MAMMALIAN CELL CULTURES

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PURPOSE

This experiment involves the growth of mammalian cell cultures established from embryos of pregnant mice. Growth parameters will be varied to determine the effect of each on cell growth. The purpose of this experiment is to determine the most economical way to establish and maintain a mouse cell line at Pembroke State University.

INTRODUCTION

Cell cultures are growths of cells that are taken from parent tissue by mechanical means. The first cell culture dates back to the early 1900's. In 1907 R. G. Harrison discovered that animal cells could grow and survive outside a living organism. Harrison was trying to prove that nerve fibers were outgrowths of single cells. He chopped up spinal cord and added it to clotted plasma in a growth chamber. The nerve cells grew and extended axons into the clot. His discovery proved cells could be grown in an artificial environment. Cell culturing involves the removal of cells from an organism and then placing them in a culture vessel. The appropriate nutrients, temperature, gaseous environment, and pH are supplied to the cells. The cells reproduce and soon release chemical signals to stop reproduction so the culture does not become overcrowded (Ahern, 83).

According to McKeehan mammalian cell culture research is a "two way study of the cell and its environment" (9). McKeehan gives four major goals in the culture of mammalian cells. These goals are (1)"To isolate certain cells that retain certain properties; (2)To determine the mechanism of action of the environmental factors that affect the behavior of the cells; (3)To re-establish the cells and their environment into a defined unit for the study of the responses and properties of the cells; (4)To re-establish the cells and their environment into tissues and organs;"(9). Cell cultures are

used in cell and molecular biology and also in the field of biotechnology. Mammalian cell cultures are used to study viruses, study the etiology of cancer, and to perform investigations in cell physiology (Moss and Solomon, 287).

Cultured cells are capable of secreting valuable by-products such as hormones, antigens, and enzymes. These products play an important role in diagnosing and treating diseases. Some cells produce interferon, a disease fighting agent. Human kidney cells produce urokinase which is an enzyme that is capable of dissolving blood clots (Woods, G4). Cell cultures are also used to measure the toxic effects of pharmaceutical compounds and pollutants that may be hazardous to the environment.

BACKGROUND

Before beginning an experiment it is beneficial to investigate how scientists are using cell cultures and any achievements being made using cell cultures. The European Collection of Animal Cell Cultures (ECACC) is a supplier of cells to people who need cells to find cures for diseases. The ECACC is doubling the size of its facilities and adding more staff in an effort to meet the demands for cells. The ECACC has a general collection of 1,000 different cultures taken from insects, fish, and mammals. The laboratory has 1,500 cultures from people with diseases caused by genetic defects. The lab also has hybridomas which are hybrid cultures that produce disease-fighting antibodies. The ECACC is located in the Public Health Laboratory Complex at Porton Down in Wiltshire (Economist, 77).

Scientists at John Hopkins University School of Medicine have established a

continuous cell culture of human cerebral cortical neurons. The cell line was established through cerebral cortical tissue taken from an eighteen month old patient with unilateral megalencephaly. Unilateral megalencephaly is a low-grade proliferation and migration disorder of neurons. A continuous cell culture is important in that it may help characterize individual types of central nervous system neurons. The great heterogeneity of the central nervous system has made it impossible to characterize the individual types of neurons until now (Ronnett, et al, 603).

Biophysicist David Stenger of the Naval Research Laboratory and his colleagues have been practicing what they call "organic gardening". They have created patterns of molecular soil that encourage cells to grow only in certain places. This means they can encourage cells to grow in any pattern or direction they decide. This capability was introduced in the late 1980's, but Stenger and his colleagues have evolved the technique to much higher precision. This capability promises the creation of devices for science and technology. The precise arrangement of cells may function in medicine because of the ability to copy the growth patterns of capillaries. This will offer a vascularized cellular gauze for repairing wounds (Amato, 1084).

The Synbiotics Corporation developed a new device called "artificial mice" in 1989. The artificial mice system is cheaper and more efficient than growing cells in living mice. The tube- like devices are used for growing animal and human cell cultures. The system involves hollow fibers strung through plastic cartridges. The

fibers simulate the body's capillary system and are used as a medium to give nourishment to the growing cells. The initial cell cultures are grown in live mice and are then transferred to the hollow fibers. A computer support system supplies the cells with oxygen, nutrients, and blood substitute. The computer support system also controls the temperature, gas exchange, and acidic balance (Kragen, B14).

Vista Biologicals, a cell-culture firm, uses stir tank and fermentation technologies to purify and produce animal cells. These cells are produced for researchers and pharmaceutical companies. The cells are used in drug-testing programs. The cells are also used in drugs such as in monoclonal antibodies (specialized immunological molecules targeted to attack cancerous cells). Unisyn Fibertec is a company that offers an alternative technology of mass production of animal cells. They use a fibrous matrix that allows cells to rapidly multiply. In 1990 Unisyn bought Vista Biologicals. Unisyn's president, Stan Yakatan, says " with both companies technologies they will be able to offer a complete service to manufacture and purify cell cultures" (Parsons, A14).

The achievements being made using cell cultures are very beneficial. Cultured cells have made it possible to reconstruct epidermis after it suffers damage. This new way to heal human burns was discovered while studying a mouse tumor called a teratoma. Howard Green of Harvard Medical School and his graduate student, James G. Rheinwald, found conditions that allow the proliferation of an unusual cell type. This cell type was colonies of squamous epithelial cells or keratinocytes. Keratinocytes are cells of the epidermis, surface of the oral cavity, pharynx,

esophagus, cornea, and vagina. After testing they found that human epidermal keratinocytes would also grow under these conditions and could be subcultured repeatedly. The cultures contained fibroblasts which are needed for the optimal proliferation of keratinocytes. Chlorea, an epidermal growth factor, was also included in the culture medium (Green, 96).

Mammalian cells are also being used to produce vaccines and diagnostic and therapeutic proteins. Cultured animal cells in vitro have replaced the long used eggs, tissue, and animals in the manufacture of viral vaccines (Hu and Peshwa, 65). According to Hu, "Among the nearly 20 protein drugs produced by biotechnology that were approved by the Federal Drug Administration during the last decade, 5 are derived from mammalian cell-based processes" (65). Mammalian cell cultivation is needed because the protein product needs " posttranslational modifications " like glycosylation and extensive disulfide bond formation (Hu and Peshwa, 65). One problem encountered with mammalian cells is the low level of productivity. This problem has been solved through the use of bioreactors. A roller bottle, the simplest type of bioreactor, is used for anchorage-dependent cells. These cells require surface attachment to survive and proliferate. Suspension cells can be cultivated in spinner flasks and then scaled up by moving to a conventional fermenter (Hu and Peshwa, 65). Conventional fermenters provide easy sterilization, inoculation, and harvesting. Twenty-five years ago a technical adaptation was introduced that allowed the growth of cells in the fermenters. Cells were mixed and allowed to adhere to DEAE-Sephadex beads. The beads were then suspended in the conventional fermenters.

Other adaptations have led to the use of gelatin and cellulose as adherent materials and collagen coating of the beads. A microcarrier fermenter allows cells to be in a homogenous environment where pH and oxygen are controlled. One microcarrier fermenter can replace a thousand roller bottles whose environments may not be uniform. A microcarrier provides a large surface area in a small area and can achieve a cell concentration of ten times that in a roller bottle (Hu and Peshwa, 66). Cell cultures are used to study diseases, produce valuable products (hormones, enzymes, vaccines), reconstruct epidermis, and many other useful investigations.

CULTURE SUCCESS

The growth of a successful culture relies on the maintenance of the culture and good aseptic techniques. To maintain a cell culture there must be periodic medium changes. The medium must be changed because cells use the nutrients in the medium for growth. Griffiths says, "In order to maintain a culture some additional feeding often has to be carried out either by complete, or partial, media changes.."(Griffiths, 38). The amount of time between these changes depends on the rate of growth and metabolism of a particular cell line. Four factors determine whether the medium needs to be replaced. A drop in pH will indicate the need to change the medium. Most cells will not grow at a pH of 7.0 to 6.5. A stable pH is maintained with 5% CO_2 .

As cells grow they generate CO_2 which leads to a CO_2 buildup. The CO_2 cannot diffuse out which produces an excess of H+ ions and a fall in pH (Griffiths, 40). Cell concentration is another factor because a high cell concentration uses up the

medium faster than a lower one. Different cell types will also determine when the medium should be changed. Normal cells will stop dividing at high cell density and will not deteriorate if left for two or three weeks. However, transformed cells and some embryonic cells will deteriorate at high cell densities if the medium is not changed daily. The cell morphology will also determine whether or not the medium needs to be changed. Any deterioration such as granularity around the nucleus, cytoplasmic vacuolation, and detachment of the cell from the substrate indicates the need for a medium change (Freshney, 130).

Another important factor in the success of a cell culture experiment is to avoid contamination. A sterile handling area is required when working with cultures. This area should be free of traffic or any other disturbances that may cause dust or drafts. If laminar flow hoods are not available a separate room or quiet corner of the lab should be used (Freshney, 15). Other ways to prevent contamination is to take any items that are not being used away form the work area. Materials should not be poured, but should be dispersed by pipette or transfer device. Any wastes should be discarded into a beaker with a funnel to avoid splash-back of materials. All solutions and instruments should be autoclaved before use. Instruments should be resterilized by alcohol burn and cool off during use. All flasks and bottles should be wiped with 70% alcohol before use. Work surface should be swabbed before and after each use with 70% alcohol and any spillage on work surface should be cleaned up immediately. Animals should be kept out of the culture lab (Freshney, 212). Talking should be kept to a minimum and a mask should be worn if you have a cold. Deep screw caps

should be used instead of stoppers. The necks and screw caps of bottles should be flamed before and after opening a bottle and before and after closing a bottle. Pipettes should also be flamed before use (Freshney, 44). A laminar flow hood should be used if available. Laminar flow hoods provide a constant flow of filtered air over work surface which prevents contamination. There are horizontal and vertical flow hoods. Horizontal give sterile protection to the culture and reagents. Vertical hoods give more protection to the operator. The primary filters of the flow hoods should be checked every 3-6 months. Flow hoods should be left running continuously to keep work area clean (Freshney, 46).

"Unwanted organisms may introduce multiple unmeasurable variables by producing metabolites, consuming nutrients or altering the metabolism or morphology of the other cells, and cause the investigator to draw erroneous conclusions, " according to Atkin (3). Some contaminants are bacteria, yeast, fungi, molds, and mycoplasms. A microbial contamination will cause a sudden change in pH and cloudiness in the medium. Contamination can be detected by checking materials by eye and microscope after each handling. The bacterial contaminants will appear in the spaces between the cells. Yeasts will appear as round particles that may bud off smaller particles. Fungi will appear as denser clumps with filamentous mycelia. Mycoplasma have no cell wall and are the smallest, simplest self-replicating procaryotes (Atkin, 3). Mycoplasmas may be detected using a fluorescent staining technique. The fluorescent dye binds to DNA revealing the mycoplasma filamentous pattern. If there is contamination pipettes should be discarded, hood should be

swabbed with 70% alcohol containing phenolic disinfectant. Contaminations should be recorded. If a new contamination occurs the culture, medium bottle, and other material should be discarded (Freshney, 207).

PROCEDURE

This project consists of taking embryos from pregnant mice. Growth parameters will be varied to determine the effect of each on cell growth. The purpose of this experiment is to determine the most economical way to establish and maintain a mouse cell line at Pembroke State University. Cell cultures will be made by mincing the embryos and incubating the cells in CO_2. The growth of the cells will be measured under standard conditions. Three growth parameters will be varied to determine the effect of each on cell growth. Nutrasweet will be added to determine the effect it has on the cells. Another variation will be the addition of serum to the media of some cells and the absence of it in the media of other cells.

One growth parameter is temperature. An increase in temperature will increase cell multiplication. Once a certain temperature is reached the temperature increase will become inhibitory to the cells. Cells that are taken from a warm-blooded animal are usually grown at a temperature of 37 C. For this particular experiment cells will be incubated at 37° C and 40 °C (Ham and McKeehan, 55).

Another parameter that will be varied is pH. The optimum pH of most cell lines is 7.4. However, there are cells that will survive at a pH range of 6.6 to 7.8. The cell type will determine the pH at which the cell line will grow best. Phenol red

is an indicator that is commonly used to test pH. The indicator is red at pH 7.4, orange at pH 7.0, yellow at pH 6.5, bluish red at pH 7.6, and purple at pH 7.8 (Freshney, 69). The pH of the cells will be raised by adding HCL to the media (Merchant, 9). The pH levels that will be included in this experiment are the pH of 6.0, the pH of 8.0, and the pH of 9.3.

CO_2 level is another parameter that will be varied. Cultured cells have a metabolic requirement for CO_2 and bicarbonate ion. Some cells are capable of producing a sufficient amount of CO_2 , but bicarbonate must be added to the medium (McAteer and Douglas, 135). The CO_2 levels of 0% and 5% are the variations in this experiment.

Nutrasweet will be added to determine the effect it has on the growth of the cells. The presence or absence of serum in the media is another variation.

HYPOTHESIS

The cells were expected to grow best under the conditions specified in a protocol that utilized a temperature of 37°C, pH of 8.0, and 5% CO_2 level. Variations in the parameters temperature, pH, and CO_2 were tested to determine whether the specified standards were the best for growth. The presence of Nutrasweet was predicted to cause an increase in cell growth. I also predicted that cells would grow best in media that had serum added. This aspect was tested as serum is a very expensive additive to the media. If cells could be grown without it, the cost of such an experiment would be greatly reduced.

PROTOCOL

1. The mice were killed by cervical dislocation. The ventral surface was swabbed with alcohol. The ventral skin was torn transversely in opposite directions to expose the abdominal wall.

2. The abdominal wall was cut to reveal the viscera. The uteri were removed and placed in a petri dish containing 10.0 ml of PBS-CMF.

3. The uterus was torn with two pairs of forceps to remove the embryos. The embryos were taken out and placed in a petri dish containing 10.0 ml of PBS-CMF. The embryos were chopped into pieces 1-2 mm in size.

4. The fragments were placed in an Erlenmeyer flask with a 10.0 ml pipet. A magnetic bar and 10-20 ml of PBS-CMF were added and the mixture washed for 8 minutes in a magnetic stirrer at low speed.

5. The tissue was allowed to settle and then the supernatant was discarded. 20 ml of trypsin was added and the solution stirred for another 8 minutes. The tissue was allowed to settle and the supernatant was discarded.

6. Step 5 was repeated for 1 hour. Then 5 ml of fetal bovine serum (FBS) was added. The mixture stirred 5 more minutes and was then aspirated to make sure the cells had separated.

7. The suspension was transferred to a 50 ml sterile centrifuge tube filtering through a piece of gauze. The suspension was placed in the centrifuge for 12 minutes. After this time the supernatant was discarded and 10 ml of E-MEM-10% FBS was added.

8. Step 7 was repeated two more times.

9. 1.0 ml of the cell suspension was placed in a cell culture flask containing 4.0 ml of E-MEM-10% FBS which was previously equilibrated at 37° C and 5% CO_2 .

10. The media was changed every 2 or 3 days or when needed as the pH level shifted to a more acid state.

11. The cells were incubated at the temperature 37° C and 40° C. The results of the variations in temperature are listed in Table 1.

12. The cells were incubated in flasks with media that had a pH of 6.0. HCl was added to the media to achieve this pH. The cells were also incubated in flasks with media that had a pH of 9.3. NaOH was added to the media to achieve this pH. The cells were also incubated in flasks that contained media with the normal pH of 8.0. The results are listed in Table 2.

13. The cells were incubated at 0% CO_2 and 5% CO_2 . The results are listed in Table 4.

14. Nutrasweet was added to the media of cells to determine the effect it would have on the cells. The results are listed in Table 5.

15. Cells were placed in flasks with media that contained no serum to determine whether cells grow best with serum in the media or without it in the media. The results are listed in Table 3.

RESULTS AND DISCUSSION

Cells were established and maintained without contamination using the particular protocol that was employed. The average weight of the cells that were incubated at 37 ° C was 0.1150. The average weight of the cells that were incubated at 40° C was 0.0425. There was a 63% decrease in cell growth of the cells that were incubated at 40° C. The average weight of the cells that were incubated at a pH of 8.0 was 0.1150. The average weight of the cells that were incubated at a pH of 6.0 was 0.0475. There was a 59% decrease in cell growth of the cells that were incubated at the pH of 6.0. The average weight of the cells that were incubated at a pH of 9.3 was 0.0625. There was a 46% decrease in cell growth of the cells that were incubated at the pH of 9.3. The average weight of the cells that were incubated with serum in the media was 0.1150. The average weight of the cells that were incubated without serum in the media was 0.0450. There was a 61% decrease in cell growth of the cells that were incubated without serum in the media. The average weight of the cells that were incubated at a CO_2 level of 5% was 0.0425. The average weight of the cells that were incubated at a CO_2 level of 0% was 0.0350. There was a 18% decrease in cell growth of the cells that were incubated at the CO_2 level of 0%. The average weight of the cells that were incubated without Nutrasweet was 0.0375. The average weight of the cells that were incubated with Nutrasweet was 0.0238. There was a 37% decrease in cell growth of the cells that were incubated with Nutrasweet. In conclusion cells grow best at a temperature of 37° C, a pH of 8.0, 5% CO_2 , with serum in the media, and without Nutrasweet.

TABLE 1. THE EFFECT OF TEMPERATURE CHANGES ON CULTURE GROWTH.

	CELL WT/GRAMS				
	REP1	REP2	REP3	REP4	AVE
• 37 ° C	0.060	0.120	0.210	0.070	.1150
• 40 ° C	0.050	0.070	0.010	0.040	.0425

TABLE 2. THE EFFECT OF pH CHANGES ON CULTURE GROWTH.

pH	CELL WT/GRAMS				
	REP1	REP2	REP3	REP4	AVE
• 6.0	0.010	0.020	0.120	0.040	.0475
• 8.0	0.060	0.120	0.210	0.070	.1150
• 9.3	0.060	0.010	0.100	0.080	.0625

TABLE 3. THE EFFECT OF SERUM LEVEL ON CULTURE GROWTH.

SERUM	CELL WT/GRAMS				
	REP1	REP2	REP3	REP4	AVE
PLUS	0.060	0.120	0.210	0.070	.1150
MINUS	0.010	0.060	0.040	0.070	.0450

TABLE 4. THE EFFECT OF CO_2 LEVELS ON CULTURE GROWTH.

CO_2	CELL WT/GRAMS				
	REP1	REP2	REP3	REP4	AVE
0%	0.030	0.030	0.040	0.040	.0350
5%	0.050	0.040	0.050	0.030	.0425

TABLE 5. THE EFFECT OF NUTRASWEET LEVEL ON CULTURE GROWTH.

NUTRA	CELL WT/GRAMS				
	REP1	REP2	REP3	REP4	AVE
PLUS	0.005	0.010	0.020	0.060	.0238
MINUS	0.020	0.040	0.040	0.050	.0375

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