette manipulation and stirring). Furthermore, serum alters the conditions at a culture substratum, allowing the adherent cells to readily proliferate there. Fetal bovine serum (FBS) is the most popular and widely applicable serum today. Other kinds of serum are used in certain situations, including calf serum (CS) and horse serum. Researchers can select an appropriate serum type on the basis of its characteristics. Fetal bovine serum generally is rich in growth factors and contains low levels of γ-globulins (which have a cell growth-inhibitory activity). Thus, it is suitable for cells that are difficult to coax to proliferate in culture and for the cloning of cells. In contrast, because CS has a weak growth-promoting activity, it has been used effectively for studies of contact inhibition on the 3T3 cell line. It is also suitable for cellular differentiation studies, in which growth factors can interfere with the results.[128] Lipid levels in serum rise with increasing calf age (ie, days after birth)[129] and therefore CS or adult bovine serum is sometimes selected, instead of FBS, when cells with high lipid requirements are cultured. Horse serum that is harvested from adult horses via a closed system of collection has a comparatively high homogeneity between lots. Its characteristics include a low concentration of polyamine oxidase,[130] which makes polyamines; the latter have a cell-proliferative effect and are metabolically degraded less readily.

3.1.2 | Alternatives to serum

When serum supplementation is inappropriate or undesirable, researchers can choose several substitutes for serum. They include serum extracts, tissue extracts or hydrolysates, growth factors, hormones, carrier proteins like albumin and transferrin, lipids, metals, vitamins, polyamines, and reductants (Table 3). The number of combinations of these supplements is nearly infinite and they often interact with each other. Their selection thus incurs an enormous effort, time, and cost: one cannot design an optimal medium by simply trying them in arbitrary combinations at random. Thorough studies on past records of successful combinations, if available, are helpful. The

following is an example of a protocol when researchers try to implement a serum-free culture by themselves:[131]

1. Try a medium where the ITS supplement is added to DMEM/F-12.
2. If using glutamine, set its concentration to 2–4 mmol L^{-1} and consider the stable form: L-alanyl-L-glutamine (see the next section).
3. Add growth factors, hormones, vitamins, trace elements, and lipids according to the requirements of the cell type under study.
4. Pay attention to the osmotic pressure.
5. Use antibiotics as little as possible.
6. When culturing adherent cells, consider using substrates, such as fibronectin and laminin, for cell attachment.
7. For a stirred culture, consider supplementing the medium with protective agents like Pluronic F-68 to minimize the shear force.
8. Adapt the cells to the new medium with great caution.
9. Verify that the cell performance has not changed in the new medium.

The serum-free and protein-free media that are sold by a variety of manufacturers largely show good performance and researchers can use them to culture their cells of interest. Nonetheless, their composition almost has never been disclosed for commercial reasons, and therefore, technically, such media cannot be termed "chemically defined."

4. | POINTS OF CAUTION WHEN USING CULTURE MEDIA

4.1 | Setting up a serum-free culture

There are two points to note when working with a serum-free culture system. First, unintended clones of cells in a culture vessel can be selected during subculturing because serum-free media can promote the proliferation of a particular cell clone or subtype of cell more often than can serum-containing media. Second, impurities in a serum-free medium, both avoidable and inevitable, might have stronger effects on the cultured cells than can serum-containing media because of a lack of the toxin-neutralizing activity that serum contains. In addition, there are other precautions for serum-free cultures: that is, the minimization of the concentration of trypsin and the careful selection of adhesion factors.[57]

4.2 | pH changes

The pH of the culture medium that is used for animal-cell cultures generally is maintained by the equilibrium relationship between sodium bicarbonate ($NaHCO_3$) in the culture medium and CO_2 in the incubator (Fig. 1). Many laboratories set the CO_2 concentration in the incubator to 5%-6% by convention, but technically, it should be adjusted according to the concentration of the $NaHCO_3$ in the culture medium. For example, 5% CO_2 is appropriate for a medium with 26 mmol L^{-1} $NaHCO_3$ added (eg, MEM medium), 2% CO_2 for a medium with 14 mmol L^{-1} added (eg, Ham's F-12 medium), and 10% CO_2 for a medium with 44 mmol L^{-1} $NaHCO_3$ added (eg, DMEM medium). These numbers are theoretical values that are calculated by using the Henderson–Hasselbalch equation (Fig. 1): in practice, checking the pH of a culture medium after it reaches equilibrium and then making minute adjustments to the CO_2 concentration are preferable.

The 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), with its strong buffering capacity, might have buffering effects in a medium. Especially when cultured cells are handled outside the CO_2 incubator, the pH of the medium with HEPES is more stable than with only bicarbonate. It also might be beneficial for cultures of high cell density; for instance, when the pH can drop rapidly due to an accumulation of metabolites, such as lactate. With serum-free media, which lack the buffering capacity of serum, the pH-buffering role of HEPES could be more relevant. Nevertheless, HEPES might have negative effects on certain types of cells, such as chick embryo epiphyseal chondrocytes and ES cells.[107,132] It also has been demonstrated that HEPES-containing media produce an increased level of cytotoxic products, mostly hydrogen peroxide, during exposure of the medium to visible light.[133] Researchers should be aware of such drawbacks of

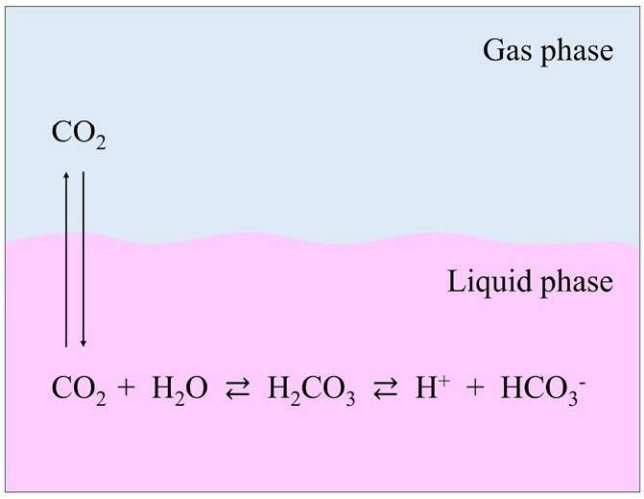

FIGURE 1 The pH control mechanism of culture media, based on the bicarbonate buffer system and the Henderson–Hasselbalch equation. When dissolved in water, sodium bicarbonate ($NaHCO_3$) dissociates to form a sodium ion (Na^+) and a bicarbonate ion (HCO_3^-). The latter reacts with H^+ in solution to form carbonic acid (H_2CO_3), which dissociates into CO_2 and H_2O. These two reactions attain their respective equilibria. The CO_2 in solution also reaches equilibrium with CO_2 in the gas phase. As a result, increasing the concentration of gas phase CO_2 increases the amount of CO_2 that is dissolved in the culture medium, in turn raising the H_2CO_3 concentration and lowering the pH. In contrast, if the concentration of the gas phase CO_2 is lowered, then the pH rises due to the reverse reaction. The relationship between the culture medium pH and the concentrations of CO_2 and $NaHCO_3$ can be expressed by the Henderson–Hasselbalch equation: $pH = pKa + \log[HCO_3^-]/[CO_2]_{Liquid\ phase}$, where: *p*Ka is the negative log of the acid dissociation constant.

HEPES and confirm that the addition of HEPES is harmless to the cells that they use.

Phenol red, added to a medium as a pH indicator, sometimes affects the proliferation of cells and shows estrogenic activity.[134,135] Therefore, investigators should not forget to test whether such characteristics of phenol red affect their cells and empirical results, especially when estrogen-sensitive cells, like mammary gland cells, are involved.

4.3 | Oxidative stress

Culture experiments in general and especially when performed under a higher oxygen concentration, such as organ culture experiments, can expose cells to oxidative stress, which negatively affects the cells and tissues in culture. Supplementation with substances with an antioxidant activity (eg, vitamins [C, E], glutathione, selenite, β-mercaptoethanol, dithiothreitol, or lipoic acid) is effective, especially when serum (it contains antioxidants) is not added to the medium. In addition, iron and copper ions in a free state promote the production of reactive oxygen species. It is therefore best for these ions to be complexed with appropriate carriers (eg, transferrin, albumin, or chelating agents) and to be supplied to the cells in this state, while they are isolated from reactive systems. Caution is also necessary with respect to free iron ions, which are readily hydroxylated and precipitate in an aqueous solution.

4.4 | Nutrient requirements

Sufficient amounts of nutrients in the medium are a prerequisite for cells to behave properly. Some cell types require higher levels of nutrients than others do, depending on their metabolic activity and proliferation rate. Such characteristics of cells should be taken into account in the selection of a medium. DMEM, for example, was designed originally to contain glucose at 5.6 mmol L^{-1}. Now, a modified version of DMEM with a high glucose concentration, 25 mmol L^{-1}, which can be used for cells requiring greater amounts of glucose, is available from various suppliers. One caveat when using this high-glucose medium for actively proliferating cells is the accumulation of metabolites, like lactate, and a plunge in the pH. Researchers are advised to replace the medium at proper intervals or to use HEPES to confer a stronger pH-buffering capacity onto the medium.

Glutamine, one of the essential amino acids, is an energy source for mammalian cells in culture, in addition to being a biosynthetic material for nucleic acids and proteins. The glutamine requirements for cells in culture are ~3-40-fold greater than those of other amino acids.[40] During the cultivation of cells with high nutrient requirements, the addition of glutamine could be helpful. In contrast, glutamine readily decomposes in culture media and generates cytotoxic ammonia. Consequently, researchers may consider adding glutamine to the medium immediately before use or to consider using glutamine derivatives, such as L-alanyl-L-glutamine or glycyl-L-glutamine, which are resistant to degradation.

Non-essential amino acids (NEAAs) can be biosynthesized in the cell. Thus, NEAAs are not included in some basal media, such as BME and MEM. Nevertheless, not every cell type can produce sufficient amounts of NEAAs in the cultured state. The addition of NEAAs to the medium, therefore, could ensure more favorable culture conditions. Even cells that can biosynthesize NEAAs at a necessary rate might benefit from the addition of NEAAs to the medium because this action alleviates the biosynthetic load of the cells.

Branched-chain amino acids (BCAAs), including valine, leucine, and isoleucine, require special attention. They all belong to the group of essential amino acids and several cell types, including human fibroblasts and mouse myeloma cells, require larger amounts of BCAAs. For those cells, increased concentrations of BCAAs or replacing the medium at proper intervals can be considered in order to obtain the best results of an experiment.

5 | CURRENT ISSUES WITH CULTURE MEDIA

5.1 | Design of experiments for the optimization of the medium components

Most of the culture media that generally are used today consist of a basal medium and several supplements. Researchers pay no attention to the possible interactions among the components of the basal medium and these supplements. The fact is that each component of a culture medium does not always act in isolation. The components can interact and their effects on the cells should be expected to be influenced by those interactions. Additionally, the concentration of a medium component and its effect on the cells might not follow a linear dependence. Cells' response to each component could be curvilinear, as shown in Figure 2. Therefore, the best way to find the optimal concentration and interactions of each component in a culture medium might be a statistical experimental strategy called a "design of experiments" (DoE), rather than the classical one-factor-at-a-time experiments. The latter approach might miss the optimal point of concentration because there are some unexamined areas in the range of experimental settings (Fig. 3A). By contrast, the DoE can allocate design points, namely the concentrations of the components to be examined, evenly throughout the area of settings. Then, the obtained empirical data will be applied to the appropriate mathematical models, such as the general linear model or the logistic regression model. In general, when the data corresponding to the response variable (also called the "objective variable") are continuous, such as the production volume of a biopharmaceutical, the general linear model is used. With binominal data, such as the survival rate of cells, the logistic regression model is preferable. By means of these models, an optimal concentration of each component (which is expected to induce the best response of the cultured cells) will be estimated (Fig. 3B). The DoE mainly consists of screening designs and response surface designs. A screening design is used first when one is trying to extract the most important components from many, say more than five, candidates of

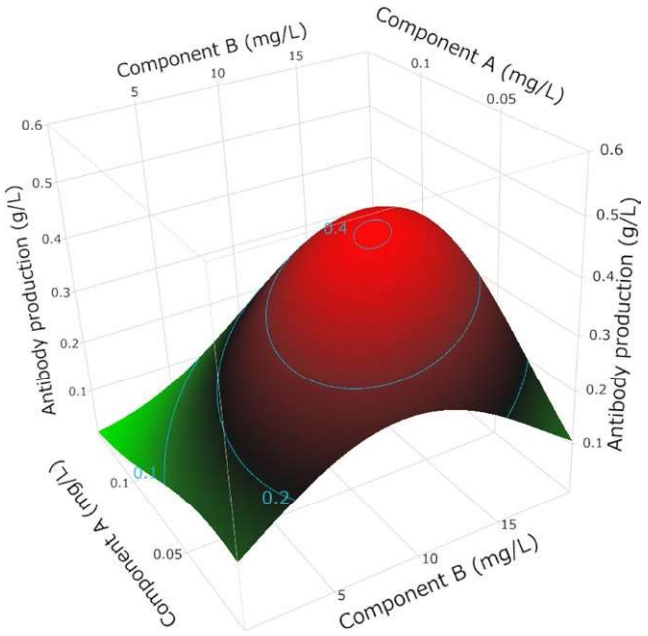

FIGURE 2 An example of a concentration–response surface. When Component B's concentration is low (eg, 0mg/L), antibody production falls as Component A's concentration rises; however, when Component B's concentration is high (eg, 20mg/L), antibody production increases as Component A's concentration rises. Such a phenomenon—one component influencing the response of another component—is called a "two-factor interaction." The relationship between the concentration and response is not necessarily linear, as is the case for Component B. An analysis of the concentration by using at least a three-level screening design is necessary to understand such responses

the initial components. The strength of the screening designs, such as fractional factorial or the Plackett–Burman design, is that they can reduce the number of experimental runs significantly, in comparison with the number that otherwise has to be done with the full factorial design, which involves every possible combination of components. For example, when trying to find the key factors in Medium 199, which consists of 60 components, only 64 experiments are needed with the Plackett–Burman design, whereas 2^{60} experiments are required with the full factorial design. Once the key factors are identified, preferably eight or fewer, a response surface design, such as the central composite design or Box–Behnken design, will be used to estimate a non-linear response of the cells and to identify the optimized concentrations and interactions of each component. These approaches have become a powerful tool for the improvement of productivity in the field of cell culture.[136] Moreover, a new screening design, called a "definitive screening design," recently was reported, with the claim that it has the advantages of both a screening design and a response surface design.[137] This design enables the estimation of not only the main effects (the effect of a certain component alone) but also the interactions of the components and the factors with non-linear effects, at a minimal number of experimental runs. In the case of Medium 199, only 121 experiments are needed. This approach could be especially useful for studies where the sample size is limited, such as a primary culture, organ culture, and embryo culture, although there are some restrictions in this design. The establishment of effective development systems for culture media that combine in vitro and in silico approaches and the optimization of the combinations of all the components in the culture medium are crucial for further improvements in cell culture performance.

5.2 | Undefined medium supplements

Supplements of biogenic origin, like serum, can be a cause of variation in the experimental results from batch to batch. They also carry a risk of microbial contamination of the culture medium. Thus, the replacement of those supplements with defined ones has been pursued in the history of culture media, as described above.[43,87] At the dawn of the technology, a human embryo was cultured in a medium containing serum. In the mid-1980s, the serum could be replaced by serum albumin.[138] Albumin is the most abundant protein in serum and is multifunctional. It binds to various water-insoluble substances like lipids. The lipids that are carried by albumin become an energy source and biosynthetic substances for an embryo.[139–141] In addition, serum albumin serves as an antioxidant, osmotic regulator, and neutralizer of toxins. These functions are key benefits that serum usually provides to the media. Nonetheless, the use of serum albumin in place of serum has not contributed much to the development of chemically defined media. First, most, if not all, commercial serum albumin versions contain >100 serum proteins, although these admixtures have very low concentrations.[142,143] Second, albumin can bind to potential toxins, like phthalates[144] (common plasticizers) or endotoxins. Thus, those toxins can be present in the commercial versions of serum albumin. These impurities in serum albumin products even were reported to vary in concentration from batch to batch, thus affecting the results of the embryo culture.[145–150] Therefore, the development of the culture media without undefined supplements is desirable, especially for human embryos. Recently, highly purified recombinant human albumin became commercially available. It is worth trying it as a substitute for serum-derived albumin. Summarized in Table 3 are the points of caution when researchers use undefined supplements.

5.3 | Contamination of a medium

Foreign substances from unidentified sources can contaminate a culture medium, thus possibly affecting the empirical results. Typically, those contaminants include viruses, bacteria, mycoplasma, and endotoxins. There are, however, other types of contaminants, like plasticizers that might be eluted from plastic instruments[144] or trace elements, even in water. These substances also can affect the cells in culture.[60] It also was reported that some toxic substances are eluted from the microfilters that are used for sterilization.[151] Some of this contamination seems to be even inevitable, but care must be taken to minimize it in order to make culture experiments reliable and highly reproducible. Thus, researchers may consider practicing the sterile technique strictly and selecting culture instruments carefully. Washing the instruments

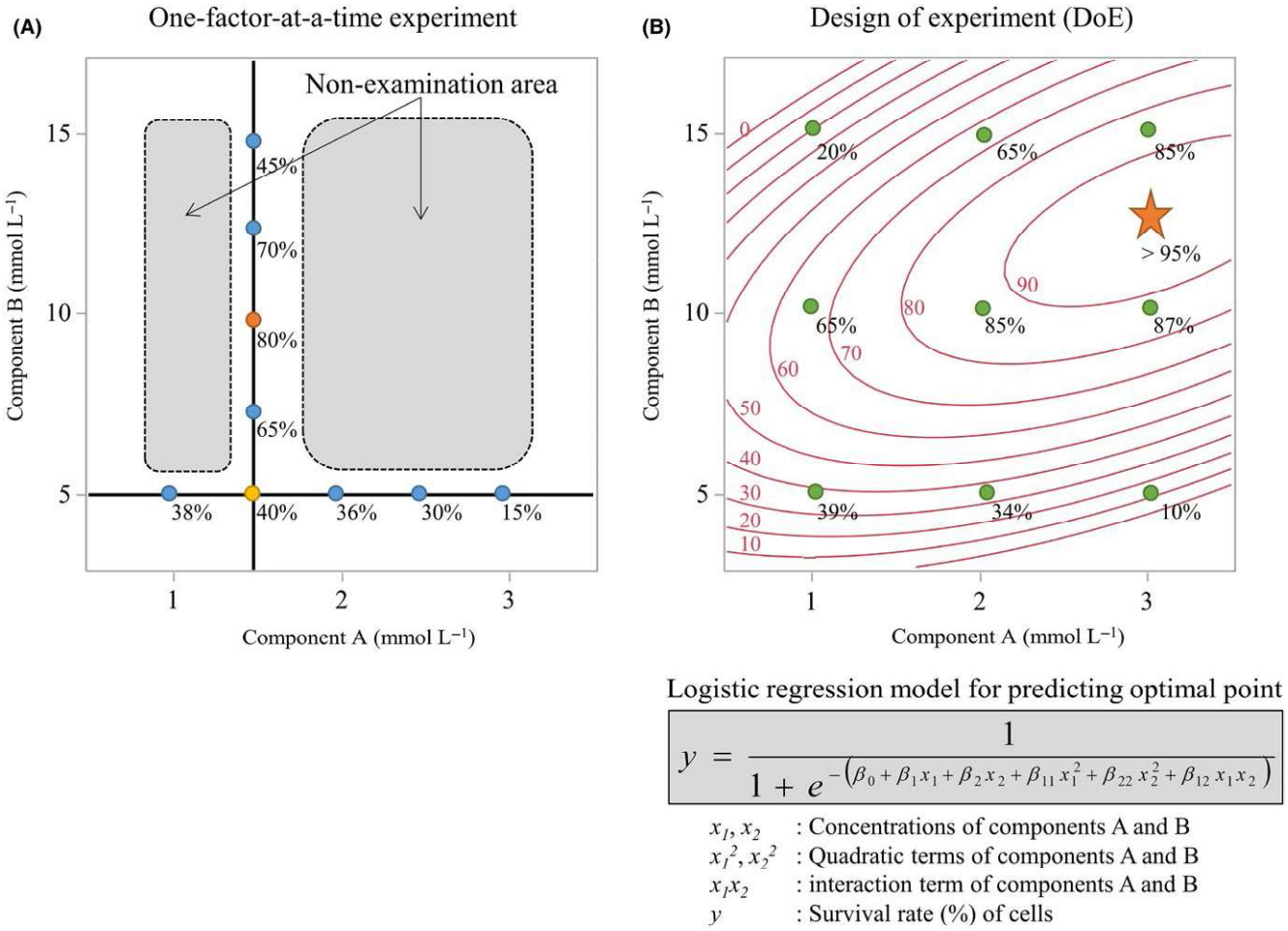

FIGURE 3 Concepts of the one-factor-at-a-time experiment and design of experiment (DoE). These figures show the difference in strategies between a one-factor-at-a-time experiment and a DoE for the same experimental runs. A, In the case of the one-factor-at-a-time experiment, the optimal concentration of one component (eg, component A) is determined at a fixed concentration of another component (eg, component B). Then, the optimal concentration of component B is determined at the optimal concentration of component A. This strategy, which usually has been used, has a big disadvantage of missing the optimal point because there are some unexamined areas in the range of parameters. B, In contrast, the DoE is a model-based statistical method that can clarify the relationship between the response of the cells and the concentrations of the tested components in the range of settings. The process of the DoE is mainly composed of four steps. First, allocate the design points evenly throughout the area. Second, record the response of the cells for each run. Third, fit the collected data to an appropriate model (eg, a logistic regression model for a binomial response) and validate the relevance of the model to decide whether it is available for the next step. Finally, use the model to optimize the concentrations of the components or to predict a response of the cells

with the culture medium immediately before use is recommended in some special cases.[151–153]

6 | CONCLUSION

Ever since Harrison's successful cultivation of animal cells, cell culture technology has developed in leaps and bounds, with many breakthroughs. With the consumables (eg, culture media) and cell culture equipment now supplied on a commercial basis, it became possible for anyone to work easily with cultured cells. As a result, there have been fewer opportunities lately to appreciate the research value of culture media, as well as their shortcomings and limitations. With progress in regenerative medicine and biopharmaceuticals, the creation of culture systems that do not require a human intervention is expected to continue: this trend will probably intensify in the future. Even with this trend, the culture medium is of paramount importance for the best quality of cell culture experiment, as well as biopharmaceutical work. Here again, it should be noted that the current culture media and their formulations have been established through the timeless efforts of innumerable researchers. From now on, investigators should drive the further evolution of culture media, with the aim of improving culture performance.

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DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. *Human and Animal Rights*: This article does not contain any study on human or animal participants that was performed by any of the authors.

REFERENCES

1. Keehan WM, Barnes D, Reid L, Stanbridge E, Murakami H, Sato G. Frontiers in mammalian cells culture. *In Vitro Cell Dev Biol*. 1990;26:9–23.
2. Brunner D, Frank J, Appl H, Schoffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. *ALTEX*. 2010;27:53–62.
3. Freshney RI. Serum-free media. In: Freshney RI, ed. *Culture of Animal Cells*. Hoboken, NJ: John Wiley & Sons, Inc.; 2010:115–132.
4. Ringer S. Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. *J Physiol*. 1882;3:380–393.
5. Ringer S. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J Physiol*. 1883;4:29–42.
6. Locke FS, Rosenheim O. Contributions to the physiology of the isolated heart: the consumption of dextrose by mammalian cardiac muscle. *J Physiol*. 1907;36:205–220.
7. Tyrode M. The mode of action of some purgative salts. *Arch Int Pharmacodyn*. 1910;20:205–223.
8. Krebs H, Henseleit K. Untersuchungen über die harnstoffbildung im tierkörper. *Klin Wochenschr*. 1932;11:757–759.
9. Gey GO, Gey MK. The maintenance of human normal cells and tumor cells in continuous culture: I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. *Am J Cancer*. 1936;27:45–76.
10. Earle W, Schilling E, Stark T, Straus N, Brown M, Shelton E. Production of malignancy in vitro; IV: the mouse fibroblast cultures and changes seen in the living cells. *J Natl Cancer Inst*. 1943;4:165–212.
11. Hanks JH, Wallace RE. Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc Soc Exp Biol Med*. 1949;71:196–200.
12. Roux W. Beitriige zur entwicklungsmechanik des embryo. *Z Biol*. 1885;21:411.
13. Loeb L. Über die enstehung von bindegewebe, leucocyten und roten blutkörperchen aus epithel und über eine methode. In: Loeb L, ed. *Isolierte Gewebsteile zu Züchten*. Chicago: Druck von Max Stern & Company; 1897:1–56.
14. Jolly J. Sur la durée de la vie et de la multiplication des cellules animales en dehors de l'organisme. *Compt Rend Soc Biol Paris*. 1903;55:1266–1268.
15. Harrison RG, Greenman MJ, Mall FP, Jackson CM. Observations of the living developing nerve fiber. *Anat Rec*. 1907;1:116–128.
16. Carrel A. A method for the physiological study of tissues in vitro. *J Exp Med*. 1923;38:407–418.
17. Burrows MT. The cultivation of tissues of the chick-embryo outside the body. *JAMA*. 1910;55:2057–2058.
18. Carrel A, Burrows MT. Cultivation of tissues in vitro and its technique. *J Exp Med*. 1911;13:387–396.
19. Carrel A. On the permanent life of tissues outside of the organism. *J Exp Med*. 1912;15:516–528.
20. Carrel A. Artificial activation of the growth in vitro of connective tissue. *J Exp Med*. 1913;17:14–19.
21. Ebeling AH. A ten year old strain of fibroblasts. *J Exp Med*. 1922;35:755–759.
22. Corner GW. Warren Harmon Lewis. *Biograph. Memoirs Natl Acad. Sci*. 1967;39:323–358.
23. Lewis MR, Lewis WH. The cultivation of tissues from chick embryos in solutions of NaCl, $CaCl_2$, KCl and $NaHCO_3$. *Anat Rec*. 1911;5:277–293.
24. Lewis WH, Lewis MR. The cultivation of chick tissues in media of known chemical constitution. *Anat Rec*. 1912;6:207–211.
25. Lewis MR. The importance of dextrose in the medium of tissue cultures. *J Exp Med*. 1922;35:317–322.
26. Baker LE, Carrel A. Action on fibroblasts of the protein fraction of embryonic tissue extract. *J Exp Med*. 1926;44:387–395.
27. Carrel A, Baker LE. The chemical nature of substances required for cell multiplication. *J Exp Med*. 1926;44:503–521.
28. Baker LE, Carrel A. Effect of the amino acids and dialyzable constituents of embryonic tissue juice on the growth of fibroblasts. *J Exp Med*. 1926;44:397–407.
29. Baker LE. The chemical nature of the substances required for cell multiplication: II. Action of glutathione, hemoglobin, and ash of liver on the growth of fibroblasts. *J Exp Med*. 1929;49:163–182.
30. Vogelaar JPM, Erlichman E. A feeding solution for cultures of human fibroblasts. *Am J Cancer*. 1933;18:28–38.
31. Baker LE. Artificial media for the cultivation of fibroblasts, epithelial cells and monocytes. *Science*. 1936;83:605–606.
32. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res*. 1961;25:585–621.
33. Sanford KK, Earle WR, Likely GD. The growth in vitro of single isolated tissue cells. *J Natl Cancer Inst*. 1948;9:229–246.
34. Gey G, Coffman W, Kubicek M. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res*. 1952;12:264–265.
35. Fischer A, Astrup T, Ehrensvard G, Oehlenschlager V. Growth of animal tissue cells in artificial media. *Proc Soc Exp Biol Med*. 1948;67:40–46.
36. Fischer A. Amino-acid metabolism of tissue cells in vitro. *Biochem J*. 1948;43:491–497.
37. Eagle H. The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. *J Biol Chem*. 1955;214:839–852.
38. Eagle H. The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. *J Exp Med*. 1955;102:37–48.
39. Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science*. 1955;122:501–514.
40. Eagle H. Amino acid metabolism in mammalian cell cultures. *Science*. 1959;130:432–437.
41. Dulbecco R, Freeman G. Plaque production by the polyoma virus. *Virology*. 1959;8:396–397.
42. Stanners CP, Eliceiri GL, Green H. Two types of ribosome in mouse–hamster hybrid cells. *Nature New Biol*. 1971;230:52–54.
43. Iscove NN, Melchers F. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive b lymphocytes. *J Exp Med*. 1978;147:923–933.
44. Neuman RE, McCoy TA. Growth-promoting properties of pyruvate, oxaloacetate, and α-ketoglutarate for isolated Walker carcinosarcoma 256 cells. *Exp Biol Med*. 1958;98:303–306.
45. McCoy TA, Maxwell M, Kruse PF. The amino acid requirements of the Jensen sarcoma in vitro. *Cancer Res*. 1959;19:591–595.
46. Moore GE, Ito E, Ulrich K, Sandberg AA. Culture of human leukemia cells. *Cancer*. 1966;19:713–723.
47. Moore GE, Gerner RE, Franklin HA. Culture of normal human leukocytes. *JAMA*. 1967;199:519–524.
48. White PR. Cultivation of animal tissues in vitro in nutrients of precisely known constitution. *Growth*. 1946;10:231–289.
49. Jacoby F, Darke SJ. Animal tissue culture with a synthetic medium. *Nature*. 1948;161:768–769.
50. Morgan JF, Morton HJ, Parker RC. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. *Exp Biol Med*. 1950;73:1–8.
51. Parker RC, Castor LN, McCulloch EA. Altered cell strains in continuous culture. A general survey. *Spec Publ NY Acad Sci*. 1957;5:303–313.

52. Parker RC. Chemically defined media. In: Parker RC, ed. *Methods of Tissue Culture*. New York: Harper & Row; 1961:62–80.
53. Evans VJ, Bryant JC, McQuilkin WT, et al. Studies of nutrient media for tissue cells in vitro II. An improved protein-free chemically defined medium for long-term cultivation of strain L-929 cells. *Cancer Res*. 1956;16:87–94.
54. Waymouth C. Rapid proliferation of sublines of nctc clone 929 (strain L) mouse cells in a simple chemically defined medium (MB 752/1). *J Natl Cancer Inst*. 1959;22:1003–1017.
55. Ham RG. Albumin replacement by fatty acids in clonal growth of mammalian cells. *Science*. 1963;140:802–803.
56. Ham RG. Putrescine and related amines as growth factors for a mammalian cell line. *Biochem Biophys Res Commun*. 1963;14:34–38.
57. Ham RG. Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proc Natl Acad Sci USA*. 1965;53:288–293.
58. Hayashi I, Sato GH. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature*. 1976;259:132–134.
59. Murakami H. Serum-free media used for cultivation of hybridomas. *Adv Biotechnol Processes*. 1989;11:107–141.
60. Hamilton WG, Ham RG. Clonal growth of Chinese hamster cell lines in protein-free media. *In Vitro Cell Dev Biol*. 1977;13:537–547.
61. Takaoka T, Katsuta H. Long-term cultivation of mammalian cell strains in protein- and lipid-free chemically defined synthetic media. *Exp Cell Res*. 1971;67:295–304.
62. Oshima M, Miyamoto I, Takaoka T, Goto K, Kagawa Y. Unsaturated fatty acids in mammalian cell strains cultured for more than 10 years in serum-free synthetic media. *Jpn J Exp Med*. 1987;57:351–354.
63. Lieberman I, Ove P. Growth factors for mammalian cells in culture. *J Biol Chem*. 1959;234:2754–2758.
64. Higuchi K. Studies on the nutrition and metabolism of animal cells in serum-free media: I. *Serum-free monolayer cultures. J Infect Dis*. 1963;112:213–220.
65. Clarke GD, Stoker MGP, Ludlow A, Thornton M. Requirement of serum for DNA synthesis in BHK 21 cells: effects of density, suspension and virus transformation. *Nature*. 1970;227:798–801.
66. Clarke GD, Stoker MGP. Conditions affecting the response of cultured cells to serum. In: Wolstenholme GEW, Knight J, eds. *Ciba Foundation Symposium – Growth Control in Cell Cultures*. Chichester: John Wiley & Sons, Ltd.; 1971:17–32.
67. Levi-Montalcini R. Effects of mouse tumor transplantation on the nervous system. *Ann NY Acad Sci*. 1952;55:330–344.
68. Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem*. 1962;237:1555–1562.
69. Taylor JM, Mitchell WM, Cohen S. Epidermal growth factor: physical and chemical properties. *J Biol Chem*. 1972;247:5928–5934.
70. Salmon WD Jr, Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J Lab Clin Med*. 1957;49:825–836.
71. Froesch ER, Buergi H, Ramseier EB, Bally P, Labhart A. Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity. *J Clin Invest*. 1963;42:1816–1834.
72. Daughaday WH, Hall K, Raben MS, Salmon WD Jr, van den Brande JL, van Wyk JJ. Somatomedin: proposed designation for sulphation factor. *Nature*. 1972;235:107.
73. Rinderknecht E, Humbel RE. Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: Isolation, chemical characterization, and some biological properties of forms I and II. *Proc Natl Acad Sci USA*. 1976;73:2365–2369.
74. Gospodarowicz D. Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3t3 cell growth. *Nature*. 1974;249:123–127.
75. Gospodarowicz D. Purification of a fibroblast growth factor from bovine pituitary. *J Biol Chem*. 1975;250:2515–2520.
76. Kohler N, Lipton A. Platelets as a source of fibroblast growth-promoting activity. *Exp Cell Res*. 1974;87:297–301.
77. Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci USA*. 1974;71:1207–1210.
78. Antoniades HN, Scher CD, Stiles CD. Purification of human platelet-derived growth factor. *Proc Natl Acad Sci USA*. 1979;76:1809–1813.
79. De Larco JE, Preston YA, Todaro GJ. Properties of a sarcoma-growth-factor-like peptide from cells transformed by a temperature-sensitive sarcoma virus. *J Cell Physiol*. 1981;109:143–152.
80. Anzano MA, Roberts AB, Meyers CA, et al. Communication: synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. *Cancer Res*. 1982;42:4776–4778.
81. Hollenberg MD, Cuatrecasas P. Epidermal growth factor: receptors in human fibroblasts and modulation of action by cholera toxin. *Proc Natl Acad Sci USA*. 1973;70:2964–2968.
82. Gospodarowicz D, Moran JS. Stimulation of division of sparse and confluent 3t3 cell populations by a fibroblast growth factor, dexamethasone, and insulin. *Proc Natl Acad Sci USA*. 1974;71:4584–4588.
83. Holley RW, Kiernan JA. Control of the initiation of DNA synthesis in 3t3 cells: serum factors. *Proc Natl Acad Sci USA*. 1974;71:2908–2911.
84. Smith GL, Temin HM. Purified multiplication-stimulating activity from rat liver cell conditioned medium: comparison of biological activities with calf serum, insulin, and somatomedin. *J Cell Physiol*. 1974;84:181–192.
85. Gospodarowicz D, Moran J. Optimal conditions for the study of growth control in balb/c 3t3 fibroblasts. *Exp Cell Res*. 1975;90:279–284.
86. McKeehan WL, Hamilton WG, Ham RG. Selenium is an essential trace nutrient for growth of wi-38 diploid human fibroblasts. *Proc Natl Acad Sci USA*. 1976;73:2023–2027.
87. Guilbert LJ, Iscove NN. Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoitec cell cultures. *Nature*. 1976;263:594–595.
88. Barnes D, Sato G. Serum-free cell culture: a unifying approach. *Cell*. 1980;22:649–655.
89. Murakami H, Masui H, Sato GH, Sueoka N, Chow TP, Kano-Sueoka T. Growth of hybridoma cells in serum-free medium: ethanolamine is an essential component. *Proc Natl Acad Sci USA*. 1982;79:1158–1162.
90. Bertheussen K. Growth of cells in a new defined protein-free medium. *Cytotechnology*. 1993;11:219–231.
91. Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in b27-supplemented neurobasal™, a new serum-free medium combination. *J Neurosci Res*. 1993;35:567–576.
92. Brewer GJ. Serum-free b27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *J Neurosci Res*. 1995;42:674–683.
93. Price PJ, Goldsborough MD, Tilkins ML. Embryonic stem cell serum replacement. International Patent Application, 1998, WO 98/30679.
94. Keen MJ, Rapson NT. Development of a serum-free culture medium for the large scale production of recombinant protein from a Chinese hamster ovary cell line. *Cytotechnology*. 1995;17:153–163.
95. Barnes D, Sato G. Growth of a human mammary tumour cell line in a serum-free medium. *Nature*. 1979;281:388–389.
96. Murakami H, Edamoto T, Nakamura H, Omura H. Growth of myeloma mpc-11 cells in serum-free growth factor supplemented medium. *Agric Biol Chem*. 1982;46:1831–1837.
97. Murakami H. Serum-free cultivation of plasmacytomas and hybridomas. In: Barnes D, Sirbasku D, Sato G, eds. *Methods for Serum-Free Culture of Neuronal and Lymphoid Cells*. New York: Alan R. Liss, Inc.; 1984:197–206.
98. Murakami H, Yamada K. Production of cancer specific monoclonal antibodies with human–human hybridomas and their serum-free, high density, perfusion culture. In: Spier R, Griffiths J, eds. *Modern*

Approaches to Animal Cell Technology. London: Butterworths; 1987:52–76.
99. Zhu MM, Mollet M, Hubert RS. Industrial production of therapeutic proteins: cell lines, cell culture, and purification. In: Kent AJ, ed. *Handbook of Industrial Chemistry and Biotechnology*. Boston: Springer; 2012:1229–1248.
100. Lu F, Toh PC, Burnett I, et al. Automated dynamic fed-batch process and media optimization for high productivity cell culture process development. *Biotechnol Bioeng*. 2013;110:191–205.
101. Aboytes KA, Allison DW, Donahue LM, Fong D, Johnson TK. Genomic and proteomic approaches for the development of cell culture medium. International Patent Application, 2004, WO 2004/101808 A2.
102. Kim JY, Kim Y-G, Lee GM. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. *Appl Microbiol Biotechnol*. 2012;93:917–930.
103. Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotech*. 2004;22:1393–1398.
104. Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol*. 2000;227:271–278.
105. Ludwig TE, Levenstein ME, Jones JM, et al. Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol*. 2006;24:185–187.
106. Yao S, Chen S, Clark J, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci USA*. 2006;103:6907–6912.
107. Furue MK, Na J, Jackson JP, et al. Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc Natl Acad Sci USA*. 2008;105:13409–13414.
108. Rajala K, Lindroos B, Hussein SM, et al. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. *PLoS ONE*. 2010;5:e10246.
109. Chen G, Gulbranson DR, Hou Z, et al. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods*. 2011;8:424–429.
110. Hasegawa K, Yasuda S, Teo J, et al. Wnt signaling orchestration with a small molecule DYRK inhibitor provides long-term xeno-free human pluripotent cell expansion. *Stem Cells Transl Med*. 2012;1:18–28.
111. Nakagawa M, Taniguchi Y, Senda S, et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. *Sci Rep*. 2014;4:3594.
112. Hasegawa K, Yasuda S, Shahsavarani H, Yoshida N. Culture medium for pluripotent stem cells. International Patent Application, 2015, WO 2015/147047 A1.
113. Thibault C, Dauzier L, Wintenberger S. Cytological study of fecundation in vitro of rabbit ovum. *CR Seances Soc Biol Fil*. 1954;148:789–790.
114. Chang MC. Fertilization of rabbit ova in vitro. *Nature*. 1959;184:466–467.
115. Whitten WK, Biggers JD. Complete development in vitro of the preimplantation stages of the mouse in a simple chemically defined medium. *J Reprod Fertil*. 1968;17:399–401.
116. Yanagimachi R, Chang MC. Fertilization of hamster eggs in vitro. *Nature*. 1963;200:281–282.
117. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet*. 1978;2:366.
118. Edwards RG, Steptoe PC, Purdy JM. Establishing full-term human pregnancies using cleaving embryos grown in vitro. *Br J Obstet Gynaecol*. 1980;87:737–756.
119. Noda Y. Embryo development in vitro. *Assist Reprod Rev*. 1992;2:9–15.
120. Goto Y, Noda Y, Mori T, Nakano M. Increased generation of reactive oxygen species in embryos cultured in vitro. *Free Radic Biol Med*. 1993;15:69–75.
121. Bastias M, McGee-Belser S, Bryan S, Vasquez J. In vitro deleterious effect of hypoxanthine in Ham's nutrient mixture F-10 culture medium on human oocyte fertilization and early embryonic development. *Fertil Steril*. 1993;60:876–880.
122. Loutradis D, Kiessling A, Kallianidis K, et al. A preliminary trial of human zygote culture in Ham's F-10 without hypoxanthine. *J Assist Reprod Genet*. 1993;10:271–275.
123. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril*. 1985;44:493–498.
124. Yao T, Asayama Y. Human preimplantation embryo culture media: past, present, and future. *J Mamm Ova Res*. 2016;33:17–34.
125. Morbeck DE, Krisher RL, Herrick JR, Baumann NA, Matern D, Moyer T. Composition of commercial media used for human embryo culture. *Fertil Steril*. 2014;102:759–766.
126. Gardner DK, Lane M. Embryo culture systems. In: Trounson AO, Gardner DK, eds. *Handbook of In Vitro Fertilization*. New York: CRC Press; 2000:205–264.
127. Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Hum Reprod Update*. 2003;9:557–582.
128. Eisenblatter T, Psathaki K, Nitz T, Galla H, Wegener J. Cell culture media: selection and standardization. In: Lehr CM, ed. *Cell Culture Models of Biological Barriers In-Vitro Test Systems for Drug Absorption and Delivery*. London: Taylor & Francis; 2002:20–40.
129. Forte TM, Bell-Quint JJ, Cheng F. Lipoproteins of fetal and newborn calves and adult steer: a study of developmental changes. *Lipids*. 1981;16:240–245.
130. Freshney RI. Defined media and supplements. In: Freshney RI, ed. *Culture of Animal Cells*. Hoboken, NJ: John Wiley & Sons, Inc.; 2010:99–114.
131. Van der Valk J, Brunner D, De Smet K, et al. Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods. *Toxicol In Vitro*. 2010;24:1053–1063.
132. Poole CA, Reilly HC, Flint MH. The adverse effects of HEPES, TES, and BES zwitterion buffers on the ultrastructure of cultured chick embryo epiphyseal chondrocytes. *In Vitro Cell Dev Biol*. 1982;18:755–765.
133. Zigler JS Jr, Lepe-Zuniga JL, Vistica B, Gery I. Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. *In Vitro Cell Dev Biol*. 1985;21:282–287.
134. Grady LH, Nonneman DJ, Rottinghaus GE, Welshons WV. pH-Dependent cytotoxicity of contaminants of phenol red for MCF-7 breast cancer cells. *Endocrinology*. 1991;129:3321–3330.
135. Walsh-Reitz MM, Toback FG. Phenol red inhibits growth of renal epithelial cells. *Am J Physiol Renal Physiol*. 1992;262:F687–F691.
136. Mandenius CF, Brundin A. Bioprocess optimization using design-of-experiments methodology. *Biotechnol Prog*. 2008;24:1191–1203.
137. Jones B, Nachtsheim CJ. A class of three-level designs for definitive screening in the presence of second-order effects. *J Qual Technol*. 2011;43:1–15.
138. Menezo Y, Testart J, Perrone D. Serum is not necessary in human in vitro fertilization, early embryo culture, and transfer. *Fertil Steril*. 1984;42:750–755.
139. Quinn P, Whittingham D. Effect of fatty acids on fertilization and development of mouse embryos in vitro. *J Androl*. 1982;3:440–444.
140. Yahia Khandoker M, Nihioka M, Tsujii H. Effect of BSA binding fatty acids on mouse and rat embryo development. *J Mamm Ova Res*. 1995;12:113–118.
141. Haggarty P, Wood M, Ferguson E, et al. Fatty acid metabolism in human preimplantation embryos. *Hum Reprod*. 2006;21:766–773.
142. Gay M, Carrascal M, Gorga M, Pares A, Abian J. Characterization of peptides and proteins in commercial HSA solutions. *Proteomics*. 2010;10:172–181.
143. Dyrlund TF, Kirkegaard K, Poulsen ET, et al. Unconditioned commercial embryo culture media contain a large variety of non-declared

proteins: a comprehensive proteomics analysis. *Hum Reprod*. 2014;29:2421–2430.
144. Takatori S, Akutsu K, Kondo F, Ishii R, Nakazawa H, Makino T. Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in media for in vitro fertilization. *Chemosphere*. 2012;86:454–459.
145. Kane MT. Variability in different lots of commercial bovine serum albumin affects cell multiplication and hatching of rabbit blastocysts in culture. *J Reprod Fertil*. 1983;69:555–558.
146. Caro CM, Trounson A. The effect of protein on preimplantation mouse embryo development in vitro. *J In Vitro Fert Embryo Transf*. 1984;1:183–187.
147. Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. *Reprod Fertil Dev*. 1991;3:601–607.
148. Leveille MC, Carnegie J, Tanphaichitr N. Effects of human sera and human serum albumin on mouse embryo culture. *J Assist Reprod Genet*. 1992;9:45–52.
149. McKiernan SH, Bavister BD. Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. *In Vitro Cell Dev Biol*. 1992;28A:154–156.
150. Rorie RW, Miller GF, Nasti KB, McNew RW. In vitro development of bovine embryos as affected by different lots of bovine serum albumin and citrate. *Theriogenology*. 1994;42:397–403.
151. Harrison K, Sherrin D, Hawthorne T, Breen T, West G, Wilson L. Embryotoxicity of micropore filters used in liquid sterilization. *J In Vitro Fert Embryo Transf*. 1990;7:347–350.
152. Quinn P, Warnes GM, Kerin JF, Kirby C. Culture factors in relation to the success of human in vitro fertilization and embryo transfer. *Fertil Steril*. 1984;41:202–209.
153. Tokoro M, Fukunaga N, Yamanaka K, et al. A simple method for transportation of mouse embryos using microtubes and a warm box. *PLoS ONE*. 2015;10:e0138854.

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Review

Basics of animal cell culture: Foundation for modern science

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The culture of animal cells is one of the major aspects of science which serves as a foundation for most of our recent discoveries. The major areas of application include cancer research, vaccine manufacturing, recombinant protein production, drug selection and improvement, gene therapy, stem cell biology, monoclonal antibody production, *in vitro* fertilization technology, cryopreservation and *in vitro* production of hormones. Cells can be propagated, expanded and divided into identical replicates, which can be characterized, purified and preserved by freezing. This article reviews the basic aspects of animal cell culture for modern day research.

Key words: Animal cell culture, cell freezing, cell preservation.

INTRODUCTION

The culture of animal cells and tissues is a generally and widely used technique that involves isolation of cells, tissues and organs from animals and growing them in an *in vitro* or artificial environment. The term culture means to keep alive and grow in an appropriate medium that simulates the natural conditions. The list of different cell types which can now be grown in culture include connective tissues such as fibroblasts, skeletal, cardiac and smooth muscle, epithelial tissues, neural cells, endocrine cells and many different types of tumour cells (Merten, 2006).

In vitro culture has been proven to be the most valuable method to study the functions and mechanism of operations of many cells. A particular group of cells can be cultured in large quantities to study their cellular activities, differentiations and proliferations. Cell culture is highly essential to biotechnology; the major areas of application of animal cell culture are; cancer research, vaccine manufacturing, recombinant protein production, drug selection and improvement, gene therapy, stem cell biology and *in vitro* fertilization technology.

Growing tissues of living organisms outside the body are made possible in an appropriate culture medium, which contains a mixture of nutrients either in solid or liquid form. Nutritional factors like serum, ca^{2+} ions, hormones etc. can be added to the medium to aid growth, differentiation and proliferation of cells. Cells can be propagated, expanded and divided into identical replicates, which can be characterized and preserved by freezing. They can also be purified phenotypically by

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growth in selective media. Historical landmarks in the development of cell culture are presented in Table 1. The objective of this review was to provide useful and basic information on animal cell culture for early career scientists.

PROCEDURE OF CELL CULTURE

Primary culture

Freshly isolated cultures are known as primary cultures until they are passaged or subculture. They are usually heterogeneous, and have a low growth fraction, but they are more representative of the cell types in the tissue from which they were derived and in the expression of tissue specific properties. The first step in obtaining the primary culture is isolation of tissues from the whole part or organ, followed by disaggregation of cells from the tissues. This is done by addition of low trypsin to the tissue for proper disintegration and isolation of cells. Trypsin is added to the tissues in order to degrade extracellular proteases and glycosidase (Huang et al., 2010). The externally exposed proteins are digested by the action of trypsin for dissociation of cells of the tissues in order to harvest individual cells. The cells obtained after trypsin digestion are incubated in the presence or absence of serum and culture in a medium.

Subculture culture

A subculture is a new culture taken from a primary culture and grown separately in the culture medium. Subculture allows the expansion of the culture (it is now known as a cell line). The advantage of sub-culturing primary culture into a cell line is the provision of large amounts of consistent material suitable for prolonged use (Nguyen et al., 2012). The subculture does not need trypsinization. The medium required is based on the type of specialized cells in culture. This subculture may not need serum for continuous propagation (Baltz and Tartia, 2010).

Monolayer culture

This is a type of culture in which the bottom of the culture plate is covered by a continuous or a single layer of cells in a culture medium (Hazen et al., 1995). They do not require enzymatic or mechanical dissociation. Growth is limited by concentration of cells in the medium, which allows easy scale-up.

Suspension culture

Suspension cultures are cells which can be grown within suspension of the medium. They are easier to passage as there is no need to detach them. They do not require enzymatic or mechanical dissociation.

Adherent culture

Cells are dissociated enzymatically before they are cultured. They require periodic passaging, but allow easy visual inspection under inverted microscope. They are referred to as anchorage dependent cells.

Seeding density and cell propagation

The conditions which favour cell proliferation are low cell density, low Ca^{2+} concentration (100 to 600uM) and the presence of growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGF), and platelet derived growth factor (PDGF). High cell density ($>10^5$ cells/ cm^2) will favour cytostasis and differentiation. Different conditions are therefore required for propagation and differentiation, and hence an experimental protocol may require a growth phase to increase cell number, this is followed by a non-growth maturation phase, which allows replication of samples and an increased in the expression of differentiated functions (De Felici et al., 2004).

In general, cultures derived from embryonic tissues will survive and grow better than those of the adult. They presumably reflect the lower level of specialization and presence of replicating precursor or stem cells in the embryo (De Felici et al., 2004). Adult tissues will usually have a lower growth fraction and high proportion of non-replicating specialized cells. Cells cultured from neo-plasma, however, can express at least partial differentiation, e.g. B16 Mouse melanoma, while retaining capacity to divide (De Felici et al., 2004; Nguyen et al., 2012). The list of main equipment required for cell culture is provided in Table 3.

TYPE OF CULTURE SYSTEMS

Batch

This can be defined as the usual type of culture in which cells are inoculated into a fixed volume of medium. As the culture grows, nutrients are consumed and metabolites are accumulated. The environment is therefore continually changing and this, in turn, enforces changes to cell metabolism, often referred to as physiological differentiation. Batch culture is suitable for both monolayer and suspension cells (Shiloach and Fass, 2005). The media are added to the culture in three different ways:

(1) By replacing a constant fraction of the culture with an equal volume of fresh medium.
(2) By increasing continuously the volume at a constant rate, but withdrawing culture aliquots at intervals.

Table 1. Historical landmarks in the development of cell culture.

Year	Historical landmarks in the development of cell culture
1878	Claude Bernard proposed that physiological systems of an organism can be maintained in a living system after the death of an organism.
1885	Roux maintained embryonic chick cells in a saline culture.
1897	Loeb demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma.
1903	Jolly observed cell division of salamander leucocytes *in vitro.*
1907	Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and observed the growth of nerve fibers *in vitro* for several weeks. He was considered by some as the father of cell culture
1910	Burrows succeeded in long term cultivation of chicken embryo cell in plasma clots. He made detailed observation of mitosis.
1911	Lewis and Lewis made the first liquid media consisted of sea water, serum, embryo extract, salts and peptones. They observed limited monolayer growth.
1913	Carrel introduced strict aseptic techniques so that cells could be cultured for long periods
1916	Rous and Jones introduced proteolytic enzyme trypsin for the subculture of adherent cells.
1923	Carrel and Baker developed 'Carrel' or T-flask as the first specifically designed cell culture vessel. They employed microscopic evaluation of cells in culture.
1927	Carrel and Rivera produced the first viral vaccine - Vaccinia.
1933	Gey developed the roller tube technique
1940	The use of the antibiotics penicillin and streptomycin in culture medium decreased the problem of contamination in cell culture
1948	Earle isolated mouse L fibroblasts which formed clones from single cells. Fischer developed a chemically defined medium, CMRL 1066.
1952	Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Helen Lane) cells. Dulbecco developed plaque assay for animal viruses using confluent monolayers of cultured cells.
1954	Abercrombie observed contact inhibition: motility of diploid cells in monolayer culture ceases when contact is made with adjacent cells.
1955	Eagle studied the nutrient requirements of selected cells in culture and established the first widely used chemically defined medium.
1961	Hayflick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite lifespan in culture.
1964	Littlefield introduced the HAT medium for cell selection.
1965	Ham introduced the first serum-free medium which was able to support the growth of some cells.
1965	Harris and Watkins were able to fuse human and mouse cells by the use of a virus.
1975	Kohler and Milstein produced the first hybridoma capable of secreting a monoclonal antibody
1978	Sato established the basis for the development of serum-free media from cocktails of hormones and growth factors.
1982	Human insulin became the first recombinant protein to be licensed as a therapeutic agent.
1985	Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
1986	Lymphoblastoidγ IFN licensed.
1987	Tissue-type plasminogen activator (tPA) from recombinant animal cells became commercially available.
1989	Recombinant erythropoietin in trial
1990	Recombinant products in clinical trial (HBsAG, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2).
1998	Production of cartilage by tissue engineered cell culture by Aigner et al.
2000	Mapping of the human genome.
2007	Use of viral vectors to reprogram adult cells to embryonic state (induced pluripotent stem cells) by Yu et al.
2008	And beyond- Era of induced pluripotent stem cells

(3) Perfusion: by the continuous addition of medium to the culture and the withdrawal of an equal volume of spent (cell-free) medium. Perfusion can be open, i.e. the complete removal of medium from the system or closed which is recirculation of the medium; usually, a secondary vessel is used to regenerate the medium by gassing and pH correction (MacMichael, 1989).

Continuous-flow culture

This system gives true homeostatic conditions with nofluctuations of nutrients, metabolites or cell number. It depends on the medium entering the culture with a corresponding withdrawal of medium with cells. It is only suitable for suspension culture cells or monolayer cells growing on micro-carriers. The system has chemostat attached to it. A fixed volume of culture in which medium is fed in, at a constant rate mixed with the cells and then leaves at the same rate (Drake et al., 2002).

GROWTH KINETICS IN CELL CULTURE

The standard growth kinetics of a culture cycle begins with a lag phase, proceeding through the logarithmic or exponential phase to a stationary phase, and finally to the decline and death of cells. The phases of cell growth in

culture are discussed below:

The lag phase: This is the period of adaptation of cells to the new environment. New enzymes are synthesized, a slight increase in cell mass and volume occurs, but there is no increase in cell number. If this phase is prolonged, there could be low inoculum volume and poor inoculum condition (that is, high percentage of dead cells).

The log or exponential phase: This is the period of balanced growth in which all cell components grow at the same rate. The cells have adjusted to their new environment and multiply rapidly (exponentially). Growth rate is independent of nutrient concentration, as nutrients are in excess. The composition of the biomass remains constant and the phase results in a straight line graph. Growth (increase in cell numbers or mass) can be defined in the following terms:

Specific growth rate (μ) or proliferation rate (r): The exponential growth rate is the first order of reaction. The rate of biomass is correlated with the specific growth rate (**μ**) and the biomass concentration or cell number, X. A measure of the rapidity of growth has dimension T^{-1}.

$$\mathbf{dx/dt = \mu.X} \quad (1)$$

Where, d_x = increase in cell mass/number; dt = time interval, and x = cell mass/number.

Doubling time (td) that is the time for a population to double in number or mass:

$$\boldsymbol{td = \frac{In2}{\mu} = \frac{0.693}{\mu}} \quad (2)$$

Degree of multiplication (n) or number of doublings (that is, the number of times the inoculum has replicated) is defined as:

$$n = 3.34 \log(x/x_0) \quad (3)$$

$$n = 3.34 \text{ x } (\log Xo - \log X) \quad (4)$$

The proliferation rate, (r) or specific growth rate (μ) = 3.32 x $(\log N_F - \log N_I)/T_2-T_1$ (Griffiths, 1972). Where, r = Proliferation rate, N_F= number of Final cell count, N_I =Number of Initial cell count, T_2= Time at harvest, T_1=Initial time.

Population doubling time (PD): This is the number of times the inoculums has replicated within 24 hours. It is defined as: (PD) = 24/(r), where r is proliferation rate.

The deceleration phase: The exponential phase is followed by deceleration phase, which is the period of unbalanced growth. The growth decelerates due to either depletion of one or more essential nutrients. There is an accumulation of toxic by-products of growth period of unbalanced nutrients in the medium. Cells undergo internal restructuring to increase their chances of survival.

The stationary phase: This phase starts when the net growth rate is zero. Cells may have an active metabolism to produce secondary metabolites. Secondary metabolites are non-growth-related which may be antibiotics like pigments. The decline and death phase is characterized by the living cell population decreasing with time, due to lack of nutrients and toxic metabolic by-products.

The rate of death is defined as =

$$\frac{dN}{dt} = -k_d' N \quad (5)$$

K_d is the cell death constant. N= Cell number concentration (cell number /L). Figure 1 shows the typical growth curve of a cell population.

MEDIA REQUIREMENTS FOR CELL CULTURE

Criteria for selecting culture media

The choice of medium to be used for culture is dependent on the cell type specifics which significantly affects the success of cell culture experiments. The selection of the media also depends on the purpose of the culture and resources available in the laboratory. Different cell types have highly specific growth requirements; therefore, the most suitable media for each cell type must be determined experimentally. In general, it's always good to start with MEM for adherent cells and RPMI-1640 for suspension. Media are the sources of nutrients for the cells in culture. They are rich in essential nutrients such as amino acids, glucose, ions, fructose, and hormones with or without serum. The varieties of artificial culture media available can be grouped into:

Serum containing media

Serum is a complex mixture of many small and large bio-molecules with different, physiologically balanced growth-promoting and growth-inhibiting activities. Serum performs major functions which are hormonal factors; stimulating cell growth and functions; attachment and spreading factors; transport proteins carrying hormones, minerals, lipids etc. Among other, the serum contains proteins which serve as carriers and protective agents for other molecules. Albumin is a protein in serum which performs a lot of functions. Albumin binds vitamins such as pyridoxal, fatty acids: such as oleic, linoleic, linolenic

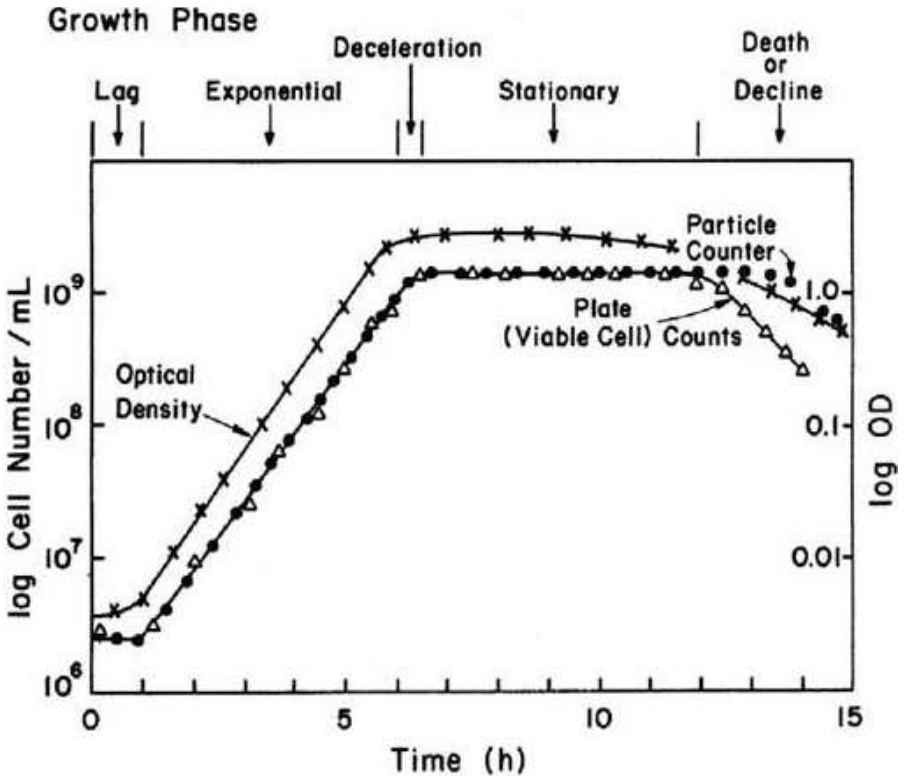

Figure 1. A typical growth curve for a cell population (Griffith, 1972).

arachidonic, myristic and palmitic acids, and ions such as copper. However, the use of serum is limited because of its complexity, expensive and a potential source of adventitious biological contaminants. Eliminating the use of serum in cell culture, it requires an understanding of what it does in *in vitro* systems. Examples of types of serum available for culture are Fetal Bovine Serum, New Calf Serum, Donor Horse Serum, Porcine Serum etc. (Theodore et al., 2005).

Serum-free media

The development of effective serum-free and serum-replacement medium formulations has become essential for the future growth of the biotechnology. The elimination of animal serum-borne components aims to advance standardization, consistency and to reduce risk of contamination by serum-borne adventitious agents in cell culture processes. Biological industries have developed serum-free formulations for all commercially significant cell and tissue cultures (Brunner et al., 2010). Examples are CHO Cell Culture Media, PER.C6 and 293 Media, Insect Cell Media, Immunology Media, Stem Cell Media, Hybridoma Media, Primary Cell Media. The two categories of serum free media are:

(i) Chemically defined media: A chemically defined medium is one in which the exact chemical composition is known e.g. Expression media.
(ii) Protein free media: The common examples are hybridoma serum free media, PFHM II is protein-free media.

Table 2 summarizes the list of commercial culture media that are commonly used (Bertheussen, 1993).

NUTRIENT UTILIZATION IN CELL CULTURE

Nutrient that is likely to be exhausted first is glutamine, and is enzymatically converted (by serum and cellular enzymes) to glutamic acid, leucine and isoleucine. L-Glutamine supports the growth of cells that have high energy demands and synthesize large amounts of proteins and nucleic acids (Le-Bacquer et al., 2001; Cardin et al., 2000). L-Glutamine is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently. Glucose and pyruvate are other nutrients needed by the cells in culture. When added to a culture at high concentrations, they are stimulatory to maturation of cells and modulation of metabolism of the substrate (Downs et al., 1997). Cysteine is also efficiently utilized by human diploid cells (Banjac et al., 2008). It must be noted that nutrients become growth-limiting before they become exhausted. As the concentration of amino acid falls, the cells find it increasingly difficult to maintain sufficient intracellular pool levels (Duboc and Von

Table 2. Commercial culture media and their compositions.

Culture medium	Composition
DMEM	High Glucose - 4 mM L-glutamine 4500 mg glucose/L 1500 mg/L sodium bicarbonate.
RPMI	Modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate
McCoy	High Glucose, L-glutamine, Bacto-peptone, Phenol Red, HEPES with or without Sodium Pyruvate.
M 2	Magnesium Sulfate , Potassium Chloride, Potassium Phosphate, Sodium Bicarbonate, Sodium Chloride, Albumin, Bovine Fraction, D-Glucose, HEPES, Phenol, Pyruvic Acid, DL-Lactic Acid.
Nutrient Mixture F-10 (Ham's F-10)	Sodium bicarbonate, without L- glutamine; supplement with 0.146 gm/L L-glutamine.
Nutrient Mixture F-12(Ham's F-12)	L-glutamine and Sodium bicarbonate
Minimum Essential Medium Eagle	Earle's salts, L-glutamine and Sodium bicarbonate, Sterile-filtered, Endotoxin tested.

Table 3. List of main equipment for cell culture.

Type of Equipment	Function
Bio Safety Cabinets	It offers protection from contaminants during culture.
CO_2 Incubators	Cells are grown in an atmosphere of 5%-10% CO_2. It keeps constant level of humidity
Microscopes	Inverted microscopes are used for this purpose.
Culture vessels	These consist of petri dishes, multi-well plates, microtitre plates, roller bottles, screw cap flasksT-25, T-75, T-150.
Centrifuges	Cells are centrifuged at low temperature and low speed.
Freezer	For freezing and short term storage
Hemocytometer	To determine the cell counts before or after culture.
Water bath with shaker	For cell dissociation and trypsinization
Liquid N_2 Cylinder	For long-term cryopreservation
pH meter	To determine the pH of the medium.

Stockar, 2000). This is exaggerated in monolayer cultures because as the cells become more tightly packed together, the surface area which is available for nutrient uptake becomes smaller. Glucose is often another limiting factor as it is destructively utilized by cell rather than adding high concentrations at the beginning, it is more beneficial to supplement after 2-3 days. In order to maintain a culture some additional feeding often has to be carried out either by complete, partial media changes or by perfusion. The efficiency of medium changes is probably due to the high extracellular concentration of nutrients it provides, thus stimulating a further replicative cycle. Many cell types are either totally dependent upon or can only perform optimally when certain growth factors are present. Cell aggregation is often a problem in suspension cultures. Media lacking calcium and magnesium ions have been designed specifically for suspension cells because of the role of these ions in attachment. This problem has also been overcome by including very low levels of trypsin in medium (Makkar et al., 2011; Lugo et al., 2008; Baumann and Doyle, 1979). The main equipment required for cell culture is listed in Table 3.

Other conditions for cell culture: effect of pH, temperature and oxygen on cell growth

In addition to nutrients, the pH of the growth medium is also important for cell growth rate and cell density. The optimal growth pH for most cells is near neutral. Cells can grow reasonably well over a range of pH 5.5 to 8.5. Extreme pH beyond this range will significantly decrease the cell growth rate and may sometimes even cause celldeath. pH is another limiting factor for cell growth in addition to nutrition exhaustion and accumulation of toxic metabolites. The medium's pH is determined by medium compositions, buffers, cellular metabolites, and aeration conditions. Cells produce large quantities of acetic acid if the growth medium contains little or no oxygen causing the growth medium to reach pH 4 or lower. Acetic acid is the major metabolic inhibitor under anaerobic growth condition. With proper aeration, cells will be able to use many organic acids as carbon sources and the pH of the growth medium will be maintained at near neutral or basic ranges. Cells cannot grow well at temperatures higher than 42°C. They can tolerate lower temperatures with lower growth rate. Temperature range from 15 to

30°C is the most optimal range for most cells depending on the cell type and species of animals. Cell growth stops when the medium is kept at 4°C or shifted above 37°C (Yang and Xiong, 2012).

CRYOPRESERVATION AND STORAGE

Liquid Nitrogen is often used to preserve tissue culture cells, either in the liquid phase (-196°C) or in the vapour phase (-156°C). Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in the concentration of electrolytes, dehydration, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent which lowers the freezing point, such as glycerol or dimethylsulfoxide (DMSO) is added. A typical freezing medium is 90% serum and 10% DMSO (Oyeleye and Omitogun, 2007).

In addition, it is best to use healthy cells that are growing in log phase and to replace the medium 24 h before freezing. Also, the cells are slowly cooled from room temperature to -80°C to allow the water to move out of the cells before it freezes. The optimal rate of cooling is 1 to 3°C per minute. Some laboratories use isopropanol at room temperature and the freezing vials containing the cells are placed in the container and the container is placed in the -80°C freezer. The effect of the isopropanol is to allow the tubes to come to the temperature of the freezer slowly, at about 1°C per minute. To maximize recovery of the cells when thawing, the cells are warmed very quickly by placing the tube directly from the liquid nitrogen container into a 37°C water bath with moderate shaking. As soon as the last ice crystal is melted, the cells are immediately diluted into pre-warmed medium. Cultures should be examined daily, by observing the morphology, the colour of the medium and the density of the cells. A tissue culture logbook should be maintained and it should contain: the name of the cell line, the medium components and any alterations to the standard medium, the dates on which the cells were split and/or fed, a calculation of the doubling time of the culture and any observations relative to the morphology (Jacob and Allison, 2009).

CELL LINE IDENTIFICATION

Cell line identification is done to determine if there is presence or absence of cross- contamination. It also confirms the origin of species of the cell line. It detects the transformed cells and evaluates if there are genetic instabilities. The following techniques discussed below are used to prove the integrity of the cultured cells.

Morphology and STR analyses

Observation of morphology is the most direct technique to characterize cell lines. The study of size, shape and structure of cells can authenticate the type and origin of a particular cell line. Most cells can be divided into five basic categories based on their morphology. The structures are fibroblastic, epithelial-like, lymphoblast-like, endothelial and Neuronal (Figures 2, 3, 4, 5 and 6). Short tandem repeats (STR) profile of a reference sample and other known cell line STR profile can be compared. STR is repetitive sequence elements 3 to 7 base pairs of DNA long scattered throughout the human genome. By amplifying and analyzing these polymorphic loci, comparing the resulting STR profile to that of a reference sample, the origin of biological samples such as cells or tissues can be identified and verified (Gill,2002; Chatterjee, 2007).

Chromosomal and Karyotyping analyses:

This is done to detect the presence of genetic abnormalities within the cell. A karyotype is the number and appearance of chromosomes in the nucleus of eukaryotic cells. The chromosomes are depicted in a standard format known as Karyogram. The karyotyping is done to determine species identification (ACOG, 2007).

Isoenzyme analysis

Isoenzyme analysis is based on the existence of enzymes with similar or identical specificity, but different molecular structure. It is used to study the pattern of migration of isoenzymes present in cell lysates following electrophoresis using agarose gels. Examples of isoenzymes available are; Aspartate aminotransferase, Glucose-6-phosphate dehydrogenase, Lactate dehydrogenase, Malate dehydrogenase (Steube et al., 1995).

ELISA

The enzyme-linked immuno-sorbent assay (ELISA) is a common laboratory technique which is used to measure the concentration of a substance (usually antibodies or antigens) in solution. The basic ELISA, or enzyme immunoassay (EIA), is distinguished from other antibody-based assays because of its separation of specific and non-specific interactions which occur via serial binding to a solid surface. Usually a polystyrene multi-well plate can be achieved due to the quantitative results that can be obtained (Braitbard et al., 2006). The steps of the ELISA result in a colored end product correlates to the amount of a substance present in the original sample. ELISAs were first developed in the early 1970s as a replacement for radio-immunoassays. They remain in wide use in their original format and in expanded format with modifications that allow multiple analyses per well, highly sensitive readouts, and direct cell-based output (Braitbard et al., 2006).

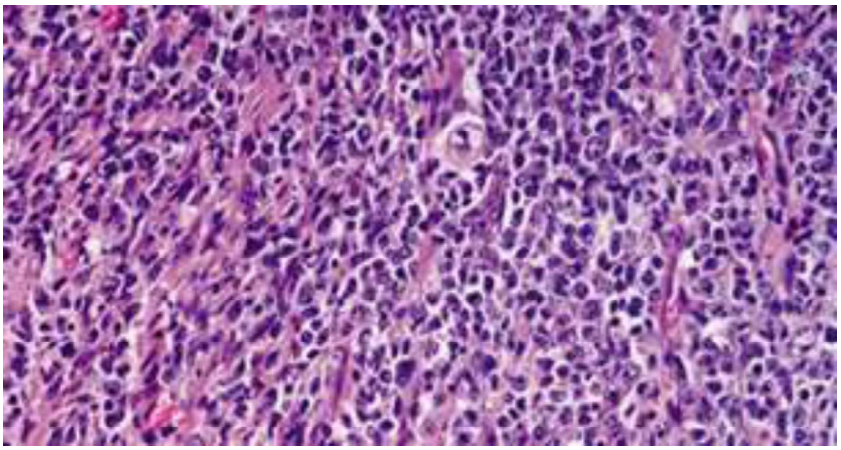

Figure 2. Epithelial cells are polygonal in shape with more regular dimensions, and attached to a substrate in discrete patches.

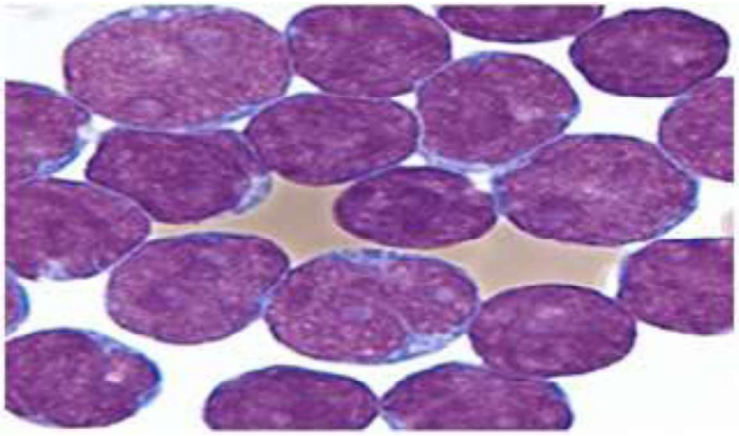

Figure 3. Lymphoblastic cells are spherical in shape and usually grown in suspension without attaching to a surface.

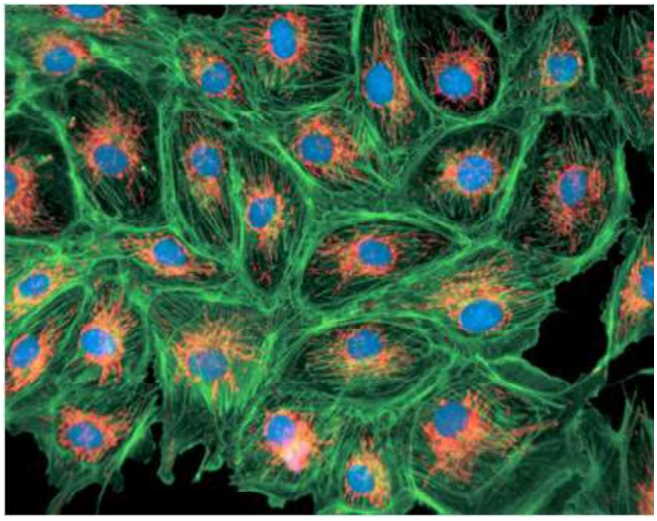

Figure 4. Endothelial cells are flat in shape having central nucleim of about 1-2 µm thick and some 10-20µm in diameter.

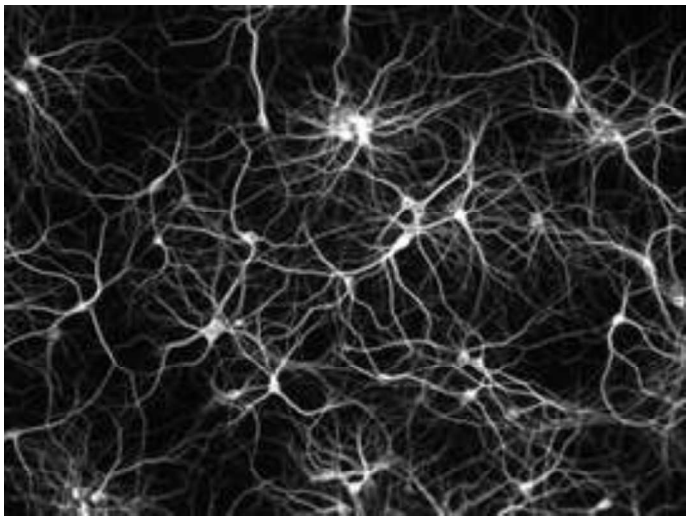

Figure 5. Neuronal cell lines can be with or without axons.

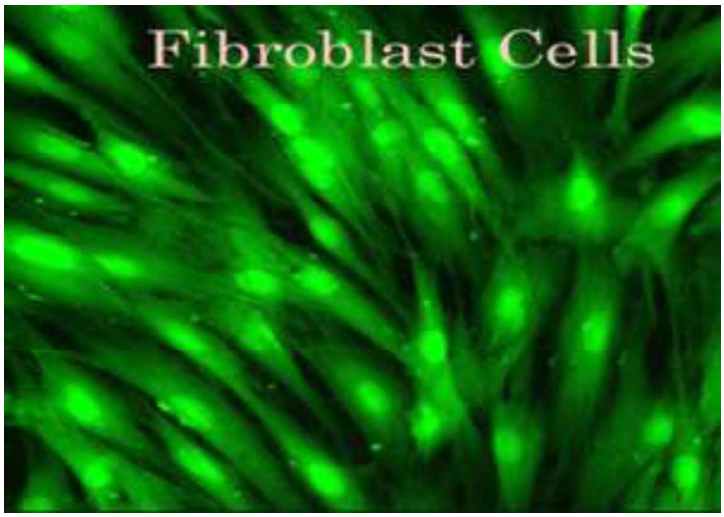

Figure 6. Fibroblastic cells are bipolar or multipolar, have elongated shapes and grown attached to a surface.

Biochemical tests

A large number of cell lines and strains can be shown to derive from a particular tissue or tumour by the presence of specific synthetic abilities or metabolic pathways. The human trophoblastic are cell lines isolated from a malignant gestational choriocarcinoma of a foetal placenta. Interestingly, the line has been shown to secrete a spectrum of placental hormones, including human chorionic gonadotrophin, placental lactogenic, oestrogen, oestradiol, oestriol and progesterone in culture (Soule et al., 1973). Biochemical tests are the tests used for the identification of cells based on the differences in the biochemical activities of different cell functions. These differences in carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, hormones and ability to utilize a particular compound, etc. help to identify them by their biochemical tests (Oyeleye et al., 2016).

Tests for microbial contamination

These tests are suitable for detection of most micro-organisms that would be expected to survive as contaminants in cell lines or culture fluids. The common examples of contaminants that could affect culture are bacteria, fungi, mycoplasma and viruses. The following

tests should be carried out to avoid any microbial contaminant: microbial environmental aseptic monitoring, container integrity testing, pre-sterilization bio-burden testing, media filtering before use and sterility testing (Sirna et al., 2010).

Tests for intra-species cross-contamination

A quiet number of cell lines are being developed; there is a high risk of intra-species cross-contaminants in the laboratory. The problem is common, especially in laboratories where many different cell lines of human and murine origins are being developed. Tests for polymorphic isoenzymes, surface marker antigens and unique karyology are all important tools to detect cellular cross-contamination within a given species (Chatterjee, 2007). The other methods for identification of cell lines include Giemsa binding, tissue-specific antigen, cell type specific marker etc.

CONCLUSION

Animal cell culture is important to all fields of bioscience; especially from medicine to agriculture. It is an important tool to study cell specific functions, physiology and biochemical components. The major advantage of cell culture is its consistency and reproducibility of results that can be obtained from using clonal cells. They serve as building blocks and stem cells for bioscience research and biological repairs. Priority should be given to developing good cell culture laboratories especially in developing countries where they are not yet fully established.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

ACOG-American College of Obstetricians and Gynecologists (2007). ACOG Practice Bulletin No. 88, December 2007. Invasive prenatal testing for aneuploidy. Obstet. Gynecol. 110(6):1459-1467.

Baltz JM, Tartia AP (2010). Cell volume regulation in oocytes and early embryos: connecting physiology to successful culture media. Hum. Reprod. Update 8(6):523-527.

Banjac T, Perisic T, Sato H, Seiler A, Bannai S, Weiss N, Kölle P, Tschoep K, Issels RD, Daniel PT, Conrad M, Bornkamm GW (2008). The cystine/cysteine cycle: a redox cycle regulating susceptibility versus resistance to cell death. Oncogene 27:1618-1628.

Baumann H, Doyle D (1979). Effect of trypsin on the cell surface proteins of the hepatoma tissue culture cells. Characterization of the carbohydrate-rich glycopeptide released from a calcium binding membrane glycoprotein. J. Biol. Chem. 254:3935-3946.

Bertheussen K (1993). Growth of cells in a new defined protein-free medium: Cytotechnology 11(3):219-231.

Braitbard O, Shieban J, Bishara G, Hava G, Miriam K, Pace U, Rund DG, SteinWilfred D (2006). An ELISA-based procedure for assaying proteins in digests of human leukocytes and cell lines, using specifically selected peptides and appropriate antibodies. Proteome Sci. 4:14.

Brunner D, Frank J, Appl H, Schöffl H, Pfaller W, Gstraunthaler G(2010). Serum-free Cell Culture: The Serum-free Media Interactive Online Database. Altex 27:1/10.

Cardin J, Carbajal ME, Vitale ML (2000). Biochemical and morphology diversity among folliculo-Stellate cells of the mink (*Mustela vison*) anterior pituitary. Gen. Comp. Endocrinol. 120:73-87.

Chatterjee R (2007). Cell biology: Cases of mistaken identity. Science 315:928-931.

Drake R, David T, Kim AB (2002). Continuous-Culture Chemostat Systems and Flowcells as Methods to Investigate Microbial Interactions. In. Polymicrobial Disease ASM Press, Washington DC.

De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D (2004). *In vitro* approaches to the study of primordial germ cell lineage and proliferation. Hum. Reprod. Update 10:197-206.

Downs SM, Houghton FD, Humpherson PG, Leese HJ (1997). Substrate utilization and maturation of cumulus cell-enclosed mouse oocytes: evidence that pyruvate oxidation doe's not mediate meiotic induction. J. Reprod. Fertil. 110:1-10.

Duboc P, Von Stockar U (2000). Modeling of oscillating cultivations of *Saccharomyces cerevisiae*: Identification of population structure and expansion kinetics based on on-line measurements. Chem. Eng. Sci. 55:149-160.

Gill P (2002). Role of short tandem repeat DNA in forensic casework in the UK-past, present, and future perspectives. Biotechniques 32(2):366-385.

Griffiths JB (1972). Scaling up in animal cell cultures. J. Cell Sci. 1986:33-69.

Hazen SA, Rowe WA, Lynch CJ (1995). Monolayer cell culture of freshly isolated adipocytes using extracellular basement membrane components. J. Lipid Reserve 36(4):868-875.

Huang HL, Hsing HW, Lai TC, Chen WY, Chan HL (2010). Trypsin-induced proteome alteration during cell subculture in mammalian cells. J. Biomed. Sci. 17:36.

Jacob H, Allison H (2009). Preservation of stem cells. J. Organogenesis 5(3):134-137.

Le-Bacquer O, Nazih H, Blottiere H, Meynial DD, Laboisse C, Darmaun D(2001). Effects of glutamine deprivation on protein synthesis in a model of human enterocytes in culture. Am. J. Physiol. 281:6-1.

Lugo JM, Rodriguez A, Helguera Y, Morales R, Gonzalez O, Acosta J, Besada V, Sanchez A, Estrada MP (2008). Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates. J. Endocrinol. 197:583-597.

MacMichael GJ (1989). The use of perfusion in mammalian cell culture. Am. Biotechnol. Lab. 6(3):34-42.

Makkar HPS, Kumar V, Oyeleye OO, Akinleye OA, Angulo-Escalante MA, Berker K (2011). *Jatropha platyphylla*, a new non-toxic Jatropha species: Physical properties and chemical constituents including toxic and antinutritional factors of seeds. Food Chem. 125:63-71.

Merten OW, (2006). Introduction to animal cell culture technology-past, present and future. Cytotechnology 50(1-3):1-7.

Nguyen HT, Geens M, Spits C (2012). Genetic and epigenetic instability in human pluripotent stem cells. Hum. Reprod. Update 19(2):187-205.

Oyeleye OO, Ola SI, Omitogun OG (2016). Ovulation induced in African catfish (*Clarias gariepinus*, Burchell 1822) by hormones produced in

the primary culture of pituitary cells. Int. J. Fish. Aquac. 8(7):67-73.
Oyeleye OO, Omitogun OG (2007). Evaluation of the motility of the cryopreserved sperm of the African giant catfish (Clarias gariepinus Burchell 1822). Ife J. Agric. 22:11-15.
Shiloach J, Fass R (2005). Growing E. coli to high cell density--a historical perspective on method development. Biotechnol. Adv. 23:345-357.
Sirna V, Garaboldi L, Papi S, Martano L, Omodeo E, Paganelli G, Chinol M (2010). Testing of microbial contamination during the preparation of the radiocompound DOTATOC for clinical trials: a process validation study by Media Fill approach. Q. J. Nucl. Med. Mol. Imaging 54(5):553-559.
Soule HD, Vazquez J, Long A, Albert S Breannan M (1973). A human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Cancer Inst. 51(5):1409-1416.Steube KG, Grunicke D, Drexler HG (1995). Isoenzyme analysis as a rapid method for the examination of the species identity of cell cultures. In vitro Cell Dev. Biol. Anim. 31(2):115-119.
Theodore XO, Timothy JH, Barsam K (2005). Understanding and interpreting serum protein electrophoresis. Am. Fam. Physician 71(1):105-112.
Yang Z, Xiong H (2012). Culture Conditions and Types of Growth Media for Mammalian Cells. Intech Open minds: Biochemistry, Genetics and Molecular Biology: "Biomedical Tissue Culture", ISBN 978-953-51-0788-0.

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An animal cell culture: Advance technology for modern research

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ABSTRACT

At the present time animal cell culture is more significant and multifarious application tool for current research streams. A lot of field assorted from animal cell culture such: stem cell biology, IVF technology, cancer cell biology, monoclonal antibody production, recombinant protein production, gene therapy, vaccine manufacturing, novel drug selection and improvement. In this review conclude animal cell culture as well as its requirements.

Keywords: Animal Cell Culture

1. INTRODUCTION

Tissue Culture is a general idiom used for the removal of cells, tissues, or organs from an animal and their next placement into an artificial environment conductive to growth. Tissue culture is capable of clear as the growth of tissue or cell separate from the organism. It is also known as techniques of keeping tissues alive and growing in an appropriate culture medium. Growing tissues of living organism outside the body is made possible in an appropriate culture medium, containing mixture of nutrient either in solid or liquid form. At present remarkable association in the field of animal cell culture done by researchers: Human insulin became the earliest recombinant protein to be approved as a therapeutic agent, Human growth hormone produced from recombinant bacteria was established in favor of beneficial use, plasminogen activator (tPA) early recombinant animal cells became commercially accessible, [1]. Historical background in the field of animal cell culture demonstrated in **Table 1**.

Table 1. Historical background of animal cell culture.

Year	Significant work	Scientist
1885	Maintained embryonic chick cells in a saline culture	Roux
1897	Demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma	Loeb
1907	Cultivated frog nerve cells in a lymph clot held by the “hanging drop” method and observed the growth of nerve fibers in vitro for several weeks	Harrisone
1911	First liquid media consisted of sea water, serum, embryo extract, salts and peptones	Lewis and Lewwis
1916	Proteolytic enzyme trypsin for the subculture of adherent cells	Rousand Jones
1923	T-flask as the first specifically designed cell culture vessel	Carrel and Baker
1948	Isolated mouse l fibroblasts which formed clones from single cells	Earle
1949	Polio virus could be grown on human embryonic cells in culture	Enders
1952	Continuous cell line from a human cervical carcinoma known as hela (helen lane) cells	Gey
1955	Nutrient requirements	Eagle
1964	Hat medium for cell selection	Littlefield
1975	First hybridoma capable of secreting a monoclonal antibody	Kohlar and Milstein

2. EQUIPMENT REQUIRED FOR CELL CULTURE

2.1. Laminar Flow Hoods

There are two types of laminar flow hoods, vertical and horizontal. The vertical hood, also well-known as a biology safety cabinet, is effective for harmful organisms since Horizontal hoods are designed such that the air flows directly at the operator hence they are not useful for working with hazardous organisms but are the best protection for cultures. Both types of hoods have continuous displacement of air that passes through a HEPA (high efficiency particle) filter use for the purpose of removes particulates from the air. In a vertical hood, the filtered air blows down from the top of the cabinet; in a horizontal hood, the filtered air blows out at the operator in a horizontal fashion. The hoods are equipped with a short-wave UV light that can be turned on for a few minutes to sterilize the surfaces of the hood, but be aware that only exposed surfaces will be accessible to the UV light. Do not put your hands or face near the hood when the UV light is on as the short wave light can cause skin and eye damage. The hoods should be turned on about 10 - 20 minutes before being used (**Figure 1**).

2.2. CO_2 Incubators

Cells are grown-up in an atmosphere of 5% - 10% CO_2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be firmly maintained. Cells are thought to left out of the incubator for as undersized time as possible and the incubator doors should not be opened for very long. The humidity must also be maintained for individuals cells' growing in tissue culture dishes so a pot of water is kept filled at the entire times (**Figure 2**).

2.3. Microscopes

Inverted phase contrast microscopes used for visualizing the cells. Microscopes must be kept enclosed and the lights turned down at the same time as not in use. Because the cells are found on bottom of the tissue culture flask that is by Use of an inverted microscope is important to absorb cell culture *in vitro*. The culture media remains above the growing cells plats. If such plates are put over the stage of an ordinary microscope, the growing cells, at bottom cannot be observed. Therefore, the inverted microscope is used for the intention (**Figure 3**).

Figure 1. Laminar flow hoods.

2.4. Vessels

Anchorage dependent cells have compulsory of a non-toxic, biologically inert, and optically visible surface that will allow cells to attach and allow improvement for the duration of growth. These consist of petri dishes, multi-well plates, microtiter plates, roller bottles, and screwcap flasks—T-25, T-75, T-150 (cm^2 of surface area) (**Figure 4**).

Figure 2. CO_2 incubators.

Figure 3. Inverted microscopes.

Figure 4. Vessels.

2.5. Centrifuges

There are different types of centrifuges based on speed. A low speed centrifuge is needed for most of the cell culture. The separated beads of cells are disrupted simply by a gentle breaking action. Frequently cells are centrifuged at 20˚C because of motor evolves heat which rises the temperature; therefore make use of low temperature centrifugation is preferred so that the cells should not be exposed to elevated temperature (**Figure 5**).

2.6. Freeze

Freezing or solidification is a phase change in which a liquid turns into a solid when its temperature is lowered under its freezing point. The render null and void procedure is melting. Human gametes and embryos can survive freezing and are viable for up to 10 years, a process known as cryopreservation. Investigational attempts to freeze human beings for later revitalization are known as cryonics (**Figure 6**).

3. SUBSTRATES IN FAVOR OF CELL DEVELOPMENT

There are numerous types of vertebrate cell that have need of support for their development in vitro otherwise they will not grow appropriately. Such cell are called anchorage-dependent cell. Used for that reason a large number of substrate which possibly will necessitate for their enlargement (e.g. glass, palladium, metallic surfaces), non-adhesive (e.g. agar agrose, etc.).

4. MEDIA REQUIREMENT FOR CELL CULTURE

When artificial environment formed in the laboratory is in generally known at the same time as media. A media

Figure 5. Centrifuges.

Figure 6. Freezer.

comprises an appropriate source of energy for the cells which they can easily utilize and compounds which regulate the cell cycle. The choice of media is cell type specific and often empirical and there is no "all purpose" medium. It should provide many nutrients, buffering capacity, isotonic, and should be sterile. Characteristics and compositions of the cell culture media vary depending on the particular cellular Requirements. Important parameters include osmolarity, pH, and nutrient formulations.

4.1. Basic Components in the Culture Media

Most animal cell culture media are generally having following 10 basic components and they are as follows: Energy sources: Glucose, Fructose, Amino acids, Nitrogen sources: Amino acids.

The various types of media used for tissue culture may be grouped into two broad categories:

1) Natural media;
2) Artificial media.

4.1.1. Natural Media

These media consist solely of naturally occurring biological fluids and are of the following three types:

1) Clots;
2) Biological fluids;
3) Tissue extracts.

4.1.2. Artificial Media

Different artificial media have been devised to serve one of the following purposes:

1) Immediate survival (a balanced salt solution, with specified pH and osmotic pressure is adequate);
2) Prolonged survival (a balanced salt solution supplemented with serum, or with suitable formulation of organic compounds);
3) Indefinite growth;
4) Specialized functions.

4.2. A Variety of Artificial Media Developed for Cell Cultures May Be Grouped into the Subsequent Four Classes

1) Serum containing media;
2) Serum free media;
3) Chemically defined media;
4) Protein free media.

5. CULTURE ENVIRONMENTS

One of the major advantages of cell culture is the capability to manipulate the physicochemical (*i.e.*, temperature, pH, osmotic pressure, O_2 and CO_2 tension) and the physiological environment (*i.e.*, hormone and nutriaent concentrations) in which the cells proliferate. Culture environment is a very responsible for cell growth and their maintenance. (**Invitrogen Cell Culture Basics**). Some specific part discussed below:

5.1. pH

Most normal mammalian cell lines grow well at pH 7.4, and there is very little variability among different cell strains. However, some transformed cell lines have been shown to grow better at slightly more acidic environments (pH 7.0 - 7.4), and some normal fibroblast cell lines prefer slightly more basic environments (pH 7.4 - 7.7). In laboratory pH control by pH meter (**Figure 7**).

5.2. CO_2

CO_2-bicarbonate based buffer. For the reason that the pH of the medium is dependent on the delicate balance of dissolved carbon dioxide (CO_2) and bicarbonate (HCO_3), changes in the atmospheric CO_2 can alter the pH of the medium. Most researchers usually use 5% - 7% CO_2 in air; 4% - 10% CO_2 is common for most cell culture experiments. However, each medium has a recommended CO_2 tension and bicarbonate concentration to achieve the correct pH and osmolality; refer to the media manufacturer's instructions for more information. Inside laboratory condition CO_2 concentration controlled by CO_2 incubator shaker (**Figure 8**).

5.3. Temperature

The majority human and mammalian cell lines are maintained at 36˚C to 37˚C for optimal growth while Avian cell lines need 38.5˚C in favor of maximum growth. Even though these cells can also be maintained at 37˚C, they will grow further slowly but Cell lines derived from cold-blooded animals (e.g., amphibians, cold-water fish) bear an extensive temperature vary between 15˚C and 26˚C.

6. TYPES OF CELLS

On the basis of morphology or functional characteristics

Figure 7. pH meter.

Figure 8. Incubator shaker.

three type cell considered for cell culture.

6.1. Epithelial Cell

Attached to a substrate and appears flattened and polygonal in shape (**Figure 9**).

6.2. Lymphoblast Cell

Cells do not attach; remain in suspension with a spherical shape (**Figure 10**).

6.3. Fibroblast Cell

Cells attached to a substrate; appears elongated and bipolar (**Figure 11**).

7. PROCEDURE OF CELL CULTURE

7.1. Primary Cell Culture

Primary cell culture is first cultivation of cell in synthetic condition [2]. Primary cultures, which be obtained straight forwardly from an animal furthermore be capable of maintain the Differentiated state for an undersized period (2). Three Basic Steps of primary tissue culture.

- Isolation of tissue.

Disaggregation of cells—[1] Chemical disaggregation (2) Mechanical disaggregation (**Figure 12**).

- Incubation in addition to growth.

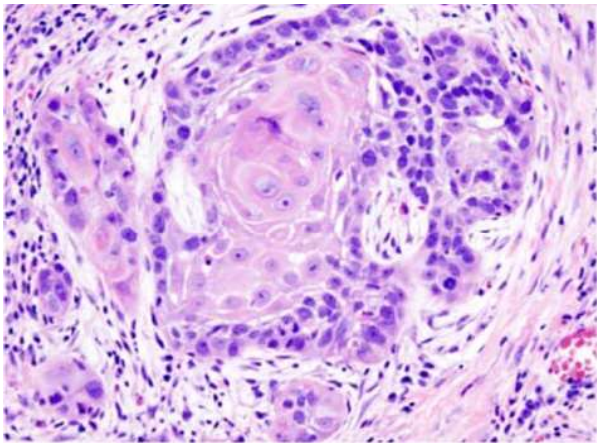

Figure 9. Epithelial cell.

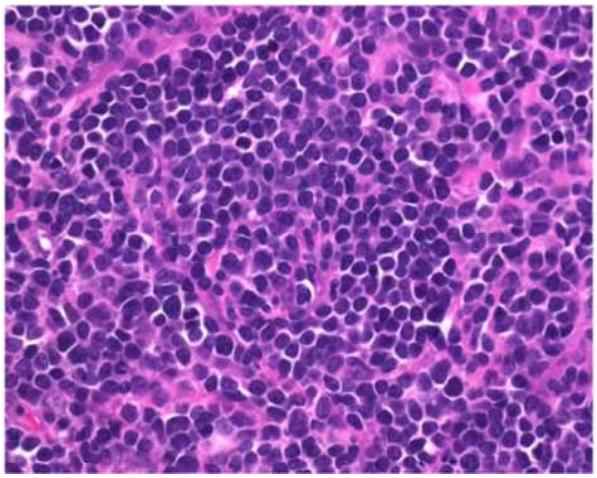

Figure 10. Lymphoblast cell.

7.2. Subculture (Passaging)

In animal cell culture a subculture is a new cell culture made by transferring some or all cells from a previous culture to fresh growth medium. This action is called subculturing or passaging the cells [3].

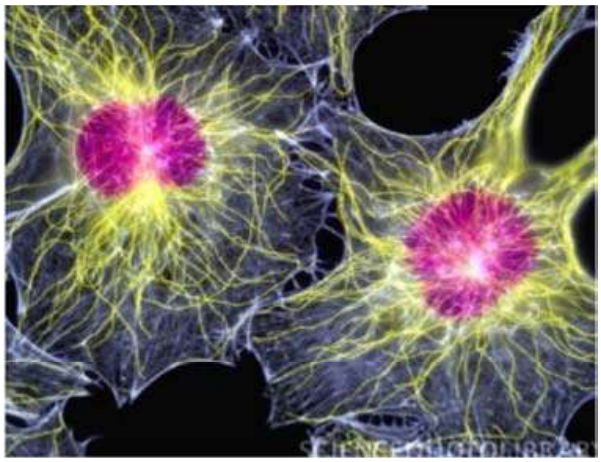

Figure 11. Fibroblast cell.

Figure 12. Tissue homogenizing.

7.3. Monolayer Culture

At what time the bottom of the culture vessel is covered by means of a continuous layer of cells, frequently one cell in thickness, they are referred to as monolayer cultures [4].

7.4. Suspension Cultures

A few of the cells which are non-adhesive e.g. cells of leukemia or convinced cells which can be mechanically kept within suspension, can exist propagated in suspendsion. There are certain applications in propagation of cells by suspension culture process.

7.5. Type of Cell Culture

7.5.1. Anchorage Dependent Cell Culture

Cells shown to necessitate attachment for growth are set to be Anchorage Dependent cells. The Adherent cells are typically derived from tissues of organs such as kidney where they are immobile in addition to embedded in connective tissue. They cultivate adhering to the cell culture.

7.5.2. Anchorage Independent Cell Culture

Cells which make not required attachment for growth or do not attach to the surface of the culture vessels are anchorage independent cells/suspension cells [5]. Each and every one suspension cultures are derived from cells of the blood system for the reason that these cells are furthermore suspended in plasma in vitro e.g. lymphocytes [6].

8. CELL LINE

A cell line arises from a primary culture at the time of the first successful subculture. The term cell line implies that cultures from it consist of lineages of cells originally present in the primary culture [3].

On the basis of the life span of culture, the cell lines are categorized into two types.

8.1. Finite Cell Lines

Cell lines which encompass a restricted life span and exit from beginning to end a restricted number of cell generations (frequently 20 - 80 population doublings) be well-known as finite cell lines.

8.2. Continuous Cell Lines

Cell lines transformed under laboratory surroundings or in vitro culture environment give rise in the direction of continuous cell lines. The cell lines demonstrate the property of ploidy (aneupliody or heteroploidy), lack of contact inhibition and anchorage dependence. They produce in monolayer or suspension type. The growth rate is fast and doubling-up time is 12 - 24 hours.

9. PRESERVATION AND STORAGE

Cryopreservation

Liquid N_2 is used to preserve tissue culture cells, either in the liquid phase (–196˚C) or in the vapor phase (–156˚C). Toward minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent which lowers the freezing point, such as glycerol or DMSO, is added. A typical freezing medium is 90% serum, 10% DMSO. In addition, it is best to use healthy cells that are growing in log phase and to replace the medium 24 hours before freezing. Also, the cells are slowly cooled from room temperature to –80˚C to allow the water to move out of the cells before it freezes. The effect of the isopropanol is to allow the tubes to come to the temperature of the freezer slowly, at about 1˚C per minute. Cells are stored at liquid nitrogen temperatures because the growth of ice crystals is retarded below –130˚C [7,8] **(Figure 13)**.

10. CELL CULTURE POSSIBLE PROBLEMS

Protection of aseptic condition is one of the most complex challenges in tissue culture there are quite a lot of rout to contamination which includes malfunction in the sterilization procedures used for glassware & pipettes, particulates cross contamination of air inside the room, weakly maintained incubation, inappropriate handling.

10.1. Cell Culture and Cross-Contamination

Cell line cross-contamination can be a trouble for scientists working through cultured cells. Studies propose anywhere starting 15% - 20% of the instance; cells used in experiments have been misidentified or contaminated with another cell line [9,10] troubles with cell line cross-contamination have even been detected in lines from the NCI-60 panel, which are used regularly for drug screening

Figure 13. Liquid n_2 tank.

studies [11,12]. Number of contaminants, from microbeological, most of the fungal and bacterial contamination quickly overwhelms a culture and is usually visible to the naked eye within a short period of time.

10.2. Bacteria and Fungi Contamination

Microorganisms are the most frequent cell culture contaminators for the reason that they thrive in all environments and are effortlessly moveable from any exterior source such as laboratory instruments, gloves or clothing toward the cells. They grow fast and can be easily seen beneath the microscope. Illustration indicators of contamination include media color change because of a shift in pH, turbidity, presence of non-cellular material, cell vacuolization, or constant cell lysis and death [13].

10.3. Mycoplasma Contamination

Mycoplasma is extremely small bacteria-like organisms that are difficult contaminators of cell cultures. Mycoplasma has the capability to alter the host cell culture's morphology, function, metabolism, growth and attachment to the culture vessel. For that reason, the integrity of any experiments performed with mycoplasma-contaminated cells is doubtful because the host cells are not performing normally [14,15].

10.4. Chemical Contamination

Chemical contamination is the occurrence of any lifeless substance in the cell culture that causes unfavorable effects to the cells. This may comprise impure media, serum or even water which may contain unwanted endotoxins or organic compounds if not purified. In addition, toxic levels of even vital nutrients can be harmful, and chemical contaminants may possibly also come from unclean storage vessel.

10.5. Antibiotics

Accurate working perform, antibiotics should not be used for the routine maintenance of cell lines. In the existence of antibiotics, contamination may be suppressed, but could alter the phenotype or genotype of the cells. Antibiotics are toxic and can alter the biochemistry of the cells. If an infection is not understandable, for the reason that it has been suppressed but not eliminated by antibiotics, all other cultures in the laboratory are at hazard [16].

11. CONCLUSIONS

At present make use of animal cell culture has undergone a significant spreading out from being a purely investigational procedure to become a conventional technological module of a lot of aspects of biological research. This review summarizes conceptual background and basic techniques of culturing animal cells in a format that is readily easy to get to all researchers in the field. Animal tissue culture functions in therapeutic filed are vast. The evaluation of the cell's response to chemicals, or as a tool to produce cellular-derived protein products that really helps in medical improvement. An animal tissue culture provides an approach to manufacture monoclonal antibody that makes it potential to produce antibody that have specificity controlled toward pathogen. In the field of animal cell culture discovery leads to the opportunity of curing various diseases such as AIDS and cancer. Additional application of animal tissue culture gives a conventional, quick and approachable method for manufacture of well-tolerated and valuable vaccines. Cell culture seems to come with a lot of important towards medical progression. Other than that, there are still differences between in vitro and *in vivo* system during drug testing and organ transplantation which make it not totally trustworthy.

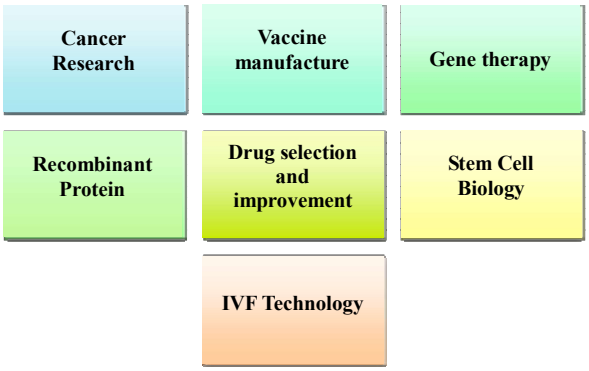

Figure 14. Animal cell culture application.

12. SIGNIFICANCE OF ANIMAL CELL CULTURE

The animal cell cultures are used for a diverse range of research and development. These areas are as **Figure 14**.

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REFERENCES

[1] Surachi, U. (1999) Basic techniques in animal cell culture. Drug Delivery System Workshop, Bangkok.

[2] Freshney, R.I. (2006) Basic principles of cell culture. John Wiley & Sons, Hoboken.

[3] Freshney, R.I. (2005) Culture of animal cells: A manual of basic technique. 5th Edition, Wiley, New York.

doi:10.1002/9780471747598

[4] Phelan, M.C. (1996) Techniques for mammalian cell tissue culture. *Current Protocols in Molecular Biology*, **Appendix 3**, Appendix 3B.

[5] Chen, J.-Y., Penco, S., Ostrowski, J., Balaguer, P., Pons, M., Starrett, J.E., Reczek, P., Chambon, P. and Gronemeyer, H. (1995) RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *The EMBO Journal*, **14**, 1187-1197

[6] Glade, P.R. and Hirschhorn, K. (1970) Products of lymphoid cells in continuous culture. *The American Journal of Pathology*, **60**, 483.

[7] Hay, R.J. (1978) Preservation of cell culture stocks in liquid nitrogen. *TCA Manual* 4, 787-790. doi:10.1007/BF00918397

[8] Freshney, R.I. (1994) Culture of animal cells. In: Freshney, R.I., Ed., *A Manual of Basic Technique*, Wiley-Liss, New York, 387-389

[9] Drexler, H.G. and Uphoff, C.C. (2000) Contamination of cell culture, mycoplasma. *Encyclopedia of Cell Technology*, **1**, 609-627.

[10] Cabrera, C.M., *et al.* (2006) Identity test: Determination of cell line cross-contamination. *Cytotechnology*, **51**, 45-50. doi:10.1007/s10616-006-9013-8

[11] Chatterjee, R. (2007) Cell biology: Cases of mistaken identity. *Science*, **315**, 928-931. doi:10.1126/science.315.5814.928

[12] Liscovitch, M. and Ravid, D. (2006) A case study in misidentification of cancer cell lines: MCF-7/AdrR cells (re-designated NCI/ADR/RES) are derived from OVCAR-8 human ovarian carcinoma cells. *Cancer Letters*, **245**, 350-352. doi:10.1016/j.canlet.2006.01.013

[13] Cobo, F., Stacey, G.N., Hunt, C., *et al.* (2005). Microbiological control in stem cell banks: Approaches to standardization. *Applied Microbiology and Biotechnology*, **68**, 456-466. doi:10.1007/s00253-005-0062-2

[14] McGarrity, G.J. (1976) Spread and control of mycoplasmal infection of cell cultures. *In Vitro Cellular & Developmental Biology—Plant*, **12**, 643-647. doi:10.1007/BF02797464

[15] McGarrity, G.J., Vanaman, V. and Sarama, J. (1984) Cytogenetic effects of mycoplasmal infection of cell cultures: A review. *In Vitro Cellular & Developmental Biology—Plant*, **20**, 1-18. doi:10.1007/BF02633326

[16] Masters, J.R. and Stacey, G.N. (2007) Changing medium and passaging cell lines. *Nature Protocols*, **2**, 2276-2284. doi:10.1038/nprot.2007.319

