LESSON 4: ANIMAL TISSUE CULTURE MEDIA

Dear students, In today's class our discussion will be on animal tissue culture media which are used for the preparation of plant and animal cell cultures, the similarity lies between these two techniques is that these two are the media is used.

Without media we cannot culture plant cell and animal cell.

Objectives

- Introduction to animal tissue culture media,
- Media ingredients, types of media,
- Different commonly used Medias.

Introduction

Culture Media Containing Naturally Occurring Ingredients

Inspite of vast use of chemically defined media in tissue culture, it is still necessary inmost undertakings to depend on naturally occurring substances derived from the organism. The various kinds of such media, which are used, may be:

(i) Blood plasma, (ii) blood serum, (iii) tissue extract and (iv) complex natural media.

Blood Plasma

The first tissue culture was done by Harrison (1907) in clotted frog lymph. Burrows (1910) substituted a coagulum prepared from chicken plasma. After many years it was found that plasma provided a complete nutrient in which cells could survive and multiply slowly for extended periods under conditions that resembled in many respect those found in body. Plasma is still being used to advantage for the following purposes:

1. To provide a nutritive substrate and a supporting structure for many types of cultures, Just as it also provides a matrix for new cells during' the repair of injury in the body.
2. To provide a means of conditioning the surface t>f glass for better attachment of cells.
3. To provide a means of protecting cells and tissues from excessive traumatic damage during sub culture. To provide some degree of protection from sudden changes in the environment at times fluid change.
4. To provide localized pockets of conditioned medium around cells. For culture work, plasma from the adult chicken is preferred to mammalian plasma because it forms a clear, solid coagulum even when diluted several times. Mammalian plasma is either too opaque for good optical work or else it fails to produce solid clots.

The plasma is obtained by centrifugation of whole blood before coagulation 'takes place. The tissue is then placed in a small quantity of the plasma and coagulation encouraged by addition of a small amount of tissue extract or thrombin. This is done because the cells in culture require a solid support for continued growth and activity. In case of fowl the blood is obtained from the wing, heart or carotid artery. In case of mammals it is obtained from carotid artery and heart.

Blood Serum

Blood serum (plasma minus fibrinogeri) with or without other nutritive substances may be used either as the entire culture medium or as the fluid phase of a medium consisting partly of a plasma coagulum. For many years it was assumed that whole serum was toxic, that plasma was useful only as a supportive structure and that the nutritive requirements of the cells were supplied by the embryo extract that was usually added to the medium. Eventually, however, it was found possible to cultivate tissues in serum alone without plasma or tissue extract. In 1928, des Ligneris reported the successful cultivation of many mammalian tissues in diluted serum and later Parker (1933, 1936) cultivated chick tissues in serum. Simms (1936), and Simms and Sanders (1942) introduced an

ultrafiltrate of serum that was used as a basal medium for many purposes including the propagation of viruses. Fischer and co-workers (1948) stressed the importance of the low molecular weight growth factors provided by serum. As some of the more elaborate chemically. Defined solutions were developed, it was found that they had to be supplemented with 10 to 20 per cent serum to provide completely adequate medium for the continuous propagation of established cell lines and freshly explanted tissues for extended periods. Harris (1959) concluded that medium 199 and NCTC-109, as well as the simpler basal medium of Eagle (1955) are all deficient in one or more factors that occur in serum dialysate and are essential for the growth and maintenance of chick skeletal muscle fibroblasts. Thus serum does provide some of the growth factors or some of the physical conditions, or both, that are presently lacking in synthetic media.

Preparation of Chicken Serum

The fluid plasma from which the serum is prepared should be completely coagulated. The plasma is coagulated deliberately by adding to each tube a drop or two of embryo tissue extract or an equivalent amount of thrombin and leaving the tubes to incubate for several hours at 37° C. The coagulated plasma is broken up into fragments and it is ground in a mortor with sterile quartz sand. After grinding the serum is separated by centrifugation.

Preparation of Mammalian Serum

The mammalian blood is left at room temperature for an hour. The clot is removed by a glass rod and then centrifuged for 30 minutes at 3000 rpm and the serum is separated.

Serum Free Media

The use of serum in culture media is not so common because it has following disadvantages:

1. The quality of serum varies from batch to batch and deteriorates within one year. Therefore every batch of serum needs fresh testing.
2. If more than one cell types are used, each may require different serum batch, therefore, many batches are to be maintained and co-culturing may be difficult.
3. The demand of serum usually exceeds the supply for a variety of reasons.
4. When cell culture is used for downstream processing to recover cell products, the presence of serum is an obstacle to purification; Serum increases the cost of the medium.
5. Serum may stimulate undesirable growth and may even inhibit growth in some cases.

Advantage of Serum Free Media

1. It has the ability to make a medium selective for a particular cell type, since each cell type appears to require a different recipe.
2. It has high degree of purity of reagents and water.
3. It needs high degree of cleanliness of all apparatus.

How to Develop Serum Free Media?

1. A known recipe for a related cell type may be used.
2. Individual constituents of the serum may be altered, until the medium is optimized.
3. The development and assessment constituent is a time consuming^ process. Sometimes it takes about three years for development of a new medium.4. In the second approach, an existing medium like RPMI 1640 or Ham's F12 or DMEM (Dulbecco's modified minimal essential medium) is taken and a shorter list of constituents like selenium, transferrin, albumin, insulin, androgen, hydrocortisone, estrogen, etc. is use! for manipulation.

Tissue Extracts

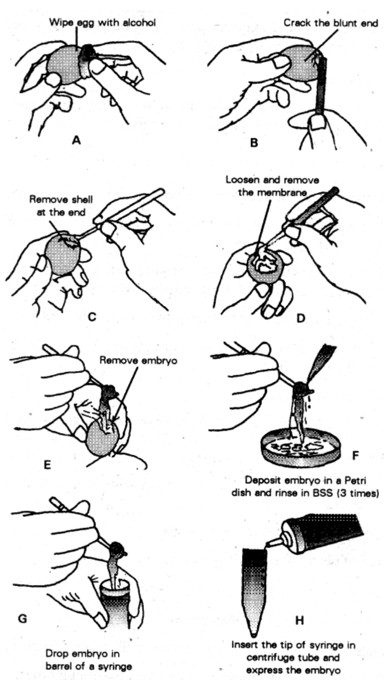

Fig.1. Different steps involved in the preparation of embryo extract.

Carrel (1912) discovered that embryo tissue extract had remarkable powers of promoting eel growth and multiplication in cultures of connective tissue cells from chick embryo heart. Since Carrel's experiments, there have been many attempts to determine the chemical nature of the substances responsible for the stimulating effect of embryo extract.

Baker and Carrel (1926) obtained active fractions of the extract by precipitation with carbon dioxide and found that the activity was concentrated largely in the protein portion containing nucleoproteins and glycoproteins. This was further observed (Carrel and Baker, 1926) that proteoses and higher molecular weight protein degradation products also had very potent growth promoting properties. Growth promoting activity appeared to be associated particularly with fractions containing predominantly nucleoproteins of the ribonucleic acid type. Fractions relatively high in deoxyribonucleic acid appeared much less active. Active nucleoprotein fractions from adult chicken heart, brain, liver and spleen have been used but no indications of organ specificity were observed. The activity of the fraction also did not depend on their total nucleic acid content or on the age of the individuals from which they were prepared.

Preparation of Embryo Extract

Chick embryo extract is made from 10 to 11 days old embryos (before the calcifying mechanisms have become too active). The embryos are removed from the egg, then homogenized in a motordriven homogenizer. Six to eight embryos and a measured quantity of balanced salt solutions (e.g., 2.0 ml per embryo) may be processed at one time. After homogenization, it is centrifuged and further diluted 10 to 20 times. Embryo extract may be stored indefinitely after it has been dried from the frozen state.

Complex Natural Media

Some of the complex natural media are as follows:

Supplemented Hanks-Simms medium

Weller and co-workers (1952) in their earlier work with polioviruses made excellent use of a combination of 3 parts Hanks's balanced salt and 1 part Simm's ox serum ultrafiltrate. For rollertube cultures of various human and animal tissues (embryonic, infant and adult), the complete medium consisted of Hanks-Simms solution (85%), beef embryo extract (10%), horse serum inactivated at 56° C for 30 minutes (5 to 20%), penicillin (50 jig/ml) and streptomycin (50 Hg/ml).

Supplemented bovine amniotic fluid medium

Milovanic and co-workers (1957, 1958) used the following medium:

Bovine amniotic fluid (37.5%), horse serum inactivated at 56° C for 30 minutes (20%), bovine embryo extract (5%), Hanks balanced salt solution (37.5%), streptomycin (lOOu /ml), penicillin (lOOu/ml) and mycostatin (lOOu/ml).

Serum-Supplemented Yeast Extract Medium

Various human cell lines and strains from other species have been successfully grown in this medium. The medium consists of:

Yeast extract medium (76 parts) [composition: 10 parts of 1% solution of Difco's yeastolate, 2.5 parts of 10% glucose solution and 87.5 parts of Hanks balanced salt solution], human serum (20 parts), and 1.4 per cent sodium bicarbonate solution (4 parts).

Serum-supplemented lactalbumin hydrolysate and yeast extract medium

This medium consisted of: Earle's saline containing lactalbumin hydrolysate (0.5%), yeast extract (0.1%), and human or ox serum (10-20%).

Chemically Defined Media

Earlier, the nutritive media for the cultivation of animal cells in vitro were derived from the organisms and consisted of blood plasma, blood serum, tissue extracts, etc. The complexity and variability of these naturally occurring materials made it difficult to use them in experiments designed to determine the nutritive substances required by the cells and the effect of particular substance upon them.

The first attempts to devise chemically defined media were rnade by Lewis and Lewis, Baker and Carrel, Vbgelaar and Erlichman. Carrel's discovery (1912) that extracts of embryonic tissues contained an abundance of growth promoting substances made it possible for the first time to propagate animal cells indefinitely and stimulated the search for the particular substances responsible.

Correl and Baker (1926) reported that Witt's peptone, proteoses and other degradation products when used with serum or plasma provided essential nutrients for fibroblasts, epithelial cells and blood monocytes. Vogelaar and Erlichman (1933) prepared a feeding solution consisting of irradiated beef plasma, Witte's peptone, hemin, cystine, insulin, thyroxine, glucose and the salts of tyrode's solution. They kept a strain of fibroblasts from the human thyroid in a state of active proliferation for 3 months. Baker later on added vitamin A and D, ascorbic acid, glutathione and 10% serum for the cultivation of epithelial cells. For blood monocytes certain B-vitamins were also added.

Fischer's supplementary medium, V-614 consisted of dialyzed plasma, dialyzed embryo extract, Tyrode's solution, glucose, fructose diphosphate, glutamine, cystine, glutathione, tryptophan, phenylalanine, threonine, isoleucine, leucine, valine, arginine, histidine, and lysine. Fischer further suggested that different cell types may have different amino acid requirements. Thus, osteoblasts were more sensitive than myoblasts to lysine deficiency. He also stated that animal tissue cultivated outside the body require certain nutrients not required by the intact animal.

White (1946) for the first time made serious attempt to cultivate animal tissues in a complete solution of known composition. He reported a feeding solution of 20 ingredients of known composition that supported relatively large masses of chick embryo heart tissue in a state of functional survival for several weeks.

Morgan et ai. (1950) published the composition of a more adequate medium No. 199. The medium included almost complete complement of amino acids and vitamins as well as several nucleic acid constituents and certain intermediary metabolites and accessory growth factors. Fischer's group included Glutamine because of its importance place in the medium. Tween-80 was used as a water-soluble source of fatty acid and as means of dissolving the fat soluble vitamins and cholesterol in a minimal concentration of ethyl alcohol. The medium also contained the salts of Erie's balanced salt solution, glucose, ferric nitrate and phenol red.

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Table 24.1 . Different commonly used media (with serum) their constituents (all quantities in mg/l) , for animal cell and tissue culture, and

Component	Eagle's MEM	Dulbecco's modification	Ham's F12	CMRL 1066	RPMI 1640	199	L15 Fischer's Way-mouth MB • 752
Amino acids							
L-alanine	-	-	8.90	25.0	-	25.0	225
L-arginine (free base)	-	-	-	-	200	-	550
L-arginine-HCl	126	84.0	211	70.0	-	70.0	15.0 75.0
L-asparagine	-		-	-	50.0	-	-
L-asparagine-H_2O	-	-15.0	-	-	-	260	11.4
L-aspartic acid	-	-13.3	30.0	20.0	30.0	-	60.0
L-cysteine (free base)	-	-	-	-	-	-	120 61.0
L-cysteine	24	48.0	-	20.0	50.0		15.0
L-cysteine. 2Na	-	-	-	-	-	23.7	23.7
L-cysteine. $HCl.H_2O$	-		35.1	260	-	0.0987	-
L-glutamic acid	-	-14.7	75.0	20.0	66.8	-	150
L-glutamine 292	584	146	100	300	100	300 .	200 350
Glycine	-	30.0	7.50	50.0	10.0	50.0	200 50.0
L-histidine (free base) L-histidine $HCl.H_2O$	42.0	42.0	21.0	20.0	15.0	21.9	250 128 / 81.1
L-hydroxyproline	-	-	-	10.0	20.0	10.0	-
L-isoleucine	52.0	105	3.94	20.0	50.0	20.0	125 75.0 25.0
L-leucine	52.0	105	13.1	60.0	50.0	60.0	125~ 30.0 50.0
L-lysine. HC1	73.1	146	36.5	70.0	40.0	70.0	93 50.0 240
L-methionine	15.0	30.0	4.48	15.0	15.0	15.0	75.0 100 50.0
L-phenylalanine	33.0	66.0	4.96	25.0	15.0	25.0	125 67.0 50.0
L-proline	-	-	34.5	40.0	20.0	40.0	50.0
L-serine	-	42.0	10.5	25.0	30.0	25.0	200 15.0
L-threonine	48.0	95.0	11.9	30.0	20.0	30.0	300 40.0 75.0
L-tryptophan	10.0	16.0	2.04	10.0"	5.0	10.0	20.0 10.0 40.0
L-tyrosine	36.0	72.0	5.40	40.0	20.0	-	40.0

Component	Eagle's MEM	Dulbecco's modi-fication	Ham's F12	CMRL 1066	RPMI 1640	199	US	Fischer's	Way-mouth MB 752
L-tyrosine. 2Na		.		-	-	49.7	373	74.6	
L-valine	47.0	94.0	11.7	25.0	20.0	25.0	100	70.0	65.0
Vitamins									
L-ascorbic acid		...		50.0	.	0.05		.	17.5
						0			
Biotin			0.0073	0.010	0.200	0.01		0.010	0.02
						0			
D-Ca-	1.00	4.00	0.480	0.010	0.250	0.01	1.00	0.500	1.00
pantothenate						0			
Calciferol		...		.	.	0.10		.	.
						0			
Choline chloride	1.00	4.00	14.0	0.500	3.00	0.50	1.00	1.50	250
						0			
Folic acid	1.00	4.00	1.30	0.010	1.00	0.01	1.00	10.0	0.40
						0			
i-inositol	2.00	7.20	18.0	0.050	35.0	0.05	2.00	1.50	1.00
						0			
Nicotinamide	1.00	4.00	0.04	0.025	1.00	0.02	1.00	0.50	1.00
						5			
Pyridoxal. HC1	1.00	4.00	0.062	0.025	.	0.02		0.50	
						5			
Riboflavin	0.10	0.40	0.038	0.010	0.20	0.01		0.50	1.00
						0			
Thiamin. HC1	1.00	4.00	0.34	0.010	1.00	0.01		1.00	10.0
						0			
Vitamin Bj2			1.36	-	0.005	-		-	0.20
Pyridoxine HC1			0.062	0.025	1.00	0.02		.	1.00
						5			
Cholesterol		-		0.200	-	-		-	
Para-amino		...		0.050	1.00	0.05		.	.
benzoic acid						0			
fJicotinic acid		...		.	. •	0.02		.	.
						5			
Menaphthone		...		.	.	0.01		.	.
sodium bisulphite						9			
$3H_20$									
Dl-A tocopherol		...		-	-	0.01		-	-
PO_4.2Na									

Component	Eagle's MEM	Dulbecco's modi-fication	Ham's F12	CMRL 199	1066	RPMI 1640	L15	Fischer's	Way-mouth MB 752
1 tain A acetate		.	.		0.11		.	.	
					5				
I Riboflavin		-	-				0.10	-	
1 $P0_4$.2NaThiamin 1 Mio$P0_4$,$2H_20$		-	-		-		1.00	• -	
I Inorganic salts									
I $CaCI_2$(anhyd.)	200	200	-		200			-	
I $CaCI_2 2H_20$		-	44.0		186		186	91.0	. 120
1 $Fe(N03)3.9H_20$		0.10	-		0.10		-	-	
KCI	400	400	224	400	400	400	400	400	150
KH_2P0_4		-	-		60.0		60.0	80.0	
$MgCI_2.6H_20$		-	122		. ,		-	240	
1 $MgS0_4.7H_20$	200	200	-	200	100	200	400	121	200
' NaCI	6,800	6,400	7,599	6,799	6,000	8,00	8,00	8,000	6,000
					0		0		
$NaHC0_3$	2,200	3,700	1,176	2,200	2,200	350	-	1,125	2,240
$Na_2H_2P0_4.H_20$	140	125	-		140		-	78.0	
Na_2HP0_4 (anhyd)		-	-		47.5		190	60.0	
$Na_2HP0_4.7H_20$		-	268		1,512		-	566	
$CuS0_4.5H_20$		-	0.0024 9	-	-	-	-	'	
$FeS0_4.7H_20$		-	0.834		.		-	-	
$ZnS0_4.7H_20$		-	0.863		.		-	-	
$CaN0_3.4H_20$		-.	-		100			-	
Other components									
D-glucose	1,000	4,500	1,802	1,000	2,000	1,000		1,000	5,000
D-galactose		-	-		-		9,00 0	-	
Lipoic acid		-	0.21		-		-	-	
Phenol red	10.0	15.0	12.0	20.0	5.00	17.0	10.0	5.00	10.0
Sodium pyruvate		110	110		.		550	-	
Hypoxanthine		-	4.10		0.30		-	-	
Linoleic acid		0.084	-		' -		-	25.0	

Examples of selective media (modified from Freshney, 1987)

Cells of cell line Medium

LHC

MCDB 130 MCDB 202 MCDB 110 MCDB 153 MCDB 170 WAJC 404 WAJC 404 KITES

Bronchial epithelium

Endothelium

Fibroblasts

Fibroblasts

Keratinocytes

Mammary epithelium

Prostate epithelium (human)

Prostate epithelium (rat)

Small cell lung cancer

Selecting a suitable medium* (modified from Freshney, 1987)

• Medium with serum

Cells or cell line	Medium	Ser	Serum-free
Chick embryo	Eagle's	CS	MCDB 202
Chinese hamster	Eagle's	CS	MCDB 301
Chondrocytes	F12	FB	Adolphe et.
Continuous cell lines	Eagle's MEM	CS	199, Waymouth, 1984
			MB752/1.M D7505/1
			CMRL 1066
Endothelium	DME, 199,	FB	MCDB 130
Fibroblasts	Eagle's	CS	MCDB 11
Glial cells	MEM, SF12	FB	Michler et al.,
Glioma	MEM, SF12	FB	
HeLa cells	Eagle's	CS	Blakeretal.,
Hemopoietic cells	RPMI 1640, Fischer's	FB	Iscover, 1984
Human diploid	Eagle's MEM	CS	MCDB 11 0,202
Human leukemia	RPMI 1640	FB	Breitman et al., 1984;
'			Iscover, 1984

Other Tissue Culture Media

Medium No. 612

It was observed that medium 199 had a very high oxidation-reduction potential. Healy and co-workers attempted to bring it near the physiological range by increasing very considerably the levels of three reducing agents present in the medium namely cysteine, glutathione, and ascorbic acid. Cysteine and glutathione increase the rate of cell multiplication and improve the appearance of the cells; the ascorbic acid has no apparent effect. Healy increased the levels of cysteine and glutathione 2,600 and 200 times respectively. He designated this medium as medium No. 612.

Medium No. 635

This medium was devised by omitting from medium No. 612 all the purées, pyrimidines as well as adenosinetriphosphate, adenylic acid, ribose and dexyribose. This medium gives a better growth response than that of medium No. 612, but the cultures rarely survive more than 40 days.

Medium No. 858

This medium includes all the deoxyribonucleosides, coenzymes, and sodium glucoronate in addition to medium No. 635. This medium contains only L-form amino acids, and yields ten-fold increase in the population of culture cells in 7 days. Addition of 10 to 20 per cent of horse serum yields 20 to 30 fold increases in 7 days.

Medium No. 866

Medium No. 866 is similia to that of medium No. 858 supplemented with three fatty acids namely linoleic acid, loosened acid and arachidonic acid. This medium does not improve the growth of culture cells.

Medium CMRL-1066

This medium is identical to medium No. 858 except that the fat-soluble vitamins (A, D, E and K), ferric nitrate and sodium bicarbonate included in medium No. 858 have been omitted. Five B vitamins (thiamine, riboflavin, niacin, niacinamide and sodium pantothenate) were added. It also contains 0.02 mg per cent n-butyl parahydroxybenzoate, which offers protection from certain moulds. Antibiotics are added according to the need.

Eagle found that 12 amino acids are essential for the growth of both L-strain and Hela-strain cultures even in the presence of small quantities of whole or diazlyzed serum. These amino acids are arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. Recent attempts have been made to determine the vitamin requirements of cells cultivated in chemically defined media containing dialzed serum.

Glucose is generally added to tissue culture media as an energy source, other carbohydrates have also been tested. Harris and Kutsky have shown that chick heart fibroblasts can utilize D-fructose or D-mannose as well as D-glucose.

Interesting studies have also been made of the inorganic requirements of cells in vitro. Harris found that carbon dioxide is beneficial for the outgrowth of cells from explanted chick embryo tissues. Eagle has shown that sodium, potassium, magnesium, calcium, chloride and H2P04 ions are essential for the survival of L-and HeLa cells.

Laboratory Facilities for Tissue Culture

For animal tissue culture, the following laboratory facilities/ methods are needed,

1. Szterile handling
2. 0.Incubation
3. P 4reparation of glassware, media and tissue

4. Wash-up
5. Sterilization
6. Storage

The clean area for handling should be available at one end of the room and the wash up and sterilization at the other end, with preparation, incubation and storage in between.

Tissue culture facilities (modified from Freshney, 1987)

Minimum requirements (essential)
Importantfeatures required (beneficial)

Other useful additions required

Sterile area: clean and quiet, no thorough traffic, separate from animal house and microbiological labs	Filtered air (air conditioning) Piped CC>2 and compressed air
Preparation area	Hot room with temperature recorder Store room for bulk plastic
Storage areas:	Microscope room Containment room for biohazard work.
liquids-ambient, 4°-20°C	Dark-room Liquid N_2 storage tank (500 1)
glassware (shelving)	Service bench adjacent to culture area

Current research work on animal tissue culture

Students now we knew about animal tissue culture but we need practical oriented research work whats going in world, Iam giving some web site address of journals:

Tissue engineering
Aris Persidis Description: Tissue and organ failure, produced as a result of injury or other type of...... CONTEXT: Tissue and organ failure, produced as a result of injury or other type of damage, is a major health problem, accounting for about half of the total annual expenditure in health care in the US. Treatment options include transplantation...... Nature Biotechnology 17, 508 - 510 (01 May 1999) Resources

Technological trends
SUMMARY: Products with a biotech bias, including a separator good for over 100 l.p.h.... CONTEXT: ...and high sample throughput. Stericup-VP filtersMillipore http://www.millipore.comRemove mycoplasma from tissue culture media Millipore claims that the Stericup-VP filter unit is the first vacuum filter cup that both removes...... Nature 415, 559 - 560 (31 Jan 2002) New on the Market

Note