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# Investigations of CHO 1-15 and Silk Gland Cell Line Development

Susan Kathleen Robinson
*Utah State University*

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INVESTIGATIONS OF CHO 1-15 AND SILK GLAND CELL LINE DEVELOPMENT

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Susan Kathleen Robinson

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Approved:

_____              _____
Dr. Jon Takemoto                      Dr. Jixun Zhan
Major Professor                       Committee Member


_____              _____
Dr. Michelle Grilley                  Mark McLellan
Committee Member                      Vice President for Research and
                                      Dean of the School of Graduate Studies


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ABSTRACT

Investigations of CHO-1-15 and Silk Gland Cell Line Development

by

Susan K. Robinson, Master of Science

Utah State University, 2014

Major Professor: Dr. Jon Takemoto
Department: Biology

The BioProcessing Demonstration and Training Laboratory was established with

a collaboration between Utah State University and Thermo Fisher- Scientific, Inc. and

developed into an operational tissue culture facility. Demonstration and establishment of

tissue culture protocols were necessary to be industry-fully qualified. The CHO 1-15 cell

line protocols were optimized by establishing conditions for reproducible growth curve

profiles in shaker flasks and bioreactors (2 to 250 L capacity). CHO 1-15 is the cell line

of choice for protein production in the bio-manufacturing industry. Oxidative stress is

problematic in industry because it causes a decrease in protein production.

Mesobiliverdin IXα and biliverdin IXα possess similar antioxidant properties. The effects

of the antioxidant nature of these compounds were investigated on the CHO 1-15 cell

line. Cultures from silkworm and spider silk producing cells were also pursued. Methods

to produce a primary cell line from spider silk gland cells were developed. Cell lines from

spider and silkworm silk producing glands appeared to have the capacity to secrete full-

length native proteins ranging in size from 200 to 500 kDa, and possibly larger.

(132 pages)

PUBLIC ABSTRACT

Investigations of CHO-1-15 and Silk Gland Cell Line Development

Susan K. Robinson

The BioProcessing Demonstration and Training Laboratory was established with a collaboration between Utah State University and Thermo Fisher-Scientific, Inc. This lab was developed into a fully functioning tissue culture facility. Demonstration of tissue culture procedures were necessary for the lab to be industry-fully qualified. The CHO 1-15 cell line protocols were optimized by establishing conditions for reproducibility in shaker flasks and bioreactors (2 to 250 L capacity). CHO 1-15 is the cell line of choice for protein production in the bio-manufacturing industry. Oxidative stress is a problem in the industry because it can cause a decrease in protein production. Mesobiliverdin IXα and biliverdin IXα possess antioxidant capabilities. The effects of the antioxidant nature of these compounds were tested on the CHO 1-15 cell line. Cell cultures from silkworm and spider silk producing cells were also pursued. Methods to produce a primary cell line from spider silk gland cells were developed. Cell lines from spider and silkworm silk producing glands appeared to have the capacity to secrete full-length native proteins ranging in size from 200 to 500 kDa, and possibly larger.

This thesis is dedicated to my little boy.
Jaxon, I love you to Pluto and back, times infinity.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

| Abbreviation | Meaning | Page |
|---|---|---|
| AM HyCell | antioxidant modified HyCell CHO media | 40 |
| AP | alkaline phosphatase | 55 |
| BPC | bioprocessing container | 5 |
| BV | biliverdin IXα | 7 |
| CHO | Chinese Hamster Ovarian | 1 |
| CV | coefficient of variation | 30 |
| DO | dissolved oxygen | 25 |
| FBS | fetal bovine serum | 52 |
| GSH | glutathione | 6 |
| ·HO | hydroxyl radical | 6 |
| $H_2O_2$ | hydrogen peroxide | 11 |
| MaSp1 | major ampullate spidroin 1 | 13 |
| MaSp2 | major ampullate spidroin 2 | 13 |
| mesoBV | mesobiliverdin IXα | 7 |
| MiSp1 | minor ampullate spidroin 1 | 15 |
| MiSp2 | minor ampullate spidroin 2 | 15 |
| MNNG | N-methyl-N´-nitro-N-nitrosoguanidine | 19 |
| MNU | N-nitroso-N-methyl urea | 19 |
| PCB | phycocyanobilin | 9 |
| $O_2^-$ | super oxide anion | 6 |
| ROS | reactive oxygen species | 6 |
| SABU | sample buffer with urea | 55 |

| Abbreviation | Meaning | Page |
|---|---|---|
| SOD | super-oxide dismutase | 6 |
| SSC | sodium citrate | 53 |
| S.U.B. | single use bioreactor | 5 |
| TFS | Thermo Fisher Scientific Inc. | 4 |
| tPA | human tissue plasminogen activator | 1 |
| USU | Utah State University | 5 |

CHAPTER 1

INTRODUCTION


**PART 1: CHO 1-15 GROWTH STUDIES**

The Chinese hamster (*Cricetulus griseus*) belongs to a rodent family that is native

to the deserts of Northern China and Mongolia (1). This species was first catalogued in

1773 (2). Since 1919, the Chinese hamster has been used in biomedical research and is

presently credited with saving many lives from a variety of illnesses, including cancer,

each year (1). In the mid-20<sup>th</sup> century the hamsters were mainly used in epidemiological

research. That changed in 1957 when Theodore Puck from the University of Colorado

took cells isolated from an ovary of a Chinese hamster and was able to establish them in

culture plates (1). That isolation later became known as the CHO (Chinese Hamster

Ovarian) cell line.

CHO cells have been termed the mammalian equivalent to the model bacterium,

*Escherichia coli*, because of their frequent use in biological studies (1). There are several

mutant strains of CHO cells because they are highly resilient, are easily manipulated, and

are capable of foreign gene amplification (1) (3), which makes CHO cells the mammalian

expression system of choice for bio-manufacturers (2). For the past 20 years, CHO cells

have been a major bio-manufacturing platform for protein pharmaceuticals including

anticancer therapeutics, hormone treatments, and the widely used anti-blood clotting

drug, human tissue plasminogen activator (tPA) (1). It has also been established during

the past 20 years that CHO cells do not allow for the propagation of many pathogens that

affect humans, making these cells a safe host for therapeutic protein production (1). CHO

cells are not the expression system of choice just because they are safe hosts, but also

because they are adaptable and can be genetically manipulated with ease (1). CHO cells can be adapted for growth in serum free conditions. This adaptation allows for the cells to be scaled up to high densities in suspension using large scale bioreactors (10,000 L plus). Increased cell density makes it possible for increased quantity of product (1) (3). Bioreactors provide a low stress environment by controlling agitation and aeration in large tanks. The use of bioreactors to grow industrial cell lines makes it possible to culture high volumes of mammalian cells, which will lead to the increased production of proteins (4).

In 1986, the FDA approved the first therapeutic protein, tPA, from recombinant mammalian (CHO) cells (3). The use of CHO cells to secrete tPA triggered the development of mammalian cell culture for biopharmaceutical production (3). Seventy percent of all recombinant therapeutic proteins produced today are made in CHO cells (3). The market value for cell-produced protein therapeutics is in excess of $112 billion and is expected to grow to $143 billion by the end of 2015 (information available at http://www.rncos.com/Report/IM389.htm). Since CHO cells are the major platform for recombinant therapeutics production, the optimization and study of their growth and maintenance is critical to maximize protein production and, therefore, maximize profits.

Mammalian cells proliferate in culture with a standard growth pattern. The first phase (lag phase) of growth is a time in which cells grow slowly because they are adapting to the culture. The next phase, the log phase, is the most important phase for reaching maximum cell densities and maximum protein production because cells are proliferating exponentially. Growth medium and nutrients are critical during the log phase. When the medium is spent, the cells enter a stationary phase where proliferation

ceases. At this point, the cells can no longer live and enter the death phase. Figure 1-1

shows the characteristic growth pattern of cultured cells. The semi-logarithmic plot

shows the cell density versus the time spent in culture (information available at

http://www.invitrogen.com/cellculturebasics).
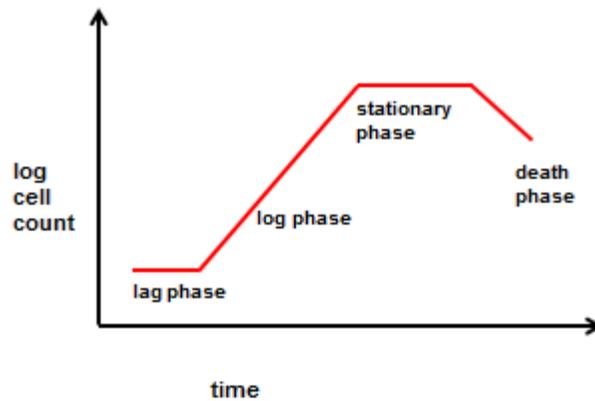


**Figure 1-1**: Cell growth phases (information available at
http://www.invitrogen.com/cellculturebasics).

Table 1-1 lists several different techniques for improving cell culture for the bio-manufacturing industry. Developing and optimizing new media is a method that will maximize protein production during the log phase of growth with various mutant strains of CHO cells. Thermo Fisher Scientific, Inc. (TFS) developed a HyCell CHO medium that is formulated specifically for CHO cell lines grown in suspension. The HyCell medium is a serum free, chemically-defined medium that was formulated using Metabolic Pathway Design (HyCell CHO information available at http://www.gelifesciences.com). This medium is just one example of the many types of media that have been developed by bio-manufacturing companies to provide higher cell densities to increase product yield and quality (HyCell CHO information available at http://www.gelifesciences.com).

**Table 1-1**: Techniques for improving cell culture on the large scale (4).

| | |
|---|---|
| **Cell line development** | **Stable or transient high producing cell lines** |
| **Metabolic fingerprinting of cell line** | **Glucose, amino acids, $NH_4$ , lactate, osmolarity, etc.** |
| **Medium optimization** | **Serum-free, protein-free, chemically defined media** |
| **Process optimization** | **Cultivation parameters process mode** |
| **Bioreactor optimization** | **Heat transfer, shear forces, mixing pattern, residence time distribution, oxygen transfer efficiency** |
| **Growing use of disposables** | **Single-use bags for medium storage, sampling, seed inoculum, disposable couplers, sensors and bioreactors** |

In 2012 the BioProcessing Demonstration and Training Lab located in the Innovations Campus Building 650 was established.  The Lab is the result of a successful collaboration between Utah State University (USU) and TFS. The lab is a full functioning tissue culture and bioprocessing lab with state-of the-art equipment. Experiments performed in the BioProcessing Demonstration and Training Lab fulfill contractual agreements with TFS. The tPA secreting CHO 1-15 cell line is grown in the Formulated HyCell CHO medium. The CHO 1-15 cell line (1) was obtained from the ATCC and then adapted to propagate for growth in suspension by TFS. Experiments demonstrating mammalian cell growth curves and protein production are necessary for developing and optimizing tissue culture and bioprocessing protocols. The adapted CHO 1-15 cell line will initially be used for growth studies ranging from small-scale shaker flask growth studies all the way to large scale bioprocessing platforms.

The three main objectives that must be completed for the Thermo Fisher collaboration, which are focused on protein quantity and production, are as follows:

1. Characterization of cell count and viability of CHO 1-15 cells grown in suspension.
2. Quantification of tPA produced by CHO 1-15 cells.
3. Upscale platforms: shaker flask, bench top bioreactor 2L, single use bioreactor (S.U.B.): 50L, 100L, and 250L.

The S.U.B. bioprocess containers provide for an environment that is optimal for protein production by CHO cells by allowing parameters such as oxygen, pH, temperature and agitation to be controlled. Also, the S.U.B. tank supports a bioprocessing bag (BPC) which comes pre-sterilized and ready to use. This provides a safe and sterile

environment for the cells to grow. The main goals in CHO cell protein production are to obtain higher protein quantity and quality. High growth yields and optimized growth conditions are best achieved in controlled bioreactors. This project focuses on using optimized conditions (S.U.B., media) to evaluate the growth conditions of CHO 1-15 cells grown in suspension. A controlled environment and specialized HyCell CHO media allow the cells to grow better and produce high yields of tPA.

**PART 2: HEME-DERIVED ANTIOXIDANT COMPOUNDS**

Oxygen is necessary for biological function; unfortunately, the events that utilize oxygen come with the byproduct, reactive oxygen species (ROS) (5). The ROS include free radicals such as the superoxide anion ($O_2^-$), the hydroxyl radical ($\cdot HO$), and the non-radical hydrogen peroxide (6). The cell has natural anti-oxidant defenses which are super-oxide dismutase (SOD), catalase, and glutathione (GSH). The cell can become overwhelmed when the concentration of ROS is too high for its antioxidant capabilities. In response to this stress, the cell will begin to down-regulate normal protein production and put efforts into producing antioxidant defenses, such as the heat shock proteins (7). The depletion of antioxidant defenses can result in oxidative damages within the cell. At the same time the normal cell cycle will be suspended in an effort to focus resources on cell survival. If the damages are severe enough it will cause cell death by apoptosis or necrosis. Oxidative stress can be observed when eukaryotic cells or tissues are exposed to agents such as peroxides, glutathione-depriving drugs, toxins, radiation, and inflammatory cytokines (5). It is important to note that oxidative stress in cells has been

shown to have pathological implications in humans such as promotion of Alzheimer's and Parkinson's diseases, cancer, and aging (7).

Other factors that can trigger oxidative stress in cells are oxidation, heat, toxic contamination, and physical stress. Once oxidative stress is triggered in a cell, there is a down-regulation of normal gene production. This happens to conserve cellular energy for use in neutralizing the damage. Low concentrations of hydrogen peroxide have been shown to lead to an arrest in cell growth and a lengthening of the cell cycle (7).

Biliverdin IXα (BV IXα) is a green bile pigment and is involved in the catabolism of heme (information obtained from http://en.wikipedia.org/wiki/Biliverdin). The oxidation of heme leads to BV IXα (8). BV IXα is further reduced to bilirubin. It is believed that the reduction of BV IXα to bilirubin is very rapid (8). Both BV IXα and bilirubin are water-insoluble pigments that possess antioxidant properties (9). In the late 1980s, it was demonstrated that the antioxidant effect of bilirubin exceeds that of vitamin E toward lipid peroxidation (10). It is thought that BV IXα participates with bilirubin in an oxidation/reduction cycle. Shown in Figure 1-2 is bilirubin acting as an antioxidant, oxidized to BV IXα and then recycled by biliverdin reductase back to bilirubin (11). BV IXα exists in trace amounts in vivo during heme metabolism (8). During one study, only small amounts of bilirubin (10 nM) were needed to protect neuronal cells from a 10,000 fold molar excess of $H_2O_2$ (8).

Mesobiliverdin IXα (mesoBV IXα) is a close analog of BV IXα (12). MesoBV IXα is produced from phycocyanobilin. The chromophore phycocyanobilin is recovered from lyophilized powders of the cyanobacteria *Spirulina platensis* (12). MesoBV IXα has demonstrated anti-oxidant capabilities (12). When mesoBV IXα was used to protect

pancreatic islet cells from oxidative stress, a very low concentration (1 µM) of mesoBV

IXα was needed (12). That cytoprotective capability was better than the commercial BV

IXα (12).
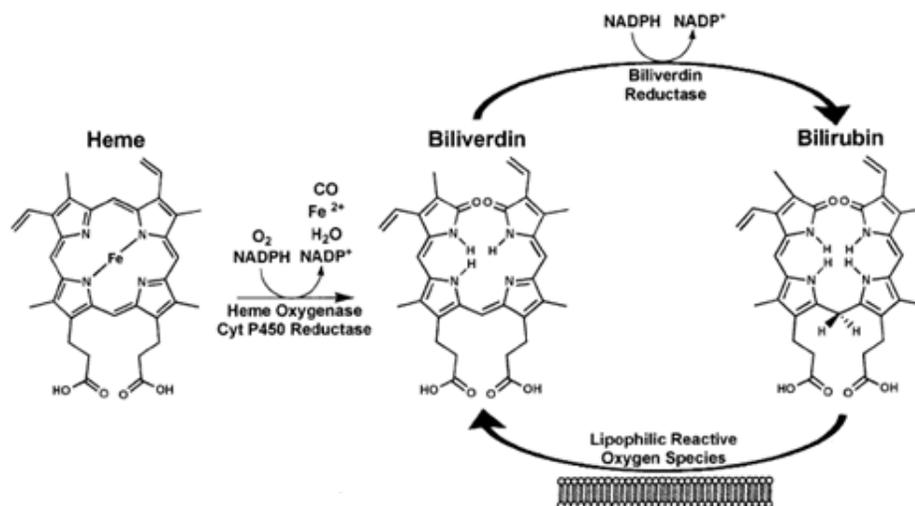


**Figure 1-2:** Oxidation and reduction cycle of BV IXα and bilirubin (10)**.**

Phycocyanin is also proven to be a strong antioxidant (13). The chromophore of phycocyanin is phycocyanobilin (PCB). PCB is similar to the chemical structure of bilirubin and is also a scavenger of ROS (13). The structure of PCB is similar to that of BV IXα and it appears to be susceptible to biliverdin reductase, which results in phycocyanorubin, an analog of bilirubin (14). Phycocyanin and PCB are isolated from *S. platensis* (14). PCB is important in the antioxidant properties of phycocyanin because it contains antioxidant and radical scavenging abilities (14) (15).
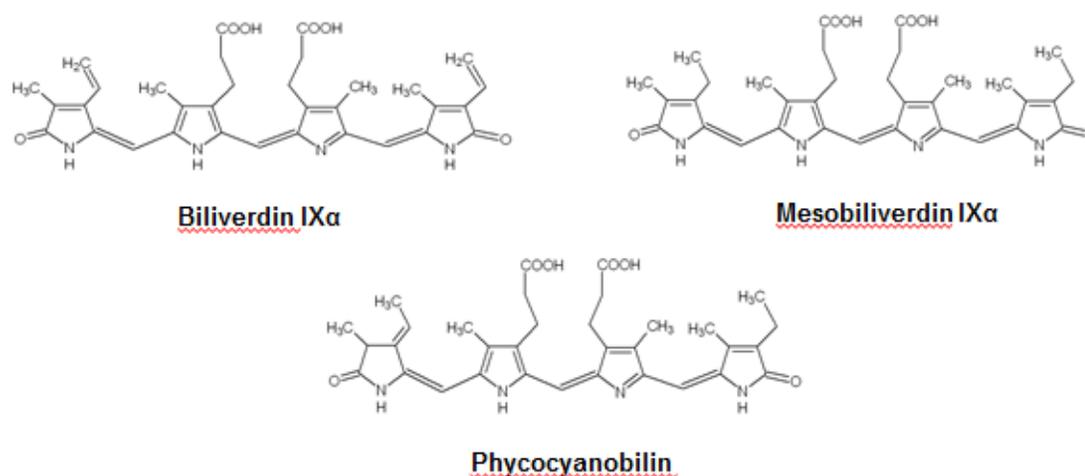


**Figure 1-3:** Structures of heme-derived compounds:  BV IXα, mesoBV IXα and PCB.

CHO cells are the main bio-manufacturing biological system for protein pharmaceuticals including anticancer therapeutics, hormone treatments, and the anti-blood clotting drug, tPA. The market value for cell produced protein therapeutics is in excess of $112 billion and is expected to grow in the future (information available at http://www.rncos.com/Report/IM389.htm). As of 2009, few studies on oxidative stress had been performed on mammalian eukaryotic cells, despite the fact that mammalian cells are often used to produce high cost, recombinant human protein products (such as tPA) (7). CHO cells are used to produce these complex proteins because they require certain modifications to be functional and prokaryotic cells cannot make those modifications (7). There is a lack of knowledge about how CHO cells react to oxidative stress when used for large-scale platforms even though oxidative stress is a huge concern and is problematic in the industry of mammalian cell growth because it causes down-regulation of protein production, slower growth, and decreased viability (7).

Menadione and hydrogen peroxide are two chemicals that have shown to cause oxidative stress and be toxic in CHO cells.  Menadione (2-methyl-1, 4-naphthoquinone) is a redox cycling quinone (7). Menadione is an artificial form of Vitamin K3 and an oxidant that raises $O_2^-$ concentrations (7). That in turn forms ROS (5). The ROS then induces a rapid oxidation of antioxidant defenses, including glutathione (GSH) (5). With concentrations ranging from 50 μM to 750 μM, menadione was found to induce oxidative stress in human skin fibroblasts after 30 min of exposure (7) (16). Menadione has not been used on CHO 1-15 cells but on a different strain of CHO cells and has worked as a strong oxidant with a rapid effect causing the majority of cells to die after 1 h of exposure (7).

Hydrogen peroxide ($H_2O_2$) is a stable compound and a regularly used oxidant that can pass freely through membranes and raise the intracellular level of peroxides. $H_2O_2$ participates in the catalytic fenton reaction (7). It is changed to a hydroxyl radical, a short lived but very potent ROS that oxidizes macromolecules inside the cell (5). The hydroxyl radicals will cause rapid oxidation of reduced GSH (5). The cell type and media composition determines the necessary concentration of hydrogen peroxide needed for oxidative stress induction. When $H_2O_2$ is added directly to the culture medium, the cells will experience a short-term exposure to a quickly decreasing concentration of $H_2O_2$ (17). The cell population density plays an important role in the rate at which the $H_2O_2$ diminishes (17). At high densities cells are less susceptible to the cytotoxic effects of $H_2O_2$; this is due to the fact that $H_2O_2$ will be detoxified when the cell activates its own antioxidant defenses (18). It is thought that glutathione peroxidase acts as the predominant defense enzyme against lower $H_2O_2$ concentrations (17).

The use of the three heme-derived compounds BV IXα, mesoBV IXα, and PCB on CHO cells could have the potential to increase protein production and quality. Due to the nature of the antioxidant properties of each compound and their similarities to each other in structure, it would be expected that all three would yield similar results when tested on a CHO cell line. The billion dollar protein therapeutics industry is constantly researching ways to maximize protein production with CHO cells, and finding compounds that minimize oxidative stress could have a significant impact.

**PART 3: SILK GLAND CELLS**

Around 4,500 years ago, an empress of China dropped a silkworm cocoon into a cup of tea. The cocoon unraveled and, with that, the empress wove the first thread of silk (19). This discovery is believed to have started the use of silks for the benefit of human societies (19). For thousands of years, silk from the silkworm, *Bombyx mori*, has been used to produce fabrics. Commercial use of this silk from *B. mori* has been developed for centuries. The silkworm is not the only animal that produces silk in nature; scorpions, mites, flies, and spiders are all examples of insects that produce silk (20). Currently, there are around 37,000 known species of spiders which all produce some kind of silk, and about half of those produce silk to spin webs (21).

The spider began its evolutionary process of using silk about 400 million years ago (22). For thousands of years, societies have been using spider silk for their benefit, two examples being fishing lines and webs to stop bleeding from wounds (23). Despite the fact that spider silk benefits have been known for centuries, spider silk has only begun to be extensively studied in the last few decades. One reason spider silk has not been studied is because farming spiders for large-scale silk production is not possible because of their highly territorial and cannibalistic nature (24).

The study and use of spider silk has great potential to make new, improved products. Spider silk is much tougher than the brittle cocoon silk of the silk worm (22). The tensile strength of spider silk is comparable to steel and the elasticity to that of rubber (25). Due to those properties, the toughness of the silk is two or three times higher than Kevlar (25). Other properties of silk that make it useful, especially for biomedical products, are that it is antimicrobial, hypoallergenic, and biodegradable (25).

Orb-weaving spiders live off of the ground and use a web to catch their prey (26). The name of these spiders comes from the orb shape of the web they produce. The orb-weavers have six different silk producing glands and one glue producing gland (26), see Figure 1-4 below. All of these glands produce a silk that is necessary for the spider's survival. Some of the functions include locomotion, prey swathing, web construction, and egg protection (21). Those different functions give the silk unique mechanical properties, which make these spiders and their different silks especially desirable to research.

Silk proteins (spidroins) produced by the six different silk producing glands generally have relatively high molecular weights of 200 to over 350 K (27). The synthesis of these spidroins occurs in columnar epithelial cells (26). The newly formed proteins appear as droplets in the cell that are exocytosed into the lumen of the gland where they can be stored until needed by the spider (26) (28). The "droplets" are thought to form a micelle structure in the lumen of the gland. Changes in the pH along with shear forces (from the spider moving its abdomen or pulling with its legs) cause a mechanical change, thus linearizing the micelle structure and allowing it to re-fold into fiber formation as the proteins travel into the duct of the gland, forming the spider silk fiber (25) (27). This method of action for silk fiber formation is thought to be the same for all of the silk producing glands.

The major ampullate glands are the most studied of all the silk producing glands, partly due to their large size (26), as a result most of the information available on spider silk is from the silk produced by the major ampullate glands. Two proteins make up the silk produced from the major ampullate glands: Major ampullate spidroin 1 (MaSp1) and Major ampullate spidroin 2 (MaSp2). These proteins are extremely large, with molecular

weights between 275 K and 320 K (22). The major ampullate glands produce silk that is used for the structural frame lines of webs and for dragline silk (the spiders' life-line, should they fall) (25) (26). The dragline silk, made up of the two MaSp proteins, is extremely tough and has a tensile strength comparable to Kevlar (24).
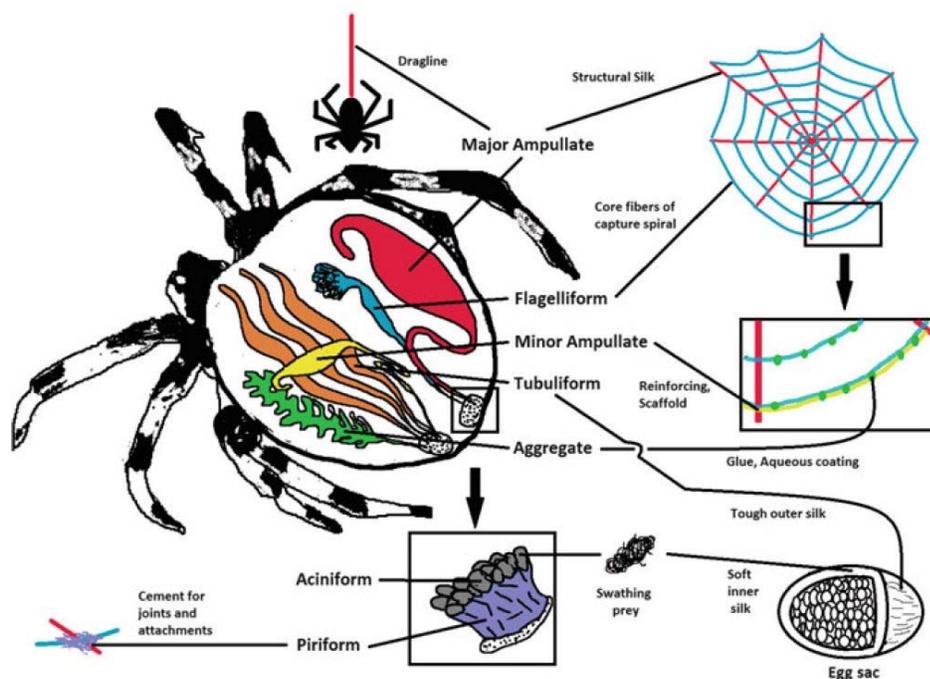


**Figure 1-4**: Spider silk glands and their products (29).

The silk from the minor ampullate glands is used for the auxiliary spiral silk construct (25), and is used as the structural reinforcement in the web. The minor ampullate glands produce at least two proteins: Minor Ampullate Spidroin 1 (MiSp1) and Minor Ampullate Spidroin 2 (MiSp2) (26). The molecular weights of these proteins are also very large, around 250 K (26). The MiSp2 protein is similar to the major ampullate silk and is often spun at the same time (30). One major difference between major ampullate silk and minor ampullate silk is that minor silk does not have the elasticity that the major silk has, one reason being that minor silk has virtually no proline (26) (30).

The aciniform gland produces a silk that is used for wrapping the prey silk and the inner egg case (31). The main protein stored in the numerous aciniform glands is AcSp1 (26). The mechanical properties of aciniform silk are quite different than the silk produced from the other glands. The main difference is the toughness of the fiber compared to the other silks. This includes the overall strength, stiffness, and elasticity of the fiber (32).

The piriform gland secretes the attachment cement silk for the web (25). There is only one known piriform spidroin, PySp1 (28), but it is thought that the silk from the piriform gland is comprised of more than one protein (33). This gland produces accessory proteins, which are also considered to be glue proteins.

Flagelliform glands secrete the capture spiral silk. The silk made by the flagelliform glands is a tremendous component of elastic support for the capture of prey in the orb-web (22) and is dotted with the glue-like silk from the aggregate gland (34). It is the extensibility feature of this silk, combined with the tensile strength, that make it

one of the hardest silks to break (34). The gland is thought to secrete one extremely large

spidroin (Flag) 500 kDa in size (26).

Tubuliform glands are unique among orb-weavers (26). The silk is known as egg

case silk and is only produced during the reproductive season (35) from sexually mature

females. There has been one protein identified, secreted by the glands, as TuSp1 (26).

The silk is very similar to minor ampullate silk because it has high strength but low

elasticity (36), although it is brittle and the amino acid sequence is very different.

The glue producing aggregate glands help the spiders with many tasks: web

construction, prey capture, and locomotion (31). Not much is known about the glue

proteins, but there are two specific known proteins from the aggregate gland: AgSf1 and

AgSf2, which are around 40 to 60 kDa (31).

*Nephila clavipes* is known as the golden orb weaver (26) because it is an orb-web

spinning spider with golden silk (37). These spiders are one of the most studied spiders

(24) because they make good lab animals and are large in size.  The dragline silk from the

major ampullate glands of the *N. clavipes* was the first spider silk to ever be studied (37).

Attempts have been made with several different expression systems for the

synthetic production of spider silk (38), all with pros and cons. A common disadvantage

that these different expression systems have is that none can produce native full-length

synthetic spider silk proteins, whether on a small scale or a large-scale basis. A reliable

system for the large-scale production of spider silk is necessary if spider silk is going to

be used at a commercial level. Figure 1-5 is a summary of the attempts at recombinant

spider silk production.

**Table 1-2**: Spider silk function (25).

| Gland | Function |
|---|---|
| **Major Ampullate Silk Gland** | Structural and dragline silk |
| **Minor Ampullate Silk Gland** | Auxillary spiral thread |
| **Flagelliform Silk Gland** | Capture spiral thread |
| **Piriform Silk Gland** | Attachment cement |
| **Tubuliform Silk Gland** | Tough outer egg case |
| **Aciniform Silk Gland** | Soft inner egg and wrapping |

An expression system in the bacteria *E. coli* has been developed. This system has been good for large-scale production via fermentation. Maintenance cost of bacterial cultures is comparatively low. Unfortunately, spider silk production in bacteria results in unstable DNA fragments, low protein yields, and solubility problems. Also, endotoxins must be removed before use in biomedical applications (38). Yeast cells can perform post-translational modifications and produce high yields; however, modifications in yeast can be different than the more evolved eukaryotic cells. Also, undesirable glycosylations can be present, which increase the chances of an allergic reaction when the silk is used for biomedical purposes (38). When the expression system is a plant, there can be toxic substances in plant vacuoles, and plant antigens can cause allergic reactions which would be a problem when using silk produced from plants for biomedical purposes (38). There have also been attempts to produce silk in mammalian cells. Hamster, bovine, and goat mammary cells have been used, but contamination and complex media might be a problems (38) (39). Transgenic animals have also been used, one example being goats.

The spider silk proteins MaSp1 and MaSp2 are excreted from the mammary glands into the milk of the goat, but the yields are low and the development time is long, (38) because the milk is only produced for a few months out of the year.
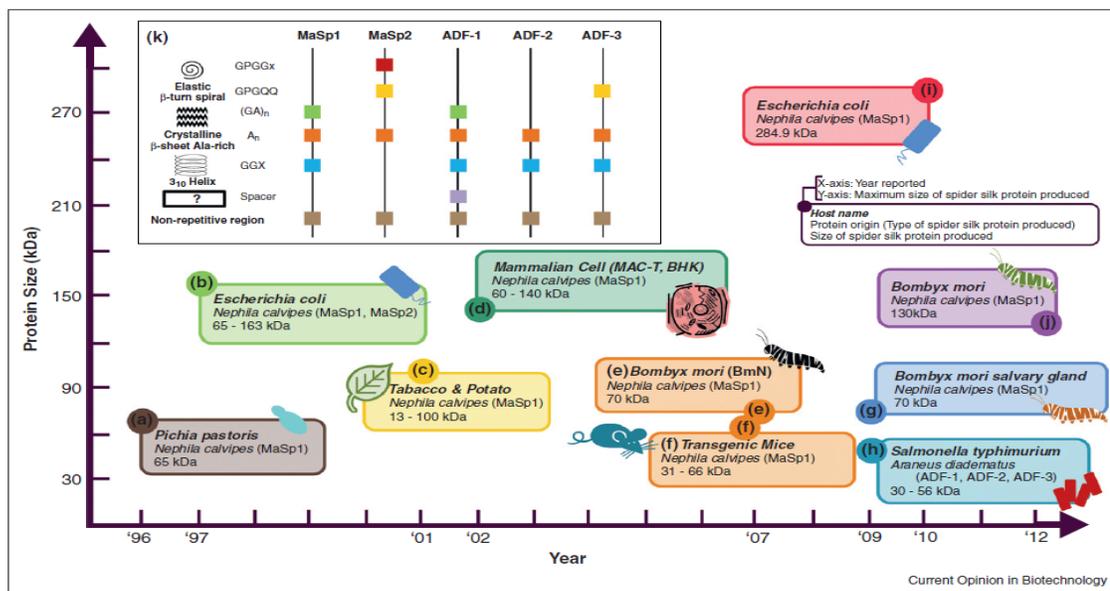


**Figure 1-5**: Spider silk expression systems (40).

A continuous insect cell line was first established in 1962 when Thomas Grace was able to grow female moth ovaries from the *Antherea eucalypti* (41). Since the year 2000, over 500 insect cell lines have been established. Around 80% of those came from *Lepidoptera* and *Diptera*, with only 20% from all other invertebrates and only 5% of those being from non-insect (42). Around half of the insect cell lines have come from embryonic tissue and, as a result, have mixed morphologies. That can be an issue when the desire is to answer a specific tissue function question.

Insect cell culture success is dependent upon antibiotics, improved medium, and patience (41). The small size of the animals and the "dirty environment" they live in make it difficult for a successful culture (41). Fortunately, there has been some success despite the obstacles. For example, cultures with the "dirty" house fly and with a very small wasp have been achieved (41). These successes were achieved largely due to the advances and development of antibiotics and the use of growth supplements in the media. The methods for establishing primary (finite) and secondary (continuous) insect cell lines are still poorly developed (43). The use of transformation, the process of immortalizing a cell line or transforming it from a primary cell line to a secondary cell line, are well studied within mammalian cells (43). The chemical, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), a mono-functional alkylating agent that causes chromosomal DNA damage, has been used as a chemical transformation means for immortalizing mammalian cells and has been used successfully for transforming insect cells from a primary to a secondary culture (43). Unfortunately, MNNG is illegal in the U.S., but a similar mutagen N-Nitroso N-methylurea (MNU) has been recommended by Sigma Aldrich and is available for use in the U.S.

There are no published successful attempts at developing a cell line from the glandular epithelial spider silk producing cells. There are multiple reasons for the need of a spider silk producing cell line. One reason being that it would benefit and further the research and understanding of the spider silk production itself. These cells not only could be a means of spider silk production, but they may also answer questions like, do different cell types secrete the different spider silk proteins or is it one cell type per gland? A cell line from one of the spider silk producing glands should have the capacity to secrete full-length native proteins, which could range in size from 200 kDa to 500 kDa, possibly larger. Also this cell line could be used to further insect cell recombinant technologies production. It has already been demonstrated that insect cell lines are good choices for expression of recombinant molecules, including antibodies (44). In some cases expression levels with insects are higher than with mammalian cells (44). Also insect cells have the capability of post-translational modifications that would be provided by a eukaryotic cell (44). Antibodies are fairly large proteins, and a cell line that is made primarily to secrete very large proteins could be extremely beneficial to the multi-billion dollar industry of recombinant therapeutics (45) that is expected to have a surge of growth in the next 10-20 yr (46). For example, a cell line developed from the *N. clavipes* major ampullate glands should be ready-made cell factories that are already programmed to secrete very large spidroins ~350kD. That gene could be spliced out and a protein of interest inserted, and the cell should have all the capabilities to produce that protein. Most of what is known about silk is from dragline silk which comes from the major ampullate (26). An immortalized cell line available that can secrete full-length spidroin proteins could be the solution for the large-scale production of spider silk.

**SUMMARY OF RESEARCH OBJECTIVES**

The research contained in this thesis is devoted to the growth, maintenance and optimization of cells adept at producing protein in high yield and of optimal quality. Cells expressing proteins of interest are isolated, maintained, quality assured, and supplied to meet the objectives. The following are those main objectives:

1. Establish operational qualification bioreactor runs for Thermo Fisher Scientific Inc.:

   a. Quantify the cell number and viability of CHO 1-15 cells grown in HyCell CHO media from shaker flasks to bench top bioreactor (2 L) and single use bioreactors (50L).

   b. Quantify human tissue plasminogen activator (tPA) produced by CHO 1-15 cells.

   c. Adapt protocols and establish for use in BioProcessing Demonstration and Training Lab (Appendices A, B, C, D, E, F, G and H).

2. Determine the antioxidant capability of heme-derived compounds (mesoBV IXα, BV IXα, and PCB), by using CHO 1-15 cells and measuring cell growth and viability via the trypan blue exclusion dye method.

3. Establish a silk gland cell line into primary culture.

   a. Establish protocols for silk gland isolation for primary cultures by optimizing medium and methods.

   b. Test the MNU compound for immortalizing primary cell cultures.

CHAPTER 2

CHO 1-15 GROWTH STUDIES

More than two thirds of the recombinant therapeutic proteins of the biotechnology industry are produced using the CHO cell line. The main goals in CHO cell protein production are higher protein quantity and quality. The ongoing efforts for the growth and maintenance of the mammalian cell line CHO 1-15 was assessed and characterized in the BioProcessing Demonstration and Training Lab. This lab was a result of collaborative efforts with Thermo-Fisher Scientific (TFS). The research on the CHO 1-15 has been used to establish in-house protocols, growth curves, and tissue plasminogen activator (tPA) production patterns. The growth studies presented in this chapter start with a shaker flask and are scaled up to a 50 L single use bioreactor (S.U.B.).

The growth study experiments for the TFS collaboration are completed with the CHO 1-15 cell line grown in HyCell CHO media, a specially formulated animal derived - component free media from TFS designed to maximize recombinant protein production from CHO cells. The growth study objectives are:

1.  Quantify the cell number and viability of CHO 1-15 cells grown in suspension.

2. Quantify human tissue plasminogen activator (tPA) produced by CHO 1-15
   cells.

 3. Increase growth platform scales from shaker flasks to a bench top bioreactor (2 L) and single use bioreactors (S.U.B.s) (50L and 250 L).

**MATERIALS AND METHODS**

       The CHO 1-15 cell line was obtained from TFS, which adapted the cell line for

growth in suspension using selection. The CHO 1-15 cells were brought out of

cryopreservation storage and scaled up for growth following the protocols in Appendix A

and B. A working stock of CHO 1-15 cells were cryopreserved following Appendix C.

The CHO 1-15 cells were grown in suspension at 37 °C, 95% humidity, 5% $CO_2$ at 125

RPM . The HyCell CHO media was obtained from TFS in powder form and prepared

following the protocol in Appendix D.

       The total viable cell concentration and the percent viability were determined using

the trypan blue exclusion method via the Vi-CELL Cell Viability Analyzer from

Beckman Coulter. Trypan blue is a molecule (FW: 960.8) that can only penetrate cells

that have damaged membranes (5). This principle allows the percentage of the stained

(dead) cells in relation to the unstained (alive) cells to be calculated (Figure 2-1). The Vi-

CELL takes 50 images of a 0.5 mL to 2.5 mL sample. The Vi-CELL averages the 50

images, yielding the % viability and the total viable cell count via the stained cells and

unstained cells. The cell counter also has other data available including the average

diameter and circularity of the cells (Figure 2-1). The Vi-CELL protocol found in

Appendix E is used when collecting samples for viability and concentration.

       Under healthy growth conditions the CHO 1-15 cells secrete tissue plasminogen

activator (tPA) into the media. One mL cell samples were collected for the tPA ELISA

and centrifuged at 1,000 RPM for 4 min. Samples are stored at -20°C until it is time to

perform an ELISA. The concentration of recombinant  tPA (in the supernatant) is

analyzed using the kinetic sandwich ELISA method (www.enzymeresearch.com) and the

SpectraMax spectrophotometer. The ELISA kit was purchased from Enzyme Research

Laboratories and comes with the capture antibody and the detecting antibody. The

standard was purchased from Calbiochem (cat #612200) for 100 µg of human tPA. The

standard was re-suspended in sample diluent to a final concentration of 10 µg/mL. The

ELISA protocol in Appendix F was used for the analysis. The CHO 1-15 cells secrete 22

mg per L when grown in a standard CHO media (CD-CHO Invitrogen) (47). That value,

21.9 mg, will be used as a reference value for the tPA produced in the following

experiments.



| Settings | |
|---|---|
| Cell type | JC CHO |
| Minimum diameter (microns) | 10 |
| Maximum diameter (microns) | 25 |
| Dilution factor | 1.0 |
| Cell brightness (%) | 85 |
| Cell sharpness | 100 |
| Viable cell spot brightness (%) | 65 |
| Viable cell spot area (%) | 5 |
| | |
| Results | |
| Total cells | 2099 |
| Viable cells | 2061 |
| Viability (%) | 98.2 |
| Total cells / ml (x10^6) | 3.47 |
| Viable cells / ml (x10^6) | 3.41 |
| Average diameter (microns) | 14.54 |
| Average circularity | 0.87 |
| Images | 50 |
| Average cells / image | 42.0 |
| Average background intensity | 205 |

**Figure 2-1**: Image of CHO 1-15 cells from the Vi-CELL counter along with the data that is available when used.

**Shaker Flask Platform.** The shaker flask growth study was started using CHO 1-15 cells that had been in culture for at least 1 month. Each study was run in triplicate. After performing a cell count (Appendix E), the three PETG Erlenmeyer 250 flasks were seeded to a final volume of 100 mL. The target density for all shaker growth studies on day 0, or "start seed" was 250,000 cells/mL. Cells were grown in sterile filtered HyCell CHO media (TFS) on MAXQ 2000 platform shakers (TFS) at 125 RPM, incubated at 37°C, and 5% $CO_2$ in a Reach in $CO_2$ incubator (3950) from TFS.

Two 1 mL samples were collected on days: 3, 5, 7, 10, and 12 for the Vi-CELL Analyzer and for tPA quantification. The 1 mL sample collected for tPA determination was centrifuged at 1,000 RPM for 4 min. The supernatant was collected and stored at -20°C until analysis with the tPA ELISA protocol could be completed (Appendix F).

**Bioreactor Platform (2 L).** The 2 L bioreactor growth study was conducted with the APPLIKON Bio Console ADI 1025 bioreactor control unit and a 2 L glass vessel. The temperature was controlled with a heating jacket and maintained at 37°C. The pH was set at 7 and maintained using $CO_2$ through gentle sparging and the addition of sodium carbonate monohydrate. The dissolved oxygen (DO) was maintained at 50% of air saturation by gentle sparging of air and $O_2$. The CHO 1-15 cells used for the experiments were kept in culture 1-3 months. The 2 L bioreactor setup and maintenance protocol is found in Appendix G. Each bioreactor study was seeded from a PETG Erlenmeyer flask of CHO 1-15 cells in logarithmic growth phase. Logarithmic growth phase was determined from the shaker growth studies which was between day 3 and 6 of

growth. The target seed density for all three runs was 250,000 cells/mL, which was determined using the Vi-CELL protocol (Appendix E).

A 1 mL sample was analyzed on the Vi-CELL each day of the study, starting at day 0 and continuing through day 11. Samples were collected around the same time of day for consistency. The sample was centrifuged at 1,000 RPM for 4 min. The supernatant was collected in a new microfuge tube to be used for tPA quantification. The tPA sample was analyzed with ELISA (Appendix F). A 1 mL sample was collected for the bioprofile analysis using the BioFLEX instrument. The analysis was done by TFS using an in house protocol.

**S.U.B. Bioreactor Platform (50 L).** Large scale bioreactors are mainly used to produce mass quantities of recombinant proteins. These reactors can provide for an environment that is optimal for protein production. The main goal in using large scale bioreactors to grow CHO 1-15 cells would be to yield higher protein quantity and quality. The 50 L S.U.B. bioreactor growth study was conducted with the Pendotech bioreactor control system (PCS-BRBA) and a 50 L S.U.B. with the appropriate size of bioprocessing container bag (BPC). The control unit, the S.U.B., and the BPC bags were provided by TFS as part of USU's collaboration with them. The S.U.B. bioreactor along with the supporting BPC bags are a new technology designed for large scale bioprocessing. The S.U.B.'s have been developed by TFS to reduce the risk of contamination and cross-contamination, reduce cleaning time, and allows for process success and flexibility. The BPC bags come completely sterilized and ready for use. The BPC bag fits inside the supporting 50 L S.U.B. tank. The bag is designed around the conventional stirred tank bioreactor and comes with ports that are provided for liquid

transfer, gas control, monitoring, and sampling. The ports make it easier to maintain sterility in the system. The protocol for loading the BPC bag into the 50 L S.U.B. tank is found in Appendix H.

CHO 1-15 cells were used that had been growing in culture for 1-3 months. The 2 L bioreactor was used to prepare a seed culture for the 50 L S.U.B. in order to ensure enough cells for the target cell seeding density (250,000 cells/mL) was achieved. When the 50 L bioreactor was seeded the cells used were in logarithmic growth phase to ensure that the cells would continue to grow normally and were not under stress. The protocols used to upscale the CHO 1-15 cell line and the 2 L bioreactor are found in the Appendices B and G. The BPC should be filled with media one day prior to seeding because the electric heating system took 4-6 h to get 50 L of media up to 37°C. The protocol for filling the S.U.B. with media is found in Appendix H. While the bioreactor was filling with media, the pH probe was calibrated according to the user manual. The pH and the DO probes had to be autoclaved in the Kleenpak connector ports to maintain sterility. Once the bioreactor was filled with media, the system was warmed to 37°C. The sterile probes were then inserted into the appropriate ports with aseptic technique and per user guide instructions. The DO probe was allowed to polarize in the warmed media overnight (minimum of 6 h). Table 2-1 lists the parameters and set point values for the 50 L bioreactor.

**Table 2-1**: The parameters and set point values for the 2 L and 50 L bioreactor.

| Parameters | DO | pH | Temperature | Agitation | Anti-foam C |
|---|---|---|---|---|---|
| Set Points 2L | 50% | 7.0 | 37°C | 300 RPM | 13 mL |
| Set Points 50L | 50% | 7.0 | 37°C | 180 RPM | 350 mL |

The next morning the DO probe was calibrated. The protocol found in Appendix H was used for seeding the 50 L S.U.B. The target starting density for the two 50 L bioreactor runs was 250,000 cells/mL. The advantage of using bioreactors is being able to control parameters that affect protein production from cells. This kind of control is not available with flasks and incubators. The set point values for the 2 L and 50 L bioreactor are found in Table 2-1. The temperature was controlled with an electric heating system and was maintained at 37°C. The pH was set at 7 and maintained using $CO_2$ through sparging at the base of the bioreactor and the addition of sodium carbonate monohydrate. The dissolved oxygen (DO) was maintained at 50% air saturation by the use of macro and micro sparging of air and $O_2$. Figure 2-2 shows the maintenance capability that the Pendotech controller system performed with the pH and DO value set points during run 1.
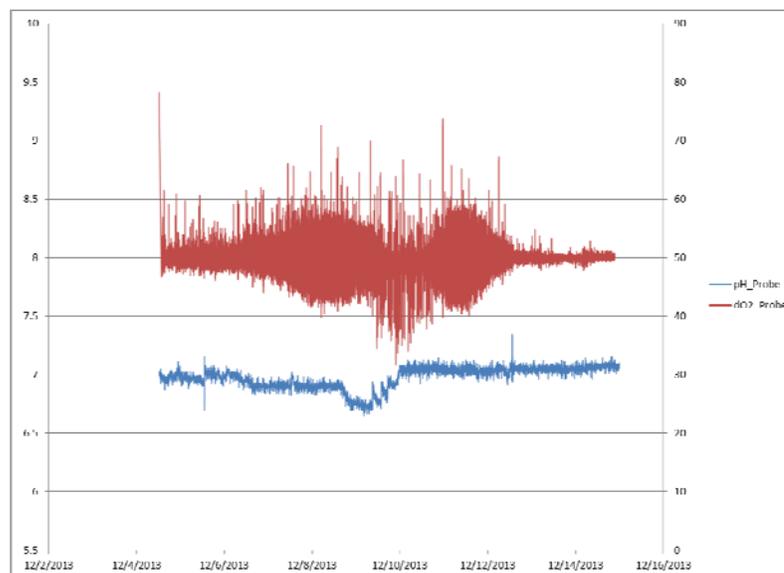


**Figure 2-2**: The pH and % dissolved oxygen maintenance by the pendotech controller during run 1.

During the course of a S.U.B. run, the most probable chance of contamination is during sampling. By using sterile technique, the risk of contamination while sampling can be drastically reduced. This can be done by wearing gloves, using 70% EtOH liberally, and keeping positive pressure on the syringe. The sampling protocol found in Appendix H was used. A 1 mL sample was analyzed on the Vi-CELL each day of the study, starting with day 0 and continuing through day 11.  The sample collection was conducted around the same time of day to keep consistency. The 1 mL sample was centrifuged at 1,000 RPM for 4 min. The supernatant was collected in a new microfuge tube to be used for tPA quantification. The tPA sample was stored at -20°C until ELISA analysis (Appendix F).  Once the run was completed (day 12), the waste was sterilized and disposed of following the protocol in Appendix H.

**RESULTS**

       **Shaker Flask Platform.** For the shaker platform study, the cell density peaked on day 7, ranging from 8.5 to 10.5 million cells/mL. The density was linked to the passage number of the CHO 1-15 cell line. When comparing the three runs, run one had the lowest passage number  (or had been in culture the shortest amount of time (1 month) and had only reached 8.5 million cells/ mL on day 7 as the maximum density (Figure 2-3)). Run 3 reached 10.5 million cells/ mL and had been in culture the longest, closer to 3 months. Concluding that, the higher the passage, the higher the cell density on day 7. The doubling times of the CHO 1-15 cells increase the longer they are kept in culture, however, three months is the maximum amount of time the cells should be kept in culture. The viability of the cells was maintained above 90%, which is consider healthy,

until the maximum cell density was reached.  Upon reaching maximum cell density, the

cell viabiltiy dramaticallly decreased as the cells entered the death phase.

The tPA quantity for the shaker study was measured using the established ELISA

assay (Appendix F). The maximum quantity of tPA produced was 2 mg per 100 mL,

which occurred between days 7 and 12 (Figure 2-4). That amount is right where the

standard tPA production level is produced at high quantites (47). The variation towards

the end of the growth curve was in part due to the % coefficient of variation (CV) values

in the ELISA assay.  Anything less than 10% CV was acceptable to the TFS parameters

set for the collaboration objectives.
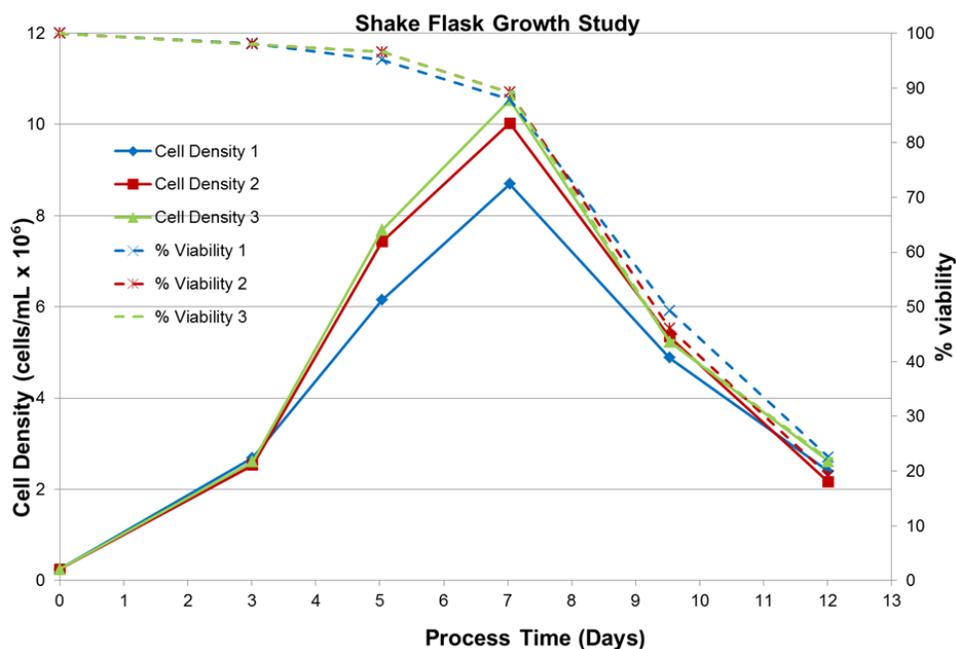


**Figure 2-3**: Cell density and viability growth curves for the shaker flask growth study.
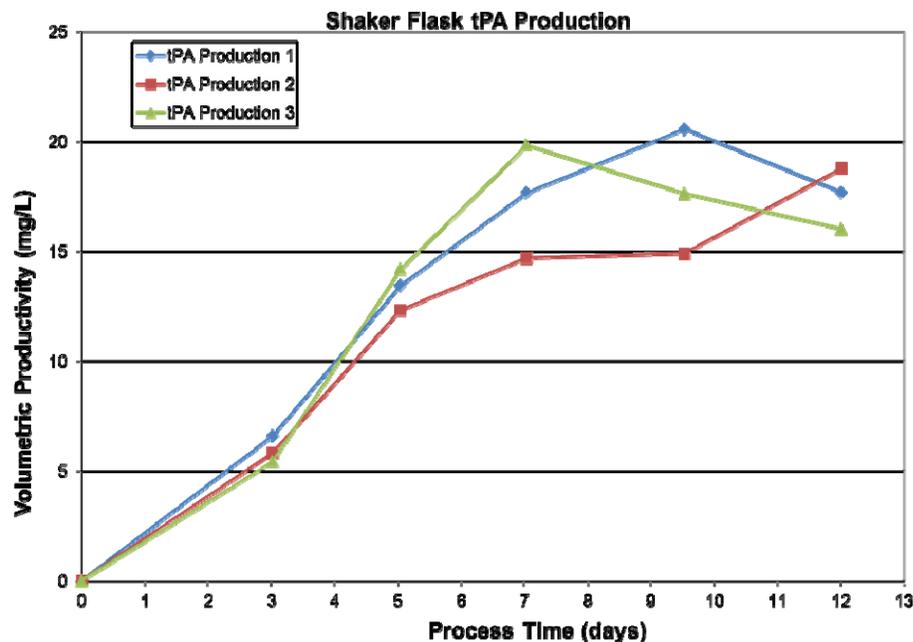
**Figure 2-4**: tPA production for the shaker flask growth study.


**Bioreactor Platform (2 L).** For the 2 L bioreactor growth studies, the cell density

peaked at day 5, with densities ranging from 14 to 17 million cells/mL (Figure 2-5). It

was observed that the maximum cell density increases the longer cells are in culture

(higher passage number). The viability was stable, remaining above 90% until the

nutrients were exhausted (Figure 2-6) and the cells reached stationary phase. The

viability decreased rapidly during the death phase of the growth curve. The glucose was

completely depleted from the media once the cells hit their maximum growth on day 5

(Figure 2-6).  The lactate increased until day 5 showing that the glucose was being

metabolized by the cells (Figure 2-6). Table 2-2 shows an example (run 1) of the data that

is obtained from the bioprofile analysis.
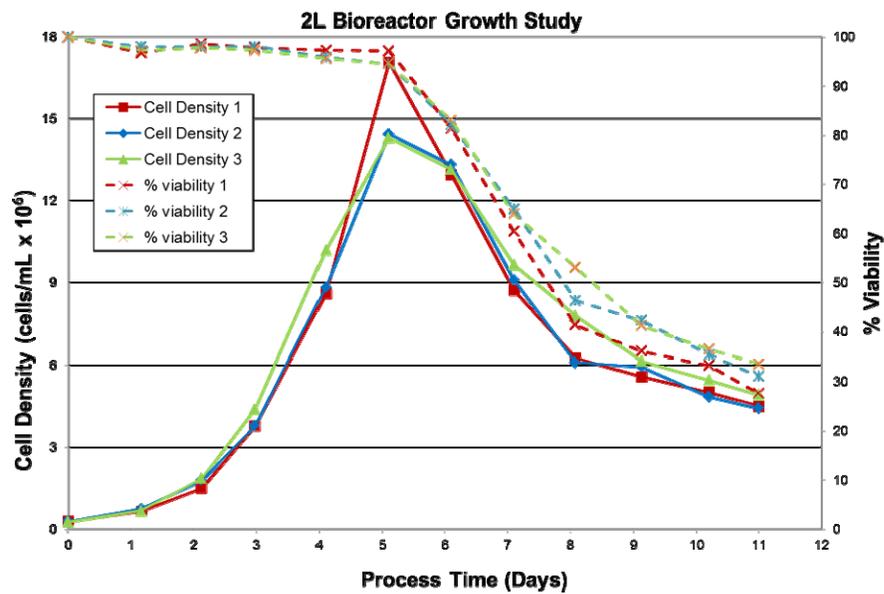
**Figure 2-5**: Cell density and viability growth curves for the 2 L bioreactor study.
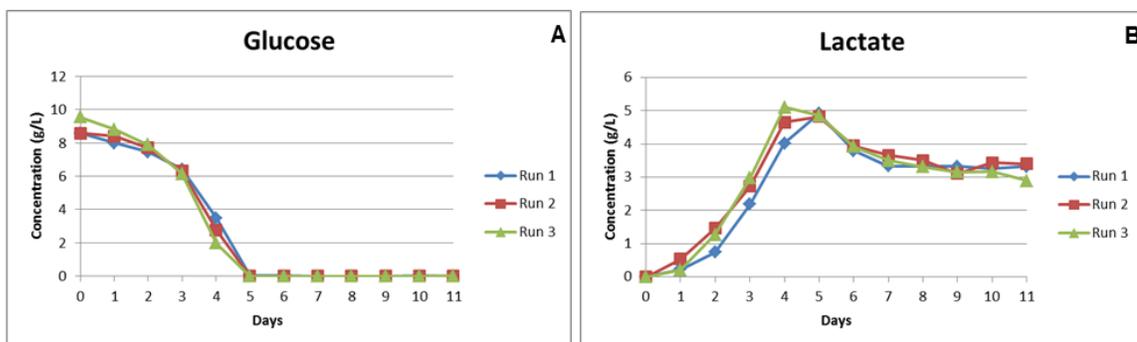


**Figure 2-6:** Glucose and Lactate amounts during the course of the 2 L bioreactor CHO 1-15 growth studies.

The tPA quantity was measured using the ELISA assay (Appendix F). The

maximum tPA quantity produced was around 24-26 mg per liter (Figure 2-7). The

maximum quantity of tPA produced occurred between days 5 and 6. The variation

towards the end of the growth curve was in part due to the % CV values in the ELISA

assay. Anything less than 10% CV was acceptable to the TFS parameters set for the

collaboration objectives.

**Table 2-2**: Example of the BioFLEX data from the 2 L bioreactor run 1 samples.

| Day | pH | Glutamine (mmol/L) | Glutamic Acid (mmol/L) | Glucose (g/L) | Lactate (g/L) | NH4+ (mmol/L) | Na+ (g/L) | K+ (mmol/L) | Ca++ (mmol/L) |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 7.49 | 4.42 | 2.19 | 8.58 | 0.01 | 3.27 | 88.4 | 7.72 | 0.16 |
| 1 | 7.40 | 3.84 | 2.21 | 8.01 | 0.22 | 4.02 | 90.4 | 7.88 | 0.17 |
| 2 | 7.35 | 3.29 | 2.32 | 7.47 | 0.74 | 4.69 | 90.9 | 7.68 | 0.17 |
| 3 | 7.30 | 2.28 | 2.58 | 6.43 | 2.19 | 5.65 | 106.8 | 7.51 | 0.17 |
| 4 | 7.32 | 0.86 | 2.72 | 3.50 | 4.02 | 5.40 | 122.9 | 6.30 | 0.16 |
| 5 | 7.58 | 0.62 | 2.77 | 0.02 | 4.92 | 3.81 | 139.0 | 5.25 | 0.14 |
| 6 | 7.81 | 0.92 | 3.72 | 0.01 | 3.80 | 5.66 | 136.7 | 6.68 | 0.15 |
| 7 | 7.79 | 1.15 | 4.19 | 0.00 | 3.34 | 6.30 | 132.9 | 6.97 | 0.18 |
| 8 | 7.80 | 1.25 | 4.49 | 0.00 | 3.33 | 6.75 | 136.1 | 7.25 | 0.21 |
| 9 | 7.70 | 1.24 | 4.56 | 0.00 | 3.32 | 6.95 | 136.4 | 7.32 | 0.22 |
| 10 | 7.78 | 1.24 | 4.60 | 0.01 | 3.27 | 7.19 | 141.8 | 7.61 | 0.24 |
| 11 | 7.81 | 1.21 | 4.59 | 0.00 | 3.33 | 7.26 | 142.6 | 7.65 | 0.24 |
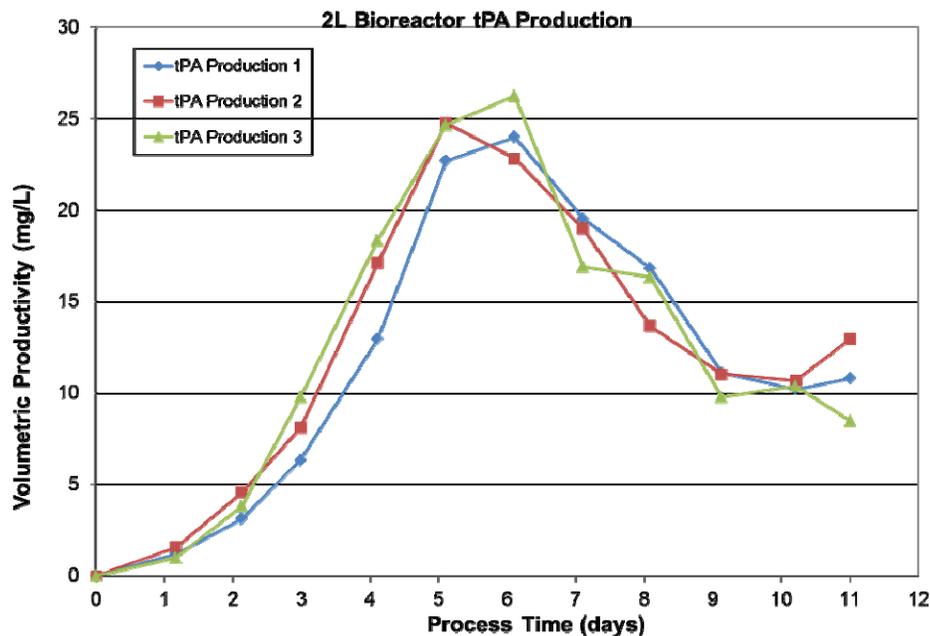
**Figure 2-7**: tPA production for the 2 L bioreactor study.


**S.U.B. Bioreactor Platform (50 L).** For the 50 L bioreactor growth studies, the cell density peaked between days 5 and 6, with maximum densities the ranging from 12 to 15 million cells/mL (Figure 2-8). The discrepencies between the two runs is believed to be due to the aggitation being shut off for 24 h on day 4 of the second run. Although the oxygen was depleted without aggitation, the continual sparging of air allowed for some stirring, which could explain why the cells did not die during that time. The cells only grew from 8 million on day 4 to 12.4 million on day 5. It took 24 h to see the effect the stress had on the cells, which presented as decreased tPA production (Figure 2-9). It was believed that the cells were unable to recover fully because of the immense stress and that is why they peaked on day 5 instead of continuing in logarithmic growth phase and reaching closer to the expected to 16 million on day 6, as was the case during run 1. The death phase started on day 6 and the cell density decreased dramatically, following the trends of the previous run (run 1) and other runs in the 50 L bioreactor. In spite of the

stress on the cells, the viability remained stable and healthy (above 90%) until the cells

reached death phase.
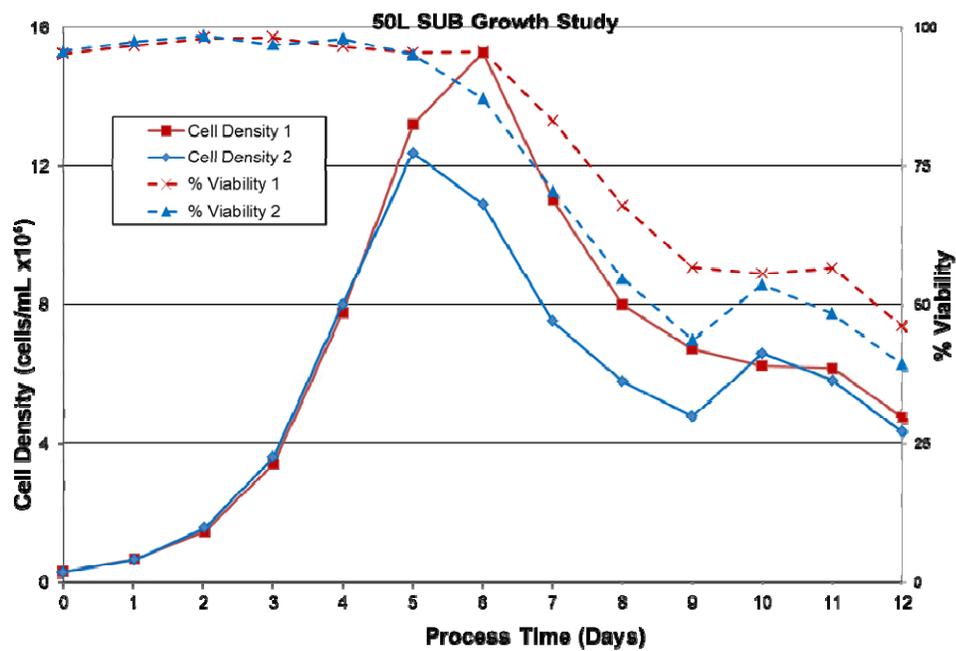


**Figure 2-8**: Cell density and viability growth curves for the 50 L bioreactor study.

The maximum tPA quantity produced was 21-27 mg per liter (Figure 2-9). This was higher than the expected 22 mg per liter (47) from CHO 1-15 cells in different media and the base production of 20 mg per L from the shaker flasks. The maximum quantity of tPA produced occurred between days 5 and 6. The variations between the runs can be attributed to the error on day 4. Up to day 4, the amount of tPA production was very similar between the two runs (Figure 2-9). The variation toward the end of the growth curve was in part due to the % CV values. Nothing over a 10% difference was allowed from TFS protocol standards. Also, comparing the 50 L S.U.B. runs to the 2 L bioreactor runs, it can be observed that the tPA production can fluctuate, increasing and decreasing slightly at the very end of the death phase. Although the second 50 L run did not reach the level of cell density the first run did, it was successful as an operational qualification run. The growth curve is still visible and the amount of tPA produced was substantial.
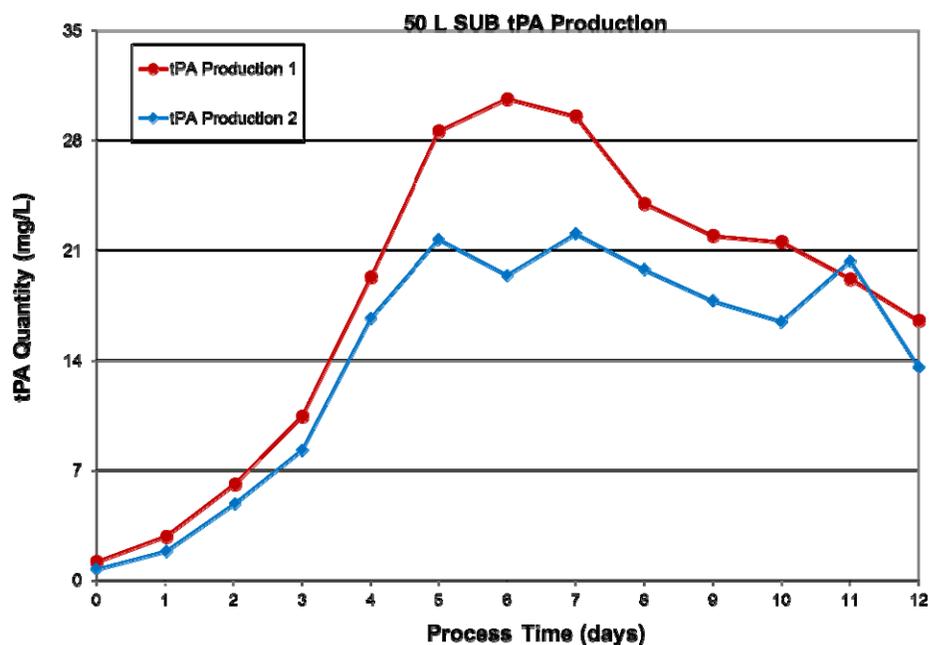


**Figure 2-9**: tPA production for the 50 L bioreactor study.

**DISCUSSION AND CONCLUSIONS**

The average maximum amount of tPA quantity from the shaker platform was

19.8 mg per L. This would be the base value to compare the results to the platforms. The

tPA increases to 24.3 mg per L for the 2 L bioreactor and then to 30 mg per L for the 50

L (run 1 only). The cells reached the peak of the logarithmic growth phase faster when

using the bioreactors, day 5 for the 2 L and day 6 for the 50 L. This might be due to the

set up involved and the stress cells initally have when seeding such large volumes.

However, the ability to have more control over growth parameters with bioreactors does

increase the amount of protein production in cell lines.

The use of large scale bioreactors in the biomanufacturing industry is rising.

Large scale platforms allow for mass quantities of protein production,which leads to

increased profits for companies. For example, one run of a 2 L bioreactor with CHO 1-15

cells can produce 52 mg of tPA, while one run of a 50L S.U.B. can produce 1.35 g. The

cost of tPA is around $285 per 100μg (information found on http://sigmaaldrich.com), so

the difference in dollar amout of tPA between the 2 L and 50 L system is nearly $ 3.7

million.

Large scale bioreactors are sensitive to many factors. Protocols established in the

BioProcessing and Training Demonstration Lab will ensure that trained personnel with

access to quality equipment and materials will be able to achieve desirable results in their

own large scale bioreactor runs. The ability to control parameters such as % DO, pH,

agitation, and temperature allows the user to maximize protein production. Also the

convinience of working with disposable BPC's allows for large scale bioreactors to be

used successfully in smaler scale labs with minimal equipment. It is recommended that

this project move forward with one or two more runs using the 50 L S.U.B. in order to

verify the established protocols before scaling up to the 250 L S.U.B.

CHAPTER 3

HEME-DERIVED ANTIOXIDANT COMPOUNDS

Oxidative stress is problematic in the industry of mammalian cell growth because it causes down-regulation of protein production, slower growth, and potential for decreased viability. Mesobiliverdin IXα (mesoBV IXα) is a compound similar to biliverdin IXα (BV IXα) and has demonstrated anti-oxidant capabilities. Phycocyanobilin (PCB) is another heme-derived compound that has anti-oxidant capabilities. This chapter focuses on testing the anti-oxidant nature of the three heme-derived compounds: mesoBV IXα, BV IXα, and PCB. Oxidative stress also causes a down-regulation of protein production in cells. CHO cells are considered reliable and robust for high value recombinant protein production. It is important to know how the heme-derived compounds affect the growth, viability and protein production in the CHO 1-15 cell line. The CHO 1-15 cell line expresses the protein tPA (human tissue plasminogen activator) and secretes tPA into the media. The objectives for this project are as follows:

- Use the trypan blue exclusion dye method to perform cytotoxicity assays which include:
  - Determine if mesoBV IXα, BV IXα, and PCB exhibit protection of CHO 1-15 cells against oxidative stress after induced with a stressor by measuring cell growth via the trypan blue method.
  - Find the concentration of hydrogen peroxide and menadione that will induce oxidative stress in the CHO 1-15 cell line by measuring cell growth via the trypan blue method.

      o   Find the optimal concentration of mesoBV IXα that displays no toxicity to the CHO 1-15 cell growth and viability via the trypan blue method.

- Determine the effects that mesoBV IXα has on tPA production quantity via the established ELISA assay.

**MATERIALS AND METHODS**

      **CHO 1-15 cell line.** CHO 1-15 cells grown in suspension were obtained by TFS. The cells were grown in an antioxidant modified HyCell CHO media (AM HyCell) custom formulated by TFS and containing no additional antioxidant compounds. The CHO 1-15 cells were grown at 37 °C, 95% humidity, 5% $CO_2$ at 125 RPM. The protocol for CHO 1-15 cell growth and maintenance is found in Appendix A. Cytotoxicity was measured using the trypan blue exclusion dye method. Trypan blue 0.4% (TFS) was used. Trypan blue can only penetrate cells that have damaged membranes (5).  The percentages of stained (dead) cells and unstained (alive) cells were calculated and the latter reported as percent (%) viability.  A Vi-CELL Cell Viability Analyzer (Beckman Coulter) was used to determine the % viability of CHO cells and the total viable cell count per mL. A 1 mL cell sample was collected in the Vi-CELL sample cup. The Vi-CELL analyzed 50 images of the 1 mL sample and determined the average % viability and the total viable cell count. The protocol for using the Vi-CELL is found in Appendix E.

      **tPA.** The quantities of tPA produced were analyzed to examine the effects that mesoBV IXα had on tPA production. One mL cell samples were collected for the tPA ELISA and centrifuged at 1,000 RPM for 4 min. Samples were stored at -20°C until it

was time to perform an ELISA. The concentration of recombinant tPA (in the supernatant) was analyzed using the kinetic sandwich ELISA method (www.enzymeresearch.com) and a SpectraMax spectrophotometer. The ELISA kit was purchased from Enzyme Research Laboratories and included the capture and detecting antibody. The standard was purchased from Calbiochem (cat #612200) for 100 μg of tPA. The standard was re-suspended in sample diluent to a final concentration of 10 μg/mL. The ELISA protocol in Appendix F was used for the analysis.

**Cytotoxicity measurements.** Initially the cytotoxicity experiments were performed in PETG Erlenmeyer shaker flasks (E-125) with a volume of 30 mL. In order to test more variables during the same experiment and to conserve on materials and time, a method using 6-well plates instead of shaker flasks was developed. Plate attachment holders were used with the MAXQ 2000 platform shaker. A maximum volume of 4 mL was used per well. The CHO cells were adapted for 24 h after inoculation in the plate before experimental use (48). Varying concentrations of CHO cells ranging from 2.5 x $10^5$ to 2 x $10^6$ (6) (17) were also tested showing that the optimal seeding density was 5 x $10^5$ cells/mL. These concentrations allowed the cells to maintain logarithmic growth during the course of the experiment. When cells were concentrated at higher cell density, their sensitivity to induced oxidative stress decreased dramatically.

**Heme-derived compounds**. MesoBV IXα, BV IXα, and PCB were obtained from D. Chen and J. Takemoto (Synthetic Bioproducts Center, USU). All three compounds were obtained in powder form and required making solutions suitable for sterile filtration prior to tissue culture use. The heme-derived compounds are structurally similar so the methods for making solutions of them were similar. The method used was

as follows: mesoBV IXα, BV IXα or PCB was prepared from powder into a high concentration (1 – 100 mM) solution in 0.1 M - 0.2 M KOH with a pH between 10 and 12. Once in solution, the pH is lowered back to the range of 7–8 by dilution with AM HyCell growth medium. The solutions were filtered sterilized before addition to the cell suspension.

**Oxidative stress of CHO 1-15 cells.**  The solution form of hydrogen peroxide (30 wt. % in $H_2O$) was obtained from Sigma Aldrich. The concentration range of $H_2O_2$ that induced oxidative stress in the CHO 1-15 cells after 24 h is 500 μM to 5 mM. Menadione was purchased from Sigma Aldrich. Menadione is a powder that is soluble in 100% EtOH at 16 mg/mL, which is a concentration of 90 mM. After menadione was solubilized, it was filter sterilized with a syringe 0.22 micron filter. The concentration of menadione was kept higher so the amount used in each well was only a few microliters. This ensured that the induced cellular stress was from the oxidant and not the ethanol. The concentration range tested was 0 to 100 μM menadione.

**Effects of heme-derived compounds.**  In order to test the three heme-derived compounds ability to protect CHO cells from exposure to hydrogen peroxide, the cells were plated at 500,000 cells/mL in 4 mL of AM HyCell CHO media in 6-well plates. The cells were allowed to recover for 24 h while on a shaker platform in the incubator. CHO cells were pre-treated for 30 min with mesoBV IXα, BV IXα, or PCB, at varying concentrations, 0 μM- 50 μM, prior to exposure with the $H_2O_2$. A sample was taken 24 h after exposure to the $H_2O_2$ to measure cell density and viability.  The concentration range of menadione toxicity was tested on CHO 1-15 cells.  The range was 100 μM to 0 μM menadione. The CHO 1-15 cells were seeded at a density of 5 x $10^5$ cells/mL in AM

HyCell media and allowed to recover for 24 h. A sample for the Vi-CELL was taken 24 h after exposure to menadione. To test the cytoprotective capabilities of mesoBV IXα, BV IXα, and PCB to oxidative stress with menadione on CHO 1-15 cells, CHO 1-15 cells were seeded at 2.5 x $10^5$ cells/mL in AM HyCell media in 6-well plates (4 mL per well). The cells adapted to the culture in the incubator on the shaker platform for 24 h before experimental use. MesoBV IXα, BV IXα, and PCB were added to the culture at a concentration of 10 μM. After 30 min the menadione was added at a concentration of 15 μM. Cells were maintained at 37°C in an atmosphere of 95% air and 5% $CO_2$ at 120 RPM. A 1 mL sample was collected after 24 h and analyzed by the Vi-CELL to document cell growth and viability. The toxicity effects of mesoBV IXα on CHO 1-15 cells growth and viability was tested. The cells were plated at 500,000 cells/mL in 4 mL of media per well. The cells adjusted to the plating for 24 h in the incubator on the platform shaker. The mesoBV IXα was solubilized to a 1 mM concentration and added to each well with increasing concentrations. A 1 mL sample for each concentration was collected after 24 h and 48 h for the Vi-CELL analyzer. The experiment to test the effects on tPA production in CHO 1-15 cells after exposure to mesoBV IXα was set up using E-125 shaker flasks. The media used was the AM HyCell CHO. Cells were seeded at 250,000 cells/mL. The three parameters tested were the control, mesoBV IXα added on day 0, and mesoBV IXα added on day 0 and again on day 5. A 1 mL sample was collected for the Vi-CELL and a 1 mL sample was centrifuged at 1,000 RPM and the supernatant was saved for the ELISA protocol (Appendix F).

**RESULTS**

The experiment shown in Figure 3-1 shows the results of testing the three heme-

derived compounds on CHO cells exposed to hydrogen peroxide. MesoBV IXα, BV

IXα, and PCB did not exhibit cytoprotective capabilities when CHO 1-15 cells were in

oxidative stress induced with $H_2O_2$ (Figure 3-1). Both the 500 µM and the 5 mM

concentrations of $H_2O_2$ induced the CHO 1-15 cells into oxidative stress by dramatically

slowing cell growth when compared to the control. When a heme-derived compound was

added in combination with hydrogen peroxide, no cytoprotective effects (seen as reversal

of the $H_2O_2$ inhibitory effects) were observed. With increasing concentrations of

mesoBV IXα, BV IXα, and PCB in the absence of $H_2O_2$, dose response decreases in cell

growth were observed.
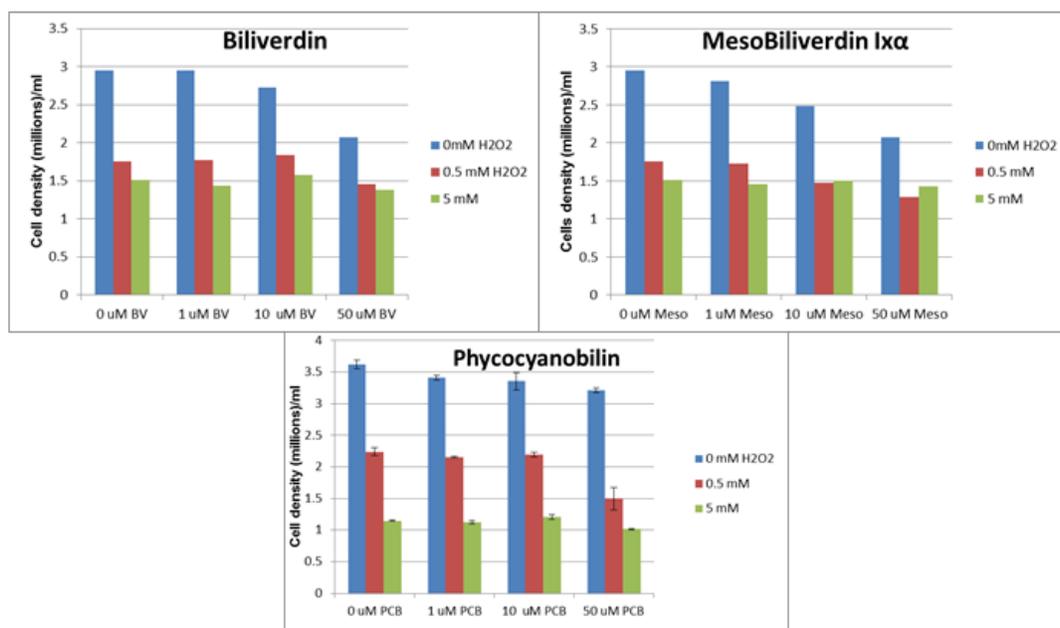


**Figure 3-1**: Cytoprotective capabilities of BV IXα, mesoBV IXα, and PCB on CHO 1-15 cells after induction of oxidative stress with $H_2O_2$.

Since cytoprotective capabilities against $H_2O_2$ were not observed, it was important to test another oxidative stressor, menadione. Menadione can cause oxidative stress through mechanisms that differ from those caused by $H_2O_2$. The optimal concentration for inducing oxidative stress with menadione was unknown in the CHO 1-15 cell line. The concentration range tested was 100 μM to 0 μM menadione. The threshold menadione concentration for causing oxidative stress was 15 μM (Figure 3-2), and this concentration was subsequently used.
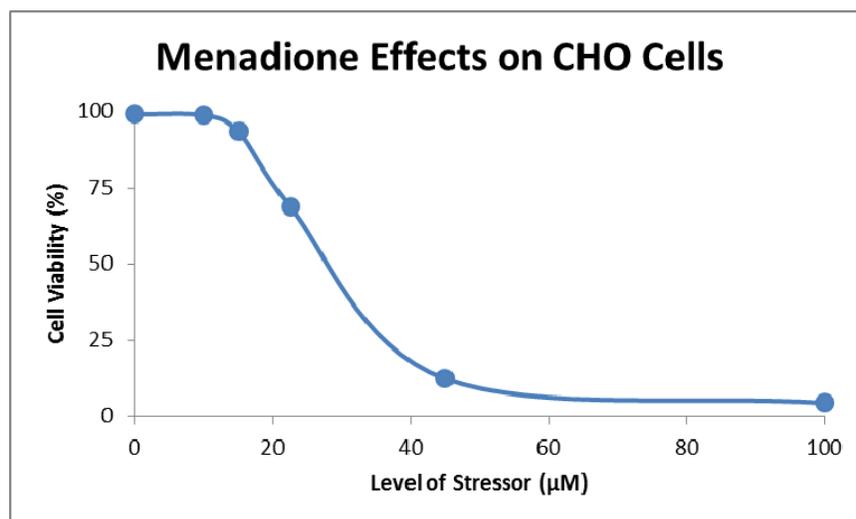


**Figure 3-2**: Exposure of CHO 1-15 cells to menadione and the effects on the viability of cells after 24 h.

The purpose of this experiment was to induce cellular oxidative stress to demonstrate the antioxidant effects of the heme-derived compounds by measuring the growth and viability. In this case the oxidant used was menadione. The results of the experiments with menadione are contained in Figure 3-3. Again it is demonstrated that the heme-derived compounds do not exhibit cytoprotective capabilities when the CHO 1-15 cell line is induced to oxidative stress. According to the Beckman Coulter Vi-CELL instrument guide, the cell analyzer has an expected coefficient of variance of 6%. This means that the counting accuracy of the Vi-CELL is + or − 6%. This is a low variance % compared to other automated counters. Therefore, it is safe to assume based on the results in Figure 3-3 that when mesoBV IXα, BV IXα, and PCB were added with menadione, the inhibition of cell growth was greater than when menadione was added to the well alone.
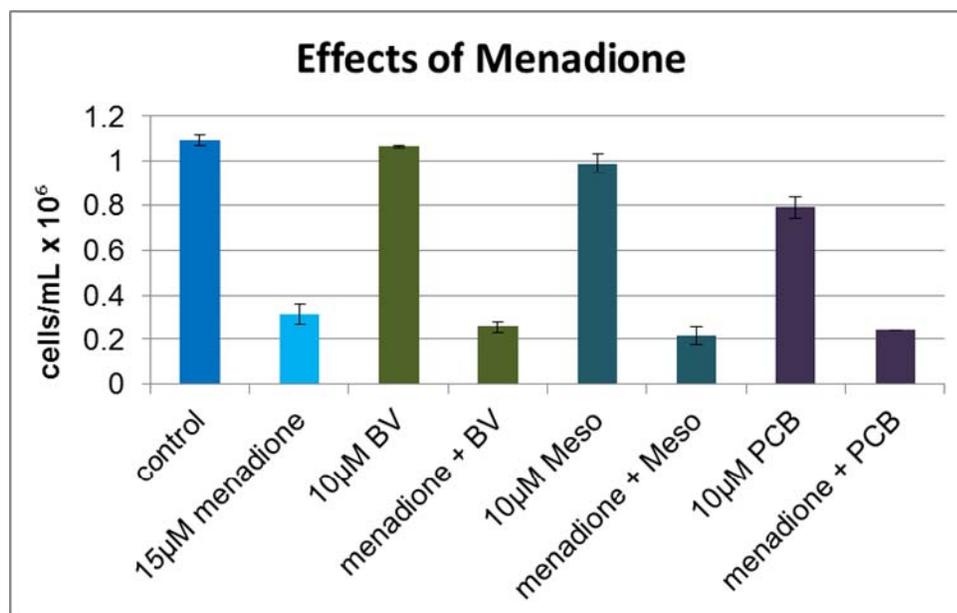


**Figure 3-3**: Cytoprotective capabilities of mesoBV IXα, BV IXα , and PCB on CHO 1-15 cell after induction to oxidative stress with menadione.

The results with $H_2O_2$ indicated that an increasing concentration of either mesoBV IXα, BV IXα, or PCB inhibits CHO 1-15 cell growth. The results with menadione demonstrate that the heme-derived compounds also exacerbated the inhibitory effects of this oxidant. The question arose: Are these heme-derived compounds alone toxic to CHO 1-15 cells?  MesoBV IXα at concentrations ranging from 0-100 μM were examined for inhibitory effects on CHO 1-15 cell growth. The cell density and viability is show in Figure 3-4.
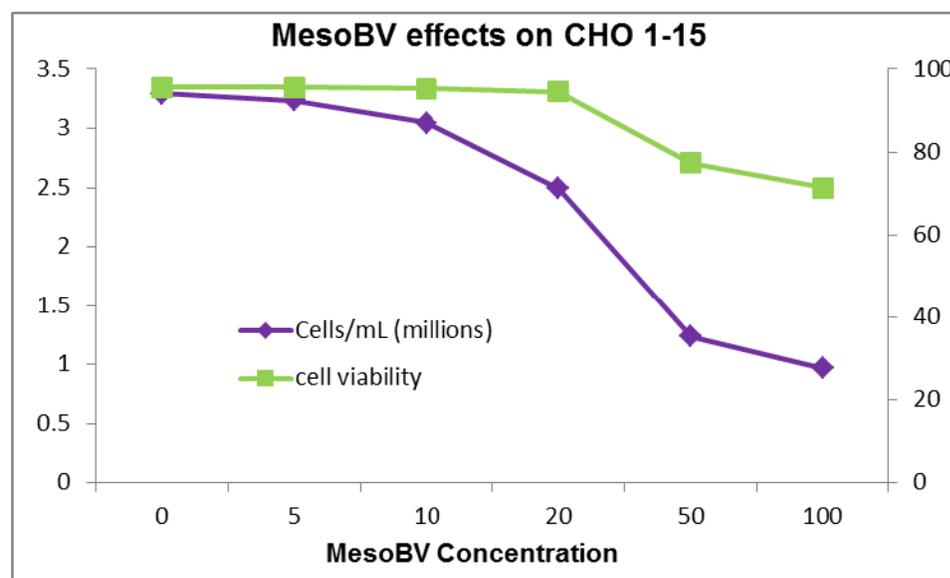


**Figure 3-4**: Toxicity effects with increasing concentrations of mesoBV IXα on CHO 1-15 cells growth and viability.

The results in Figure 3-5 show the tPA quantity from three different parameters. The flask with mesoBV IXα added on day 0 and day 5 had a decrease in tPA production after the second dose on day 5. The control and the flask with mesoBV IXα added only on day 0 had very similar levels of tPA production. The tPA quantity decreased in the flask that the mesoBV IXα was added twice because the cell density and viability decreased after the second addition of mesoBV. The cell density on day 8 for the mesoBV IXα added on day 0 and 5 was $1 \times 10^6$ cells/mL and the mesoBV IXα added only on day 0 was $8 \times 10^6$ cells/mL (Figure 3-5).
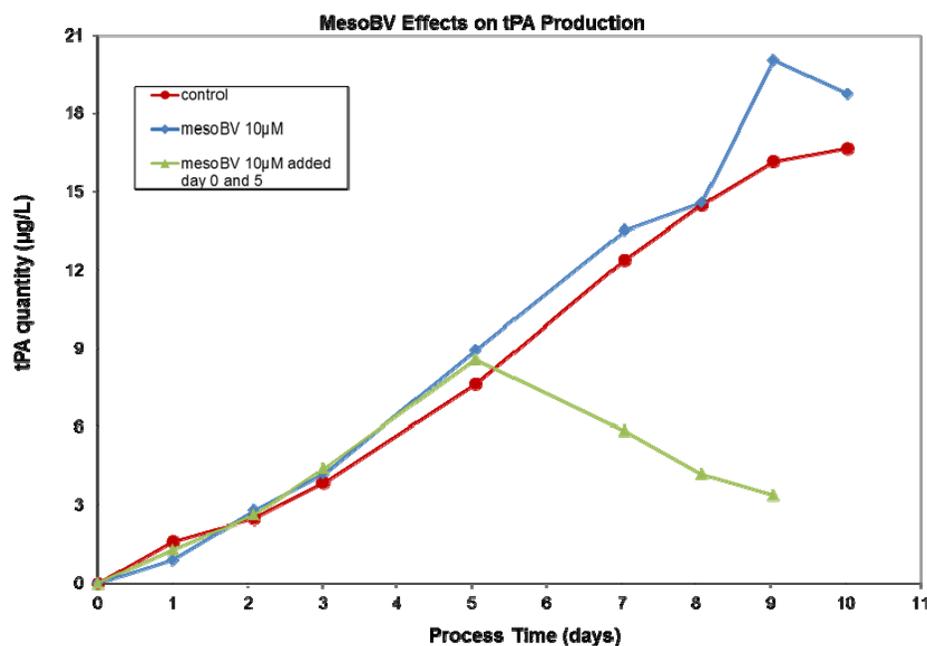


**Figure 3-5**: The effects on tPA production in CHO 1-15 cells after exposure to mesoBV IXα.

**DISCUSSION AND CONCLUSIONS**

Inducing oxidative stress in the CHO 1-15 cell line was measured by cell injury, slowed growth or death. The cytotoxicity studies focused on two different stressors: $H_2O_2$ and menadione. Those compounds have been shown to induce oxidative stress in CHO cells and can cause apoptosis or necrosis. The first oxidant tested was hydrogen peroxide. The publication that was the model of the oxidative stress experiments used primary cell cultures (55). It required higher concentrations of hydrogen peroxide to induce oxidative stress in the CHO 1-15 secondary cell line. The heme-derived compounds did not inhibit oxidative stress in CHO 1-15 cells, which are immortalized cells, when measured with the trypan blue method. Those results were opposite from those with primary cells and mesoBV IXα. The mesoBV IXα protected primary cells from oxidative stress (12) (49). This indicates that there is a major difference between secondary cells and primary cells and the way oxidative stress affects them.

Oxidative stress causes a down-regulation of protein production in cells. CHO cells are considered reliable and robust for high value recombinant protein production. It is important to know how the heme-derived compounds effect protein production in the CHO 1-15 cell line. The CHO 1-15 cell line expresses tPA and secretes the tPA into the media. The quantity of tPA was analyzed to examine the effects that mesoBV IXα had on tPA production.  MesoBV IXα at a 10μM concentration had no effect on the tPA production when exposed one time to the CHO 1-15 cells.

As a secondary cell line, CHO cells have been propagated in culture for many generations and have evolved to divide continuously. In essence, they have achieved an immortalized state of growth and existence.  Such a state provides one possible

explanation for why the heme-derived compounds did not protect CHO 1-15 cells from oxidative stress. It may be speculated that they have developed a high degree of resistance or tolerance to oxidative stress. A recent publication (51) has shown that biliverdin's antioxidant capabilities by scavenging of reactive oxygen species (ROS) inhibit the growth of immortalized cancer cells. ROS-mediated cellular signaling is critical for oncogenic pathways that promote cellular growth (51). Furthermore, increased oxidative stress in cancer cells upregulates growth factors HIF1-a and VEGF that are necessary for angiogenesis (51). Therefore, it is hypothesized that CHO 1-15 cells behave very similarly to cancer cells, which are also considered to be immortal.  This could explain why the heme-derived compounds experiments described above inhibited the growth. ROS activity was not measured in the current work; only cell growth via trypan blue method was measured. It is speculated that heme-derived compounds scavenge ROS which in turn impedes the growth of the CHO 1-15 cell line. This scenario might also explain why a high concentration of hydrogen peroxide is required to inhibit CHO 1-15 cell growth. Since immortal cells use ROS for cell signaling pathways for growth, the hydrogen peroxide at lower concentrations might have been beneficial to the cells. However, at higher concentrations there was a toxic effect. Future experiments would need to measure the ROS levels in the normal CHO cell line, the induced oxidative stress cell line, and the cell lines treated with mesoBV IXα.

CHAPTER 4

SILK GLAND CELL CULTURE

There are no publications on developing a cell line from spider silk producing cells. A cell line from one of the spider silk producing glands should have the capacity to secrete full-length native proteins, which could range in size from 200 kDa to 500 kDa, possibly larger. The common disadvantage of all heterologous expression systems attempted for producing spider silk is they produce only fragments of the spider silk proteins that have decreased mechanical properties. An immortalized silk producing cell line that produces full-length proteins could be the solution for the large-scale production of spider silk. Also, a cell line capable of producing very large proteins would have broad utility beyond simply the production of full length silk proteins.

*Nephila clavipes* is an orb-weaving spider which has six different silk producing glands and one glue producing gland (26). Silk proteins (spidroins) produced by the six different silk glands generally have relatively high molecular weights of 200 K to over 350 K (27). The synthesis of these spidroins occurs in columnar epithelial cells (26). The major ampullate glands are the most studied of the six different glands due to the mechanical ability of the silk the glands produce. The major ampullate silk has two proteins: Major ampullate spidroin 1 (MaSp1) and Major ampullate spidroin 2 (MaSp2). These proteins are extremely large, with molecular weights between 275 K and 320 K (22).

Due to the seasonal nature of spiders (only available in the fall months) the same techniques of isolating a cell line to primary culture from the silk producing glands of the

silkworm *Bombyx mori* (available year around) were also explored. Such a system

permits making silk producing cell lines when adult spiders are not available. Although

many cell lines have been developed from *B. mori,* none have ever been reported from

their silk producing glands. *B. mori* is the domesticated larva of the silkworm moth. The

silkworm produces a twin-thread of silk fibroin, secreted by gland cells. There are two

silk glands per worm. The silk gland is made up of three zones: anterior, middle and

posterior. Only the middle and posterior parts have cells that secrete very large silk

fibroins of up to 350 kDa. These cells would be useful for high-level expression of large

proteins. The results would have similar benefits in terms of silk production as a spider

silk gland derived cell line.


**MATERIALS AND METHODS**

Insect cell culture success is dependent upon antibiotics, improved medium, and

patience (41). The methods for establishing primary (finite) and secondary (continuous)

insect cell lines are still poorly developed (43). Primary insect cell culture has been

achievable. Most insect cell culture success (80%) is usually from the *Lepidoptera* and

*Diptera* species at a larva stage. There are not many published successes from adult

insects and only one from adult spiders. Since the methods are premature, trial and error

will be the way to find the optimal medium and cell line maintenance procedures. The

base media used was chosen from a paper that discusses the techniques used to start a

new insect cell line from the *Spodoptera exigua* using TNM-FH media with 10% FBS.

TNM-FH is a low cost, common media (52). Antibiotics are critical in the success of

insect cell culture because of the high risk of contamination culturing a primary insect

cell line holds. A publication that was successful in culturing epithelial venom gland cells for 48 h and muscle gland cells for up to 6 months in culture was used as the basis for supplements to the TNM-FH media (53). The supplements and antibiotics were prepared and filtered sterilized before addition to the media at the following concentrations: gentamicin (10 µg/mL), amphotericin (0.25 µg/mL), tetracycline (10 µg/mL), oxaloacetic acid (9.5mM), insulin (8.3 µg/mL), and 10% fetal bovine serum (FBS).

Various methods were attempted to generate major ampullate cells in primary culture. Below describes the best method to date. An adult *N. clavipes* spider was disinfected by submerging the entire live spider in 70% EtOH for 3-5 min. The cephalothorax was removed from the body and placed in a sterile petri dish to be used with a dissection microscope. SSC (sodium citrate) pH 7.0 was used when removing the glands from the cephalothorax. The paired major ampullate glands were removed. Figure 4-1 shows a major gland after it is isolated from the spider. Although the major ampullate glands were the focus of this study, the piriform and minor glands were harvested after the major glands were harvested. The glands were placed in a microcentrifuge tube in 400 µL of TNM-FH medium with supplements (antibiotics and serum) but no FBS, since the FBS inactivates the proteolytic enzymes. The glandular tissue was minced with dissection scissors in the tube. After the tissue was minced the tissue was digested with proteolytic enzymes in order to yield single cells. Two hundred µL of 0.05% trypsin plus 0.02% EDTA solution was added and incubated at 37°C for 5 min. Then collagenase (400 µL) was added to the microcentrifuge tube for at a final concentration of 1 mg/mL and incubated at 37°C for an additional 10 min. Medium with FBS (100 µL-300 µL) was added to the tube before the cells were centrifuged at 218 X g

for 4 min or 10 X g for 10 min (10 X g was less harsh and more successful). The supernatant was discarded and the cell pellet was gently washed with 100 µL of medium with supplements (antibiotics and serum). This wash is important to remove contamination. The wash was centrifuged again at 218 X g for 4 min or 10 X g for 10 min following the same speed from the first spinning. The supernatant was discarded and the cell pellet was re-suspended gently in medium and was then filtered with a cell strainer into a 50 mL conical vial before addition to a T25 cm² flask with a final volume of medium of 2 – 3 mL. The cells were kept at 27°C and 5 % $CO_2$.

About 500 µL to 1 mL of media was added to the flasks once every 7 days. It was found that leaving the cells alone and manipulating the flasks less often helped increase the survival of the cells. Since the venom gland paper was only successful with isolated epithelial gland cells in culture for 48 h it was thought to treat our primary cells with a mutagen as soon as possible as an attempt to preserve and mutate the cells to divide continuously (53). The chemical, MNNG, a mono-functional alkylating agent that causes chromosomal DNA damage, has been used as a chemical transformation means for immortalizing mammalian cells and has been used successfully for transforming insect cells from a primary to a secondary culture (43). Unfortunately, MNNG is unobtainable in the U.S., but a similar mutagen, MNU is recommended by Sigma Aldrich and is available for use in the U.S. Based on the literature the concentration of MNU was 3 µg/mL and added to a flask of cells for 3 days (43).

**Figure 4-1**: Major ampullate gland isolated from *Nephila clavipes*

A western blot analysis was done on the major ampullate gland cells. In order to classify these cells, a small scraping of cells plus any protein surrounding them was collected in 500 μL of medium at 19 weeks in culture. The sample was centrifuged for 18 min at 14,000 RPM. The cell pellet was re-suspended in sample buffer with urea (SABU) and prepped for a western blot analysis. The samples were prepared first by sonication for three seconds and added in a 1:1 ratio of SABU to sample. Then the samples were boiled for 10 min. Samples were loaded on a TRIS-HEPES gel at 20 μL each well. The gel was run for 8 h at 40 V then 3 h at 50 V. The gel was transferred to PVDF plus paper and blocked in milk solution for 30 min. Spider silk conserved C-termini polyclonal antibody (M4, Pacific Immunology) was added at 1:1000 dilution for 30 min. The PVDF paper was quickly washed two times and a third time for 5 min with agitation. The secondary antibody was a donkey anti-rabbit IgG labeled with alkaline phosphatase (AP) for detection at 1:2850 dilution in TBS-T20 with milk was added for 30 min with agitation. The membrane was washed quickly two times and for 5 min on the third wash. One step developer for AP was added for 2 min.

The method for isolating the silk worm silk gland cells differed from that for spider silk glands. The media was the same as the spider silk media with the only difference being the percent of FBS, 20% was used instead of 10%; 20% is typically used for primary *B. mori* cells to promote cell proliferation. The silkworm was sterilized for 3 min in 70% EtOH before dissection. All tools were sterilized and dissections took place in a sterilized laminar flow hood. The worms were pinned down (head and tail) onto sterile Styrofoam with dorsal side up. An incision in the middle of the dorsal side was made from the tail all the way to the head just under the surface of the skin making sure no other parts of the worm were cut to avoid contamination. This exposed the silk glands, and the middle and posterior zones of the glands were isolated and placed in a microcentrifuge tube containing PBS. The glands were rinsed twice and then placed in a fresh microcentrifuge tube with medium (500 μL). The glands were minced with scissors and then plated in T-25 cm² for growth (2 silk glands per flask). Another method of for harvesting the cells from the glands was attempted. The glands were minced with scissors then digested with a 0.05% trypsin plus 0.02% EDTA solution for 5 min, centrifuged at 10 x g for 10 min and filtered through a cell strainer before plating.

A "Cells to PCR" kit from Invitrogen (SuperScript II cells direct) was used to classify the cells from the silk worm gland as silk producing cells. The mRNA was isolated from the cells and first strand cDNA synthesis was performed using the kit. PCR was performed using the GoTaq kit and primers designed to prime the DNA of the *B. mori* silk fibroin (Figure 4-2) The reaction mixture (30 μL) contained 15 μL GoTaq, 1.5 μL cyber green, 1 μL of forward and reverse 10 mM primer, 6.5 μL of $H_2O$ and 5 ng of template DNA. The reactions were carried out in a thermal cycler using the following

conditions. (i) 94°C for 3 min, 1 cycle; (ii) 94°C for 1 min, 65-51°C for 30 s, 72°C for 45 s, 37 cycles; (iii) 72°C for 5 min, 1 cycle. The temperature gradient used for the PCR reaction ranging between 65°C to 51°C between eight reactions. The target size of DNA is 372 bp.

**RESULTS**

Successful cell cultures from the major ampullate gland are shown in Figure 4-3A. Major ampullate cells adhered to the tissue cultured treated T-25 cm² flask. The cells secreted a matrix that increased adherence to the flask. Unfortunately, the matrix was so strong that proteolytic enzymes used to harvest cells, such as trypsin, collagenase, and elastase, did not work to break up the matrix. As a result, cells were unable to detach from the flask for passaging. Using a cell scraper killed the cells and did not remove all of the matrix. As time passed, the cells surrounded by the matrix died (Figure 4-3B). To prevent cells from adhering to the flask and secreting their matrix, the cells were placed on a shaker platform immediately after harvested from the spider.



5′ ATCTTGTGC TGCGCTCTG CAGTATGTC GCTTATACA AATGCAACA TCAATGATT
TTGATGAGG CTATTTTGG GAGTGATGT CACTGTCCA AAGTAGTAA TACAACAGA
TGAAATAAT TAGAGATGC ATCTGGGGC AGTTATCGA AGAACAAAT TACAACTAA
AAAAATGCA ACGGAAAAA TAAAAACCA TGGAATAC TTGGAAAAA ATGAAAAAA
TGATCAAGAC GTTCGTTAT AACCACGGA TTCCGACGG TAACGAGTC CATTGTAGA
GGAAGATGT GCTCATGA AGACACTTT CCGATGGTA CTGTTGCTC AAAGTTATG
TTGCTGCTG ATGCGGGAG CATATTCTCA GAGCGGGC CATACGTAT CAA 3′

**Figure 4-2:** A DNA sequence of silk fibroin from the *Bombyx mori*. The bases shown in red are where the primers annealed.

Images of the major ampullate cells that were plated and placed immediately on the shaker platform are shown in Figure 4-4. Those cells in Figure 4-4 were treated with MNU 10 days after isolation into primary culture. The cells looked healthy and the shaking did not harm the cells. The shaking did not prevent the cells adherence to the flask, although the attachment was lighter and the matrix did not form as in Figure 4-3. These cells survived in culture for 6 months. The growth seemed to slow or stop after 6-8 weeks. The cells were maintained by adding only 1 -2 mL of fresh medium per week and replacing all the medium after 6–8 weeks.
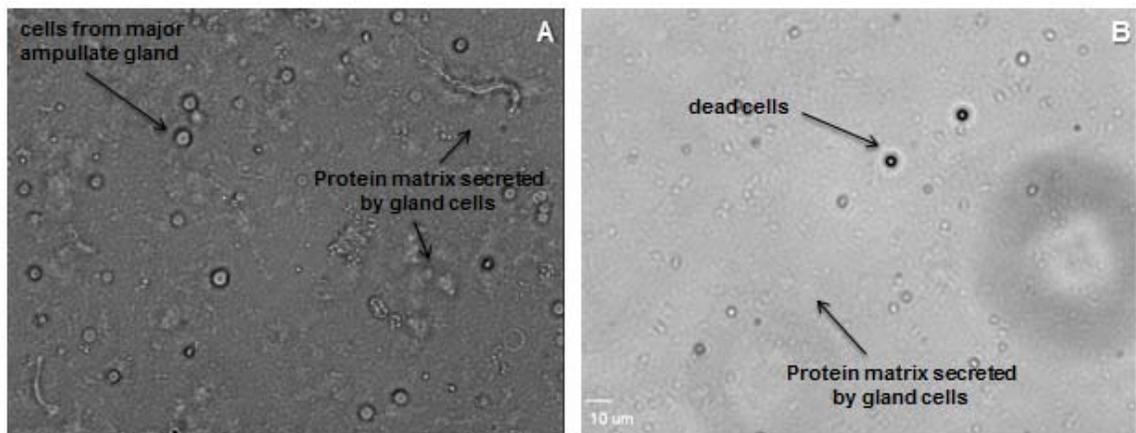


**Figure 4-3**: **A**) cells from the major ampullate gland in culture for 3 weeks shown at high magnification (40 x). **B**) Dead cells from the major ampullate gland in culture for 3 months shown at high magnification (40 x) surrounded by protein matrix secreted by cells that prevented cell-growth and sub-culturing.
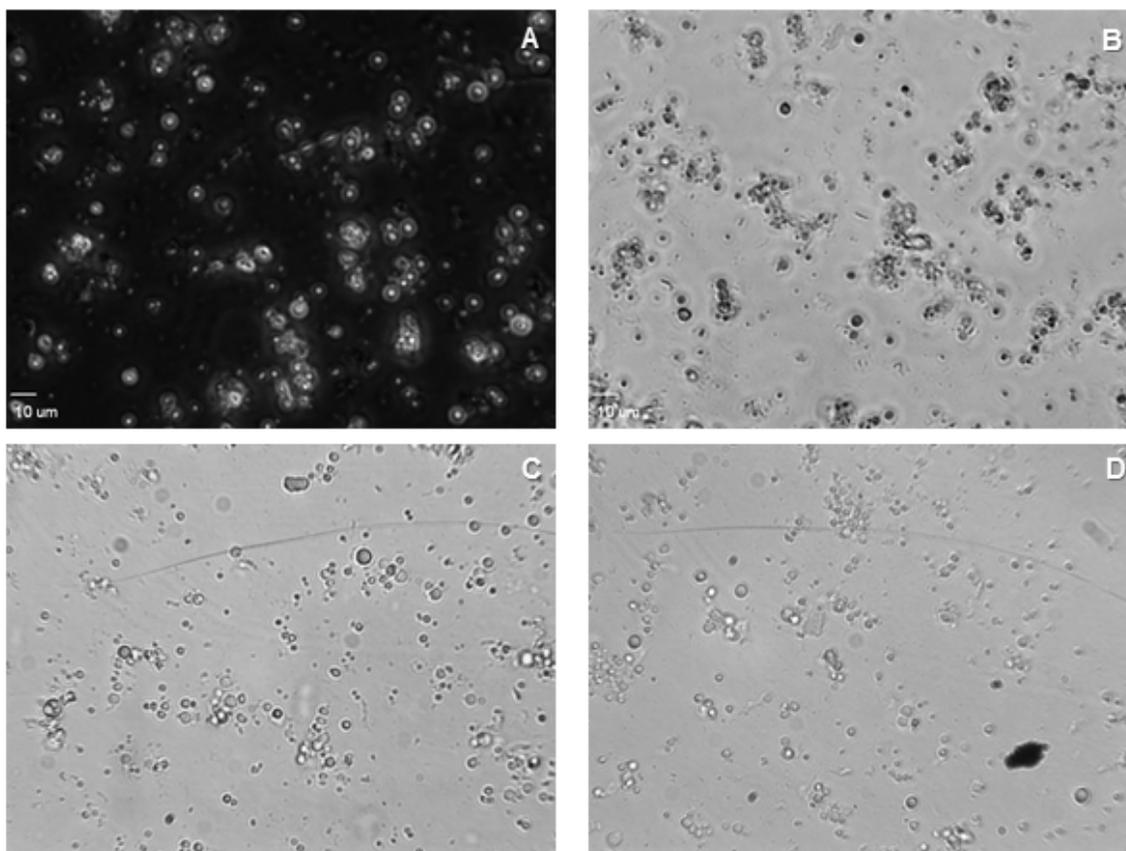
**Figure 4-4**: Major ampullate cells. **A** and **B** were images taken after the cells were 5 weeks in culture with high magnification (40 x). **C** was taken 13 weeks in culture at high magnification. **D** was taken 19 weeks in culture at high magnification (40 x).

In western blots, major ampullate cell extracts showed a purple smear throughout the entire lane. Expected sizes for full length spider silk protein were between 200 kDa to 350 kDa. Although the blot staining intensities were greater around the 250 kDa and higher areas, a discrete and cleared stained protein band was not seen (Figure 4-5). This may have resulted from the mixture of cells at different stages of protein production. Cells in culture do not behave the same as in their natural environment. Mutations may have altered the cells ability to produce full length silk proteins. The cells may have stopped producing silk and stopped growing because they were not successfully

immortalized. The purple smear might be from the proteins inside the cells that stopped

growing and what was adhered to the flask. This could explain why the cells are not

secreting the proteins into the media anymore. The positive results do indicate that the

cells grown in culture for several months were major ampullate gland cells.
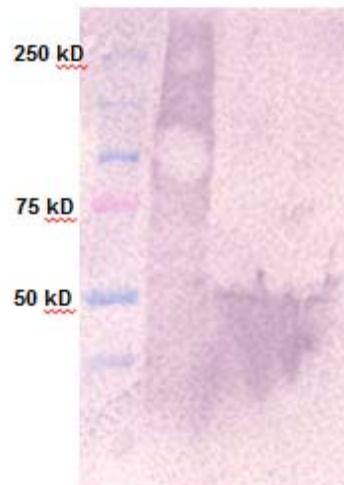


**Figure 4-5**: Western blot analysis with major ampullate cells after they had been in culture for 5 months. Lane 1- Precision Plus marker (Bio Rad), lane 2- major ampullate cell sample, lane 3-media from major ampullate cell flask, lane 4-media control.

The piriform and minor gland cells were isolated from *N. clavipes*. Once isolated into culture, neither had the abundance or potential that cells from the major ampullate demonstrated. The morphologies of the piriform and minor cell types are shown in Figure 4-6. The minor gland cells had a similar protein matrix problem, no other cell types secreted a matrix like the major ampullate cells did. The matrix problem with the minor glands was prevented by placing the flask on a shaker platform after isolation. The cells from the piriform gland did not show any cellular growth. It might be that the T-25 cm² flask was too large of a surface area for the small cells to attach and continue growing (Figure 4-6).
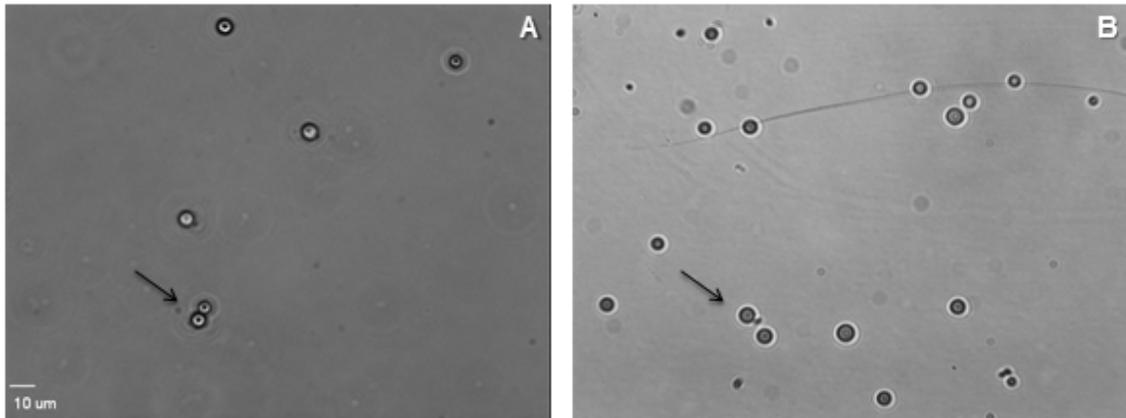


**Figure 4-6**: **A** cells from the piriform gland at high magnification (40 x). **B** cells from the minor ampullate gland at high magnification (40 x).

The results pictured in Figure 4-7 are cells from the B. *mori* silk gland. The silkworm silk gland cells are larger than the silk gland cells from the spider. The cells in Figure 4-7A are from the mincing only method and were not digested with any proteolytic enzymes. The cells were surrounded with tissue and plated with heavy amounts of tissue debris. It was difficult to yield single cells. Figure 4-7B are cells that were minced with scissors then digested with a 0.05% trypsin plus 0.02% EDTA solution for 5 min, centrifuged at 10 x g for 10 min and filtered through a cell strainer before plating. There was a decrease of unwanted tissue/debris with this method although there were still not many single cells.
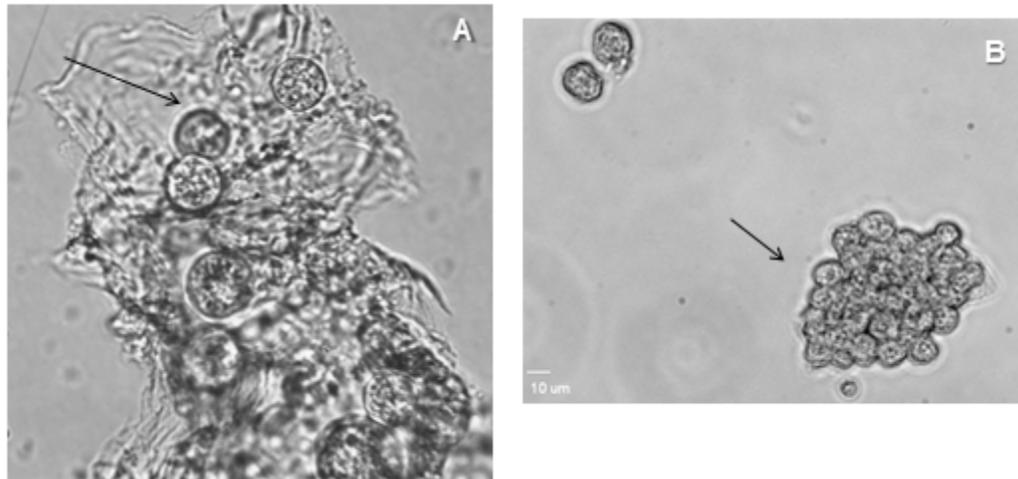


**Figure 4-7:** Living cells from the silk glands from the *B. mori.* A) gland cells plated after mincing with scissors only. B) gland cells that were digested and filtered through a cell strainer before plating.

The results in Figure 4-8 are from a gel run from a PCR reaction with template DNA from *B. mori* cells isolated in primary culture. The expected size was 372 bp and is shown positive in Figure 4-8. The mRNA was extracted from the silk gland cells in primary culture to yield template cDNA. PCR was performed with primers that are designed to amplify the DNA sequence from *B. mori* silk (Figure 4-8). The cells isolated into primary culture are from the *B. mori* silk gland. These results demonstrate the potential silk gland cells have to produce silk in culture as indicated by the presence of the messenger sequences.
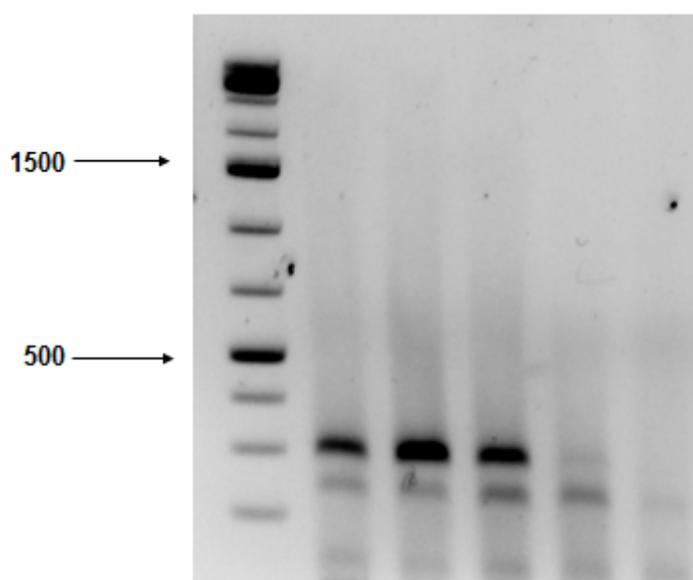


**Figure 4-8:** Gel electrophoresis results from a PCR reaction with DNA from silk gland cells. The first lane is the marker, lane 2, DNA from a PCR reaction with annealing temperature of 65°C, lane 3, annealing temperature of 64.2°C, lane 4 at 62.6°C, lane 6 at 59.9°C, lane 7 at 56.5°C.

**DISCUSSION AND CONCLUSIONS**

The primary cell line from spider major ampullate gland cells appeared viable in culture for about 6 months. Primary culture of major ampullate gland cells has never been reported in the literature. The work with getting a secondary silk producing cell line in culture should continue. Major ampullate glands should be isolated from at least 4 adult spiders before placing in a T-25 $^2$ flask. If fewer glands are isolated, then plating in smaller plates (24-12 well) would have a better chance of success. Also, keeping the cells on the shaker is beneficial and should continue. The concentration of the mutagen or its time of exposure might be increased. Other methods besides using MNU might have a positive result also.

The cells from the silkworm gland are the first native silk producing cells in primary culture. Many cell lines have been developed from *B. mori,* although it usually takes several months to years to develop a secondary cell line. The use of MNU to immortalize *B. mori* cells has not been reported in the literature. Successfully using the MNU compound to immortalize the primary cells could change the way that insect cell lines are immortalized. It is recommended to continue the research with silk worm gland cells. These animals are available all year long. Multiple cell lines have been developed from the *B. mori.* There are a lot of published protocols on starting cell lines from other parts of the silk worm. The silk gland cells from the silk worm are able to be isolated into primary culture. Methods for immortalizing it can be developed to make a secondary cell line.

Not only would a native silk producing cell impact spider silk production but also be used to further insect cell recombinant technology production. It has been demonstrated that insect cell lines are good choices for expression of recombinant molecules, including antibodies (54). In some cases, expression levels with insects are higher than with mammalian cells (54). Insect cells have the capability of post-translational modifications that would need to be provided by a eukaryotic cell (54).

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

**PART 1: CHO 1-15 GROWTH STUDIES**

The use of large scale bioreactors in the biomanufacturing industry is rising. Large scale platforms allow for mass quantities of protein production,which leads to increased profits for companies. For example, one run of a 2 L bioreactor with CHO 1-15 cells can produce 52 mg of tPA, while one run of a 50L S.U.B. can produce 1.35 g. The cost of tPA is around $285 per 100μg (reference), so the difference in dollar amout of tPA between the 2 L and 50 L system is nearly 3.7 million dollars.

Large scale bioreactors are sensitive to a varity of factors. Protocols established in the BioProcessing and Training Demonstration Lab will ensure that trained personnel with access to quality equipment and materials will be able to achieve desirable results in their own large scale bioreactor runs. The ability to control parameters such as % DO, pH, agitation, and temperature allows the user to maximize protein production. Also the convience of working with disposable BPC's allows for large scale bioreactors to be used successfully in smaler scale labs with minimal equipment.

The average maximum amount of  tPA from the shaker platform was 19.8 mg per L in HyCell CHO media. This media has never been used with the CHO 1-15 cell lines in the large scale platforms. That would make the 19.8 mg average from the shaker flask the base value to compare the results to the platforms. The tPA increases to 24.3 mg per L for the 2 L bioreactor and then to 30 mg per L for the 50 L (run 1 only).  The ability to have more control over growth parameters with bioreactors does increase the amount of

protein production in cell lines. It is recommended that this project move forward with one or two more runs using the 50 L S.U.B. to get a better idea of the amount of tPA produced in a 50 L S.U.B. from the CHO 1-15 cell line in HyCell media. It would be expected that the maximum amount of tPA in the 250 L S.U.B. would be higher than the 50 L. Experiments done in the 250 L S.U.B. can verify that claim.

## PART 2: HEME-DERIVED ANTIOXIDANT COMPOUNDS

As a secondary cell line, CHO cells have been propagated in culture for many generations and have evolved to divide continuously. In essence, they have achieved an immortalized state of growth and existence. Such a state provides one possible explanation for why the heme-derived compounds did not protect CHO 1-15 cells from oxidative stress. It may be speculated that they have developed a high degree of resistance or tolerance to oxidative stress. A recent publication (51) has shown that BV IXα's antioxidant and scavenging of ROS capabilities inhibit the growth of immortalized cancer cells. ROS-mediated cellular signaling is critical for oncogenic pathways that promote cellular growth (51). Furthermore, increased oxidative stress in cancer cells upregulates growth factors HIF1-a and VEGF that are necessary for angiogenesis (51). Therefore, it is hypothesized that CHO 1-15 cells behave very similarly to cancer cells, which are also considered to be immortal. This could explain why the heme-derived compounds experiments described above inhibited the growth. ROS activity was not measured in the current work; only cell growth via trypan blue method was measured. It is speculated that heme-derived compounds scavenge ROS which in turn impedes the growth of the CHO 1-15 cell line. This scenario might also explain why a high

concentration of hydrogen peroxide is required to inhibit CHO 1-15 cell growth. Since immortal cells use ROS for cell signaling pathways for growth, the hydrogen peroxide at lower concentrations might have been beneficial to the cells. However, at higher concentrations there was a toxic effect. Future experiments would need to measure the ROS levels in the normal CHO 1-15 cell line, the induced oxidative stress cell line, and the cell lines treated with mesoBV IXα.

**PART 3: SILK GLAND CELL CULTURE**

Not only would a native silk producing cell impact spider silk production but it could also be used to further insect cell recombinant technology production. It has been demonstrated that insect cell lines are good choices for expression of recombinant molecules, including antibodies (54). In some cases, expression levels with insects are higher than with mammalian cells (54). Insect cells have the capability of post-translational modifications that would need to be provided by a eukaryotic cell (54).

Primary culture of major ampullate gland cells has never been reported in the literature. The work with getting a secondary silk producing cell line in culture should continue. The major ampullate gland is the largest of the glands in the spider and is the most studied. The main focus should be on the major ampullate gland and not the other glands. This will make it easier when isolating into primary culture. Major ampullate glands should be isolated from at least 4 adult spiders (8 glands) before placing in a T-25$^2$ flask. If fewer glands are isolated, then plating in smaller plates (24-12 well) would have a better chance of success. Also, keeping the cells on the shaker is beneficial and is recommended. The concentration of or length of exposure to the mutagen might be

increased. Other methods besides using MNU might have a better chance of success, since the use of MNU for immortalizing insect cell lines is not well studied.

The cells from the silk worm gland reported here are the first native silk producing cells in primary culture. Many cell lines have been developed from *B. mori,* although it usually takes several months to years to develop a secondary cell line. Developing the silk gland cells of the *B. mori* has a better chance of success than from the spider. These worms are available all year long unlike the spider which is only available in the fall months. The glands come from a larval stage while the glands from the spider come from the adult. Multiple cell lines have been developed from *B. mori.* There are a lot of published protocols on starting cell lines from other parts of the silk worm. Also there are many papers and protocols to try for isolating cells from *B. mori* into culture. The use of MNU to immortalize *B. mori* cells has not been reported in the literature. Successfully using the MNU compound to immortalize the primary cells could change the way that insect cell lines are immortalized. It is recommended to continue the research with silkworm gland cells. Methods for immortalizing them can be developed to make a secondary cell line.

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APPENDICES

APPENDIX A

MAINTENANCE OF CHO 1-15 CELLS IN HYCELL MEDIUM

MAINTENANCE OF CHO 1-15 CELLS IN HYCELL MEDIUM (adapted from

Thermo Fisher-Scientific)

**Purpose.** To keep cell cultures at an optimal density for continued growth and to

stimulate further proliferation, the culture must be divided and fresh medium supplied.

**Materials.**

- PETG shaker flasks, E-125, E-250, E-500

- Pipettes (varying sizes)

- Vi-CELL sample cup

- HyCell CHO growth Media (TFS)

- Vi-CELL analyzer (Beckman Coulter)

- Laminar flow hood

**Procedure.**

1. **Passaging.** It is critical to passage the CHO 1-15 cells while they are in log phase growth. Passaging should be done routinely and take place every 3-4 days when flasks have been seeded at 250,000 cells/mL. For example: passage cells every Monday and Friday to maintain healthy cells and keep in log growth phase.

2. When the cells are ready for passaging tighten lid on flask and remove the shaker flask from the incubator and place flask in the laminar flow hood. Do not open the flask until in the sterile laminar flow hood.

3. Maintain aseptic technique at all times when handling mammalian cells. Take a 1 mL sample from the culture shaker flask using a sterile pipette and sterile technique, dispense into a Vi-CELL sample cup. Make sure the cell media mixture is uniform and that cells have not settled down on bottom of flask. If

cells have settled down before taking the sample, swirl the shaker flask to evenly distribute the cells in the media.

4. Use the Vi-CELL machine following the Vi-CELL protocol to get cell count and viability.

5. **Cell Count.** From the Viable cells/ mL, determined by the Vi-CELL sample, calculate the split ratio required to seed a new shaker flask with 250,000 cells/ mL depending on the desired final volume of new shaker flask.

    a. You can calculate your split ratio using the formula: $(V1)(C1)=(V2)(C2)$. Where;

        $V1$= the volume of spent media needed to seed new flask.

        $C1$= the density of spent media.

        $V2$= the desired final volume of media in new flask.

        $C2$= the seeding density of media in new flask.


6. Aseptically transfer the required number of cells to the new shaker flask with the appropriate volume of new media.

7. Loosen the caps of the culture flasks to allow for proper gas exchange (or use a gas-permeable cap), and return the flasks to the shaking incubator. The shaking speed for the maxQ platform shaker platform is 100-150 RPM

8. Label each PETG shaker flask with the following information; (1) date out of cryo + cell line i.e. 010101CHO1-15, (2) date of split, (3) media, (4) initials.

Note. Do not keep cells in culture longer than 3 months from out of cryo date.

APPENDIX B

THAWING CELLS FROM CRYOPRESERVATION AND CELL
CULTURE SCALE-UP

THAWING CELLS FROM CRYOPRESERVATION AND CELL CULTURE

SCALE-UP (adapted from Thermo Fisher-Scientific)

**Purpose.** To outline the procedure for cell culture scale-up.

**Materials.**

CO$_2$ Incubator

Biological Safety Cabinet

Vi-CELL analyzer

Pipettes varying sizes

T-25 Flask

Erlenmeyer flask varying sizes

**Procedure.** All work should be done in a laminar flow hood with aseptic technique.

**Thaw cells.**

1. Remove a cryo vial of frozen CHO cells from the cryo tank filled with liquid nitrogen. Wear safety glasses or a mask shield when working with cryo vials. Make note of which cryo vial you remove.

2. Immediately thaw cells under running warm tap water. Make sure not to get water by the cap of the cryo tube as to maintain sterility.

**Re-suspend cells.**

3. Once the vial is thawed spray the outside of vial with 70% EtOH and continue work inside the laminar flow hood.

4. Add 5-7 mL of HyCell CHO media to a sterile 15 mL conical tube. Add the thawed cells from the cryo vial to that 15mL tube.

5. Centrifuge the media cell mixture at 1,000 RPM for 4 min.

6. Discard the supernatant and re-suspend the cell pellet in 7 mL of HyCell media. This will remove the unwanted DMSO so cells can recover and grow in culture.

7. Immediately plate the cells in a T-25 flask by adding the 7 mL of media cell mixture to the T-25 flask.

8. Label flask as follows: (1) date out of cryo + cell line i.e. 010101CHO1-15 (2) date of split (3) media (4) initials. Immediately put flask in a $CO_2$ incubator at 37°C.

**Cells in suspension.**

9. After 2-4 days remove cells from T-25 flask and start the up-scale process by transferring cell/media mixture to a 125 shaker flask.

10. Make the final volume of media from 30 mL- 50 mL. Maintain cells following the protocol for CHO 1-15 maintenance.

**Up-scale cells.**

11. Count cells every 3 - 4 days. When cells are confluent enough to passage, seed them in the highest volume of complete media using the appropriate seeding density for the cell line being worked with. To calculate how much you will need to seed a bigger flask use the following calculation:

Seeding density you want / viable cells/mL x volume you want to achieve. This will give you the volume of culture to seed the new flask. Take that volume and subtract it from the total volume desired this will give the volume of fresh media to add. Use the table below to find the appropriate flask:

Flask

T-25 – 7 mL

125 mL Erlenmeyer – 50 mL

250 mL Erlenmeyer – 100 mL

500 mL Erlenmeyer – 200 mL

2800 mL Erlenmeyer (Fernbach Flask) – 800 mL

Repeat the process as necessary to reach the desired scale-up

APPENDIX C

CRYOPRESERVATION PROTOCOL

CRYOPRESERVATION PROTOCOL (adapted from Thermo Fisher-Scientific)

**Purpose.** The purpose of this procedure is to establish a method for preserving the CHO 1-15 cell line.

**Materials**.

Sterile conical centrifuge tubes 15ml and 50ml

Nalgene cryo vials

Cryo marker for labeling

DMSO

70% isopropanol

centrifuge

**Procedure.** All work with open cultures and media should be done under a hood. Cells to be cryopreserved should be maintained in mid-log phase and at a 90 % or higher viability.

1. Cryopreservation Medium Preparation. Use 7.5 % v/v DMSO final concentration to the cell medium being used. Make sure the DMSO is sterile.

2. Use a cryo marker to label the desired number of cryo vials with cell name, date frozen, cell concentration, and DMSO %.

3. Determine cell Determine cell concentration using the Vi-CELL protocol. Adjust the cell concentration so it is between $2 \times 10^6$ cells/mL and $7 \times 10^6$ cells/mL. Centrifuge suspended cells at 1,000 x RPM for 4 min and re-suspend in sterile cryopreservation medium. Work should proceed quickly to minimize length of time cells are exposed to the DMSO which acts as the cytoprotective agent.

4. Place 1-1.5 mL of the cell suspension in each sterile ampule by using a sterile pipet and tighten the lid.

5. Fill the Biotech apparatus with fresh isopropanol (if level is low).

6. Place sealed ampules into the Biotech apparatus and cool at an approximate rate of 1 °C/min by placing the unit into an ultra-low freezer that is kept at -70 °C or colder. The apparatus must remain in the ultra-low freezer for at least 18 h.

7. Put on protective wear, safety glasses or face shield and remove the Biotech apparatus from the ultra-low freezer.

8. Remove ampules from Biotech apparatus, wipe off alcohol, place in numbered canes and place canes in the liquid nitrogen freezer. Work quickly. Do not allow the vials to warm.

APPENDIX D

HYCELL CHO MEDIUM PREPARATION PROTOCOL

HYCELL CHO MEDIA PREPARATION PROTOCOL (adapted from Thermo

Fisher-Scientific)

**Purpose**.  To make sterile media for CHO cell growth and maintenance.

**Materials.**

- HyCell CHO

- Pluronic F68

- Sodium Bicarbonate

- L-glutamine

- 1 L PETG sterile bottle

- Filter

**Procedure.**  Use the following table to calculate the amounts needed for the

desired volume. Example below is for 1 L and 10 L.

**Table D-1:** HyCell CHO media prep calculation chart

| Component | Volume = 1.00 L Conc. | Unit | Preparation Volume= 10.00 L Conc. | Unit |
|---|---|---|---|---|
| HyCell CHO | 25.40 | G | 254.00 | g |
| Pluronic F68 | 1.00 | G | 10.00 | g |
| SODIUM BICARBONATE | 2.20 | G | 22.00 | g |
| L-Glutamine (USP Tested) | 0.88 | G | 8.78 | g |

1.  Weigh out the correction g unit for the desired volume of media.

2.  Add distilled $H_2O$ about 1 L less than the total desired volume.

3.  Mix powders until dissolved with a stir bar. Once completely in solution stop stirring and add remaining volume to bring solution up to final desired volume.

4.  Measure pH of the solution using a pH meter. pH should be between 7.0-7.2.

5.  Adjust the pH as necessary.

6.  Sterile filter mixed media into sterile 1L PETG bottles.

7.  Store media for up to 1 yr at 4-8 °C.

APPENDIX E

Vi-CELL ANALYZER

Vi-CELL ANALYZER (adapted from Thermo Fisher-Scientific)

**Purpose.** The purpose of this document is to describe the correct operation of the Vi-CELL Cell Viability Analyzer.

**Materials.**

Trypan Blue (sigma)

Coulter clenz (cleaning agent)

70% Isopropanol (disinfectant)

Diluent (buffer solution)

**Procedure.**

1. Under files choose configuration. Choose the software tab. Under paths, the data file should read: C:\(Your Name). Choose OK. Place sample cup in next available carousel position. Log in sample by clicking on the log in sample button. Select sample cup position on the carousel (if applicable).

2. Enter sample ID.

3. Choose the cell type.

4. Click OK.

5. Press start queue to begin analysis.

NOTE. Vi-CELL will automatically clean itself with each sample. Refer to product manual for all maintenance procedures such as reagent pack replacement, etc.

References. Beckman Coulter Vi-CELL Cell Viability Analyzer Manual #PN 383082A. Accessible under HELP button on Vi-CELL computer software.

APPENDIX F

tPA ELISA PROTOCOL

tPA ELISA PROTOCOL (adapted from Thermo Fisher-Scientific and Enzyme Research

Laboratories)

**Materials.**

Tissue Plasminogen Activator ELISA kit:  from Enzyme Research Laboratories

(South Bend, USA)- TPA-EIA

Coating buffer:  Sigma-Aldrich C-3041

Wash buffer:  Sigma-Aldrich T9039-10PAK

Blocking buffer:  Sigma-Aldrich T6789-10PAK

Sample diluent:  blocking buffer + Tween-20, 0.05% vol vol$^{-1}$

Ultra TMB- Thermo Fisher-Scientific catalog #34028

Stopping solution- 2.5 M $H_2SO_4$

96-well polystyrene 4-HBX clear flat bottom plate

**Procedure.**

1. **Coating of plates**.  Coat the 96-well plates with the capture antibody (provided

   from the TPA-EIA kit from Enzyme Research Laboratories).  Dilute the antibody

   1:100 in a 0.05M Carbonate-Bicarbonate buffer (Sigma-Aldrich).  This buffer

   helps stabilize the proteins by maintaining their three-dimensional structure which

   in turn helps with greater binding activity.  Add 100 μL of diluted antibody to

   each well in the plate.  Incubate for 2 h at room temperature or overnight at 2-8°C.

2. **Blocking**. After incubation with the capture antibody, the plate is treated with

   blocking buffer.  The blocking buffer used is Tris buffered saline with BSA

   (Sigma).  This will block unoccupied areas of the wells and prevent nonspecific

   binding which will reduce the background signal.  To do this, empty the contents

of the plate and add 150 µL to each well of blocking buffer (Tris buffered Saline

with BSA) and incubate for 60 min at room temperature.   After incubation, wash

the plate 3 times with wash buffer, Tris buffered saline plus Tween-20, 0.05%

(Sigma-Aldrich) (100-150 µL per well with each washing).

3.  During the incubation with blocking buffer, tPA reference standards and samples

are prepared.

a.  **Standards.** Dilute the tPA standards in sample diluent, Tris buffered

saline with BSA plus Tween-20 0.05%. Use a six reference standard with

final tPA concentrations of 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/mL.  Add

100 µL of standard to each assigned well.  Standards must be run in

duplicate.

b.  **Samples.** For the CHO 1-15 cell line grown in 100 mL shaker flask up to-

50 L S.U.B. the samples should be diluted 1:2,000.  Accomplish the

1:2,000 dilution with 2 steps.

i.  1. Perform a 1:100 dilution with 10 µL of sample into 990 µL of

sample diluent.

ii.  2. Perform a 1:20 dilution with the number 1 step (1:100 sample)

50 µL into 950 µL of sample diluent.

Add 100 µL of sample are added to each well.  Samples should be run in

duplicate.  Samples can be run in triplicate if needed.  The coated antibody

will capture the tPA present in the sample.  After samples are added,

incubate the plate at room temperature for 90 min.  After incubation, wash

the plate 3 times with wash buffer.  Be careful not to cross contaminate the

wells after samples have been incubated. Use Nunc sealing tapes to produce better results.

4. **Detecting.** Dilute the detecting antibody 1:100 in sample diluent, Tris buffered saline with BSA plus Tween-20, 0.05%. The detecting antibody binds to the captured tPA. Add 100 µL of diluted detecting antibody to each well and incubate at room temperature for 90 min. After incubation wash the plate 3 times with wash buffer.

5. **Ultra TMB**. The Ultra TMB detects peroxidase activity from the detecting antibody, changing to a blue color. Add 100 µL per well of Ultra TMB. The reaction is very quick, it is best to keep the ultra TMB cold until ready for use. Leave on the substrate on for 1 -2 min, then add 50 µL of 2.5 M $H_2SO_4$ to each well. The acid will change the color from blue to yellow and slow down the reaction. Immediately read the wavelength at 450nm.

APPENDIX G

2L BIOREACTOR INSTRUCTION PROTOCOL

2L BIOREACTOR INSTRUCTION PROTOCOL (adapted from Thermo Fisher Scientific)

**Purpose.** The purpose of this manual is to explain the operations associated with maintaining the Applikon 2L bioreactor in the BioProcessing Demonstration and Training lab. The first priority of any bioreactor process is to provide a sterile environment for cell growth. Sterilization is an important step in preparing the bioreactor prior to inoculation, and that sterile environment must be maintained throughout the entire process. Maintaining a sterile environment consists of two steps: 1) cleaning and sterilization of all equipment used in the process; and 2) the sterile transfer of media and cells in and out of the system. With the use of good lab techniques the risk of contamination can be reduced.

**A. Cleaning of glass vessel, tubing, and head plate/components.**

**Objective**. This procedure outlines the disassembly, cleaning, pre-sterilization, and rinsing of the bioreactor vessel, headplate, and all ports and probes.

**Materials.**

Tergazyme by Alconox

Sponge, pipe cleaners, toothbrush, toothpaste, large brush

Appropriate tools to disassemble the reactor

Sodium Hypochlorite (bleach)

**Procedure**.

1. Remove the DO probe from the headplate. Clean with a sponge or soft toothbrush and detergent. Rinse first with hot tap water, and then with a final rinse in DI water. Store probe in an upright position with the tip of the probe in

DO electrode storage solution or PBS.

2. Remove the pH probe from the headplate. Clean with a sponge or soft toothbrush and detergent. Rinse with hot tap water. Store in an upright position in pH electrode storage solution

3. Remove all tubing from the headplate. Do not reuse tubing and filters, with the exception of the exhaust filter set. (That filter can be re-autoclaved between 6-12 times depending on the application).

4. Remove the headplate from the glass vessel (make sure all cables, etc. are disconnected before headplate is removed). Bleach cells and dump them prior to cleaning the vessel. Take off all removable parts on the headplate to ensure thorough cleaning.

5. All parts (including the vessel) can be cleaned with a sponge or brush and the hot water detergent mix (hot water and Tergazyme). Clean the inside of all ports with a pipe cleaner. Soak the micro sparger in hot water detergent mix, then bleach to make sure that all the holes are free of debris.

6. Allow all parts to air dry on a disinfected surface (or paper towels).

**B. Assembly of apparatus.**

**Objective.** This procedure outlines the preparation of the tubing, headplate assembly, insertion of the probes, and connecting the bioreactor to the Applikon console.

**Materials.**

Silicone tubing (3/16 x 3/8 x 3/32, for harvest, air filter, and condenser lines)

Silicone tubing (1/8 x 1/4 x 1/16, for ends of harvest, feed, and condenser lines)

Tubing accessories

1/8" to 3/16" connectors

1/8" female lure lock

Male lure lock cover

Snapper clamps

Cable ties & cable tie fastener

Whatman Poyvent 16 filters (50mm)

1-1.5L of PBS

DO electrolyte

Glycerol

Whatman Polyvent 500 exhaust filter, or equivalent

**Procedure.**

1.  Once the headplate has been cleaned and dried, put 1-1.5L of the DPBS in the bioreactor.

2.  Place the rubber-sealing ring in the headplate.

3.  Make sure the impeller is firmly attached to the headplate and that all removable parts have been put back onto the reactor, and place the headplate on the glass vessel.

4.  Tighten the nuts that go on top of headplate carefully, always tightening the clamps across from each other at the same time.

5.  The tubing should be prepared similar to the existing pattern and attached to the following locations (figure 1)

**Figure G-1**: Applikon headplate configuration

- Tubes for harvesting, and sampling

    o 1 long tubing length for harvest port

    o 3'-4' of 3/8"x3/16" silicone tubing

    o 6" of 1/8"x1/4" silicone tubing

    o 1/8" to 3/16" connector

    o 1 snapper clamp

    o 3 cable ties (one at each connector end)

    o Lure lock sample piece for sample port (skinny port that goes partway

       down the vessel)

- Triport Feeding lines

    o 2-3' of 1/8"x1/4" silicone tubing for each triport spot

- o   Snapper clamp

- o   1/8" end plug

- o   Cable ties

- Condenser vent assembly

  - o   3'-4' of 3/8"x3/16" silicone tubing in 3 pieces

  - o   1/4" Y connector

  - o   Whatman Polyvent 500 and snapper clamp for one end

  - o   6" of 1/8"x1/4" silicone tubing, 118" end plug, and snapper clamp for other end

  - o   7 cable ties (one at each connector end)

- Shorter gas addition filters

  - o   3"-4" of 3/8"x3/16" silicone tubing for each line

  - o   Whatman Polyvent 16 Filter for each line

  - o   Snapper clamp for sparge line

  - o   Cable tie for each line

6.  Use two cable ties to secure the tubing to the headplate.

7.  Unscrew the end of the DO probe and inspect the mesh at the tip. Make sure the mesh membrane is not damaged. Refill the cap with DO electrolyte and screw it back in place. Insert the DO probe into its port. Adjust the probe so that it does not come in contact with the impeller, and tighten.

**C.  DO and pH Calibration and Autoclaving the Bioreactor:**

**Objective.** This procedure outlines the calibration of the DO and pH probes, as well as the sterilization of the bioreactor using the autoclave located in either QC or production.

**Materials.**

pH 7.00 and pH 4.01 calibration buffers.

Large autoclave bag

**Calibration of the pH Probe.**

1. Turn on the ADI 1010 Bio Controller and make sure all the control loops are switched off. And attach the pH probe to its cable.

2. Enable the calibration screen. Press the pH key. A list of options appears. Press the CALIB key. The calibration menu appears. Turn the dial to highlight CALIBRATION then press the CALIB key to continue.

3. Rinse the pH probe with distilled water to remove all traces of the storage solution.

4. Immerse the pH probe into the pH 7.00 buffer solution and momentarily stir with the probe to ensure proper contact. Allow a minimum of 30 seconds for the electrode to thermally equilibrate with the buffer solution before taking a pH reading. You must let the probe totally equilibrate before calibrating, it may take up to 15 minutes, do not speed the calibration along. The pH reading should be 7.00 pH± 0.33 pH at room temperature, 25°C

5. First the buffer temperature must be entered. Use the dial in order to generate the numeric buffer temperature value. Once temperature is entered press the CALIB key to continue.

6. Now the pH of the first buffer is asked for, use the dial to generate this value and press CALIB key to continue.

7. Rinse the pH probe with distilled water.

8. Immerse the pH probe in pH 4.01 buffer solution and stir with probe to ensure proper contact. Allow a minimum of 30 seconds for proper electrode/solution equilibrium before taking a pH reading, again do not speed this process along, make sure that the probe is equilibrated before calibrating.

9. Press the CALIB key to proceed with the dual point calibration and use the dial to generate the correct value of this buffer and press the CALIB key. This will return the calibration data. Press the SETP key to return to the main screen.

10. Rinse the pH probe with distilled water.

11. Insert the pH probe into its port and assure that it will not come in contact with the impeller, and tighten

**Autoclaving the Bioreactor.**

1. The bioreactor should already contain 1.5L of DPBS, which is necessary when autoclaving.

2. All of the clamps leading to vents and filters should be closed, except the clamps for the condenser vent and the overlay filter should be left open. These are left open because they are not submersed in the DPBS and it is necessary to leave an outlet open so that pressure does not build up in the reactor.

3. The top of the bioreactor with all tubing, filters and vents should be covered with a large autoclave bag. Note: make sure the DO and pH probes are both covered

with their appropriate caps and screwed tightly in the bioreactor.

4. Make sure that the bioreactor is completely disconnected from the Bio
   Controller, and Place the bioreactor in the autoclave. Autoclave on liquid cycle
   (slow exhaust) for 30 minutes.

5. When the autoclave run is over, allow the reactor to cool enough for transport,
   and then return it to the lab.

6. Allow the vessel to cool before reattaching all of the probes, inlet gas lines,
   heat jacket, temperature probe, and water hoses.

7. Hook up the vessel, all probes, and water hoses to the consol. Add
   approximately2-3ml of glycerol to the temperature well before putting the
   temperature probe in the well.

8. Turn on the machine and allow the DO probe to polarize for at least 6 hours
   before calibrating. Attach heat jacket and turn on the temperature control to
   allow the DO probe to polarize at the working temperature. Also, turn on the
   agitation to allow better heat exchange in the vessel, and turn on the air to the
   sparger to allow the vessel to saturate with air.

9. To turn on the air, use a screwdriver to turn the air valve on the console to
   sparge, and make sure that the sparge line is attached to the sparge filter.

10. Make sure the DO control loop is turned off. Press MENU key and use the dial
    to select the MANUAL CONTROL option. Press MENU again to continue.

11. Use the dial to select the AIR VALVE option and press START/STOP key to
    get to the ON/OFF column. Use the dial to toggle between the two options and
    press START/STOP again to confirm action (that valve is on). Press SETP key

to return to main screen.

**Calibration of the DO Probe.**

1. Make sure that the temperature control is turned on (press TEMP key, then START/STOP key) and that the liquid in the reactor has equilibrated to desired run temperature (generally 37°C). If set point of temperature is not at desired set point (i.e. 37°C) change it by pressing TEMP key, then SETP key, the using dial to change the set point value, it will then automatically change it after a minute to the desired value.

2. Make sure the agitation is at least 100-150 rpm. And that air is being pumped into system; see above instructions to turn it on.

3. Allow system to stabilize for approximately 10 minutes (this may take up to 30 minutes).

4. Start the calibration by pressing the dO2 key, followed by the CALIB key. Press the CALIB key again to continue.

5. Use the dial to generate the value 100 and press the CALIB key to continue.

6. The stabilized value is used to calculate the slope of the electrode, which is shown. Press the SETP key to finalize the calibration.

7. The reactor is now ready for seed.

**D. Removal of DPBS and Addition of Media and Seeding Bioreactor**

**Objective**. Use a sterile technique for removing the DPBS and the addition of the desired media prior to a bioreactor run.

**Materials**.

Peristalstic pump

Desired media

Transfer union

**Procedure.**

1. Disconnect all probes, gas lines and water lines and transfer the 2 L bioreactor to a laminar flow hood. In the laminar flow hood, using sterile technique, Pump out the DPBS with the peristaltic pump.

2. When the DPBS has been removed, turn off the pump and clamp off the tubing to maintain the sterile environment.

3. Prior to inoculation, warm media in 37°C incubator then in the laminar flow hood connect one of the feed lines to the transfer union of the media- containing liter bottle. (Use a fresh transfer union for each bottle in order to add the media to the bioreactor.)

4. Under a hood, extract at least 1 mL of cell media from the well-mixed fernbach and put into a cell counter vial.

5. Use the Vi-CELL cell counter to determine how many viable cells there are per mL.

6. Use the following equation to calculate how much of the cell media needs to be transferred to the bioreactor in order to achieve a cell concentration of approximately 250,000 cells/mL.

    a. You can calculate your split ratio using the formula: *(V1)(C1)=(V2)(C2).*

       Solve for V1

       V1= the volume of spent media needed to seed bioreactor

       C1= the density of cells/media in flask

V2=the desired final volume of media in bioreactor (2 L)

C2=the seeding density of media in bioreactor (250,000 cells/mL)

7.  Once you have determined how many mL of the cell media needs to be used to inoculate the bioreactor, transfer that amount from the fernbach flask to the 1 L PETG bottle under the hood.

8.  While still under the hood, open the autoclave bag containing the sterile transfer union. Careful not to touch the tubing below the rubber stopper with anything, insert the transfer union into the PETG bottle with cell/media mixture and the media PETG bottles.

9.  Using the laminar flow hood with aseptic technique, connect the transfer union to one of the addition ports of the bioreactor.

10. Place the tubing in the pump and make sure that the pump is set to turn in the appropriate direction to feed the cells into the bioreactor.

11. Using aseptic technique, disconnect the transfer bottle from the bioreactor, keeping the tubing sterile and the connections closed off.

12. Use the pump to add the media. Disconnect the media vessel and connect to the media/cell mixture. Pump the media/cell mixture into the bioreactor. Disconnect the vessel with sterile technique.

13. Transfer the bioreactor out of the laminar flow hood and back onto the bench top. Re-attach all the probes, cables and water lines. Turn on agitation at 300rpm and the temperature control loop. Allow the cell/media mixture to reach the desired temperature. Once at 37°C the DO, and pH, control loops must be turned on. Do this by pressing the desired parameter key (temp, DO, etc.) then pressing

START/STOP key.

**E. Adding Base**

**Background**. To keep pH at the set level, it may be necessary to add base. Base will keep the culture from going below the desired pH by adding in small increments of the base into the media. Carbon Dioxide, if it is turned on, will keep the pH from rising above the desired set point.

**Objective**. Maintain the pH parameter by adding a base that will keep the pH from drifting downward.

**Procedure**.

1. Make 10-20% (w/v) Sodium Carbonate solution or equivalent, sterile filter, and add a transfer union under a sterile environment.

2. Connect the base to the sterile tubing on the addition port under the laminar flow hood.

3. Place the base addition line into the base pump on the console, making sure that the tubing is added in the correct direction. (The pump move in a clockwise motion), and make sure the clamps are opened between the base vessel and the reactor.

4. The base will now automatically be pumped in, if the pH control loop is turned on.

**F. Connecting of Vessel to Input Gases**

**Background.**

The Applikon allows the input of gas through overlay or sparge. The only gases hooked up to the Applikon reactors are oxygen, air and $CO_2$, To control DO and pH

with these gases, the control loop has to be turned on, and the valves switched on to the desired pathway, sparge or overlay. The pH control also automatically turns on the base pump is the pH is too low, so this can be utilized to help maintain the ideal pH.

**Procedure.**

1. Attach the tubing from the direct sparge line to the filter for the direct sparge on the bioreactor. Open the clamp leading to the filter.

2. Attach the tubing from the overlay sparge line to the filter for the overlay sparge on the bioreactor. Open the clamp leading to the filter.

3. Once the Applikon console has been set to turn on the gas, the regulators (flow meters) for the oxygen, air and $CO_2$ should be adjusted, they usually are run around 300 mL/min at the start of the run.

## G. Taking Samples

**Background.** In order to monitor the progress of the run it is necessary to take a representative sample from the bioreactor on a regular basis. Under normal conditions, the appropriate sample time is once a day. In order to maintain consistency, it is important to take samples at the same time each day

**Objective.** During the course of a run the most probable cause of contamination is from sampling. For this reason the objective in sampling is to get a representative sample from the bioreactor without compromising the sterile environment. By taking extra caution while sampling, and being consistent in the sampling method the possibility for contamination from sampling can be drastically reduced.

**Materials.**

15mL sample tube with lid

Sample tube rack

2 small centrifuge tubes

10mL lure-lock syringe

Isopropanol

Bleach

Cell counter and counter vial

Centrifuge (microfuge)

**Procedure**.

1.  Apply copious amounts of isopropanol to the lure-lock sample port.

2.  Wearing gloves remove the lure-lock cap and apply more isopropanol to the cap and male lure-lock end. (Make sure that the sample port clamp is closed before removing the cap.)

3.  Attach the syringe to the sample port, release the sample port clamp, and fill the syringe with solution from the bioreactor. Make sure you keep positive pressure on the syringe, so there is no backflow into the reactor. Clamping the sample port before taking the syringe off will also insure no backflow.

4.  Discard at least 10mL of solution from the bioreactor into the discard basin to insure that the final sample is coming from the well-mixed bioreactor, Add a small amount of bleach to the unwanted solution, and dump down the drain with plenty of water.

5.  Pull approximately 10mL of sample from the bioreactor. Using the syringe, fill the two centrifuge tubes with equal amounts of solution, and then put the remaining volume of the syringe in the 15mL sample tube.

6. Apply more isopropanol to the sample port end and the cap, and then replace the cap. Make sure the clamp to the sample port is closed.

7. Count 1mL of the sample with the Vi-CELL. Centrifuge the remaining sample in the 15mL tube for 4 min at 1,000rpm.

8. Take 2 1mL samples of the supernatant and store in small centrifuge tubes clearly labeled in the freezer until needed for further use

9. Record the data collected in your lab book.

APPENDIX H

50 L S.U.B. PROTOCOLS

50 L S.U.B. PROTOCOLS (adapted from Thermo Fisher Scientific)

## A. Loading BPC into 50 L S.U.B.

**Background.** Each outer support container is designed for a specific Single-Use Bioreactor (S.U.B.) BioProcessing Container bag (BPC). Confirm the correct volume BPC is being used for the corresponding volume outer support container. The following section outlines the installation and setup of the S.U.B. BPC.

**Procedure.**

1. Remove irradiated bioreactor BPC from protective double polybags.

2. Load BPC from the top into the outer support container.

3. Orient BPC with bearing port up and toward motor drive with Kleenpak connector probe ports facing bottom access cut-out.

4. Place the bearing port into bearing port receiver, close door and close clamp.

5. Route the side and bottom ports through the side access panel and slot in tank bottom.

6. Direct the sparge, bottom drain and sampling lines through outer support container access windows. NOTE: The 500 L systems have a hatch covering the access window for the bottom sparger.

7. Connect incoming gas feed lines to both the overlay filter and the direct sparge filter.

8. Inflate BPC with air (inflation time approximately 20 min) through overlay filter, but do not exceed 0.5 psi (0.34 bar) internal BPC pressure. As BPC inflates, ensure it is properly oriented in the support container (port alignment, drain,

sparge, etc.).

9. As the bag begins to fill with air, manipulate the bag to align the sparge line in the base.

10. Use the four bottom cut-outs located at the base of the support container as a reference to align hanging tabs on the BPC.

11. Attach platform stretch hooks to each of the four bottom corners of the BPC position bottom-side drain, pulling out/downward to position port towards bottom edge of tank.

12. Align row of Kleenpak connector probe ports within access window.

    NOTE: Verify all port clamps are closed and located as close as possible to the body of the S.U.B. BPC.

13. Remove the latch pin from the safety cover over the mixing assembly and open the cover. Unscrew the threaded cap covering the hollow pass-through of the motor.

14. Assemble the upper and lower sections of the drive shaft by joining the segments and with a counterclockwise motion, hand-tighten the two sections together.

15. Remove latch pin.

16. Locate one wrench on the flat area in the lower drive shaft section, another wrench on the upper section and tighten the connection using a counterclockwise rotation. The shafts are reverse threaded to avoid loosening during operation. Caution: Do not over tighten; a "snug" fit is sufficient.

17. Once the sections are secure, return the wrenches to the tool holder.

### B. Drive Shaft Insertion

**Procedure.**

1. Use two hands to load drive shaft through top of mixing assembly; a slight back and forth twisting motion will aid in insertion.

2. When approximately 2" of shaft remains, twist slightly to engage impeller.

3. When approximately 1" of shaft remains, twist slightly to engage bearing assembly.

4. When approximately 0.25" of shaft remains, twist to align motor drive keyway with one of the four outer slots on the drive shaft head.

5. Directly couple drive shaft to motor drive.

6. Place threaded cap on hollow pass-through and hand tighten clockwise.

7. Tighten cap by placing spanner wrench on hollow pass through and tighten cap using supplied torque wrench.

8. Close safety access cover and insert latch pin.

9. The air supply to the overlay can be turned off once the drive shaft has been inserted.

10. Connect heater to controller and verify it is plugged into an appropriate 120 or 240 VAC outlet, then connect the power cord to the controller.

11. Secure exhaust vent filter on side-mounted holder

### C. Filling S.U.B. with Media

**Background.** Order media from the warehouse to be transferred to the USU lab. Media may come in bags, barrels, large space craft containers or other modes of packaging. The media packaged in these applications is sterile and only needs to be transferred to the

bioreactor for use.

**Procedure**.

1.  Select desired line set from top of S.U.B. BPC for fluid introduction.

2.  Make aseptic connection (quick connect) and begin liquid fill. This is done by using a laminar flow hood to make the connections to ensure sterility.

3.  Once approximately 10-20% of volume has been added, verify the position of the BPC in the outer support container, particularly the sparger and the drain line. Adjust positioning if necessary for proper fit.

4.  It is recommended that the top corners of the BPC be pulled upward to reduce wrinkles during filling. NOTE: The BPC must be pulled at the top corners as the bag will generally form wrinkles during liquid fill. If the film is not pulled from the top to remove wrinkles, film tensioning below the bearing port will result.

5.  The stretch hooks can be removed from bottom corners of the S.U.B. BPC when fluid volume reaches 30% volume. This will eliminate any interference with the load cell readings.

6.  Fill to desired liquid volume; 50%-100% of rated volume is recommended.

7.  The unit is also ready to be set to warm media to 37° C using the electric blanket or the TCU water bath system.

8.  Once all the media is transferred to the bioreactor by sterile methods, and is warmed to 37° C, the unit is ready to start the DO calibration.

9.  The propeller that operates the agitation of the S.U.B. bioreactor can also be initiated to desired speed.

### D. Agitation of S.U.B. Bioreactor

**Procedure.**

1. Once the media has reached half volume of the S.U.B., the agitation control can be turned on via the motor ON/OFF switch.

2. Using the arrow keys, adjust the set point speed to the desired level. Adjust desired agitation rate based on cell type and liquid volume. (For CHO 1-15 in 50 L S.U.B. use 169 RPM)

3. Allow speed to stabilize, and make fine adjustments if necessary.

**Seeding a Bioreactor**

**Materials**.

PBS

Media

Silicone Tubing

HyClone BPC

Air Filters

Bioreactor

Peristaltic Pump

**Procedure.**

1. Once the S.U.B. is operating at the targeted steady equilibrated state and has achieved temperature, the S.U.B. is ready for inoculation. Do not seed bioreactor until it has been plumbed, autoclaved, and calibrated.

2. Count and check viability of cells to be used to seed the bioreactor study. Calculate how many mL of cells you will need to seed the study. Seeding density is 250,000 cells/mL.

3. Take the seeding density and multiply it by the total volume; this is the total number of cells needed. Divide the total number of cells by the viable cell count. The result is the volume of cells you need to seed this study.

4. Connect the inoculum addition line set to the seed culture vessel (2 L) bioreactor (equipped with the proper connectors/tubing) to the inoculum line on the 50 L S.U.B. This is done with the aseptic luer lock.

5. Use a peristaltic pump to pump the desired volume of seed cells into the S.U.B. NOTE: For shear sensitive cultures, cells can be introduced by manipulating the addition port to direct the inoculum down the interior wall of the BPC and into the bulk fluid, reducing the shear on the cells.

6. Once seeded, set the controls (air, pH, and DO) for the study.

7. If necessary, add additional media as described in section to achieve desired final volume.

8. Take an initial sample to verify actual seeding density.

9. Check that the pH probe is reading correctly. If pH is off, re-adjust it by adjusting the zero on the bioreactor to match the reading of the external pH reading.

### E. Sampling a Bioreactor

**Procedure.**

1. Once the bioreactor has been seeded, samples can be taken sterilely through the sample port.

2. Ensure sample port clamp is closed, then unscrew cap.

3. Attach sterile syringe (10-20 mL size) to end of sample port.

4. Draw out cells, keeping positive pressure towards the syringe (before unclamping

sample port, make certain to maintain positive pressure on the port).

5. Fill the syringe 2-4 times, closing clamp and dumping cells each time.

6. The final time cells are drawn out, put into 15 mL centrifuge tube to save.

7. Alcohol the opening and cap of sample port before replacing the cap.

8. Collect a 1 mL sample and count the cells with the Vi-CELL to determine viability and density.

9. Centrifuge the rest of the sample at 1,000 RPM for 4 min. Save a 1 mL sample of the supernatant for ELISA analysis and freeze at -20°C until needed for use.

### F. Sterilization of Bioreactor Waste

**Background**. Chlorine is a widely accepted liquid sterilizing agent. Various chemical forms are available and acceptable for use as a sterilant. A minimum concentration of 500 ppm halogen is necessary for effective sterilization, although 3,000 ppm is recommended for spent media.

**Warning/Safety Precaution.** Do not mix hypochlorite compounds with ammonia or strong acids under any circumstances; chlorine gas, nitrogen trichloride, and / or hydrazine generation may result, which can be deadly. All chlorine compounds are considered highly corrosive, so always wear appropriate protective gear and avoid corrosion of equipment.

**Procedure.**

1. Wear appropriate personal protective equipment when performing any sanitization process.

2. Calculate amount of chlorine compound required to bring the liquid to 3000 ppm final concentration.

3. Add required amount of chlorine compound to liquid waste. Mix thoroughly, and allow to react for at least 30 min.

4. Flush sterilized liquid down drain with running tap water.

**Table H-1:** Bleach reference chart

| Compound | Available Chlorine | g/L 500ppm | g/L 3000ppm |
|---|---|---|---|
| Sodium hypochlorite | 5.25% | 9.5 | 57.1 |
| Calcium hypochlorite | 70-72% | 0.7 | 4.2 |
| Calcium hypochlorite, bleaching powder | 35% | 1.4 | 8.6 |
| Sodium dichloroisocyanurate | 60% | 0.8 | 5 |
| Sodium tosylchloramide | 25% | 2 | 12 |