The Effect of Laminin Concentration and Sildenafil Citrate on the Outgrowth and Elongation of Neurites and Effective Formation of Synaptic Structures Among Sympathetic Neurons

Cellular and Molecular Biology

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RATIONALE

A recent 2018 census estimated that the annual incidence of spinal cord injury (SCI) is roughly 54 cases per one million people in the United States, or approximately 17,700 people who experience some form of SCI every year (National, 2018). According to the World Health Organization, individuals with a spinal cord injury are five times more likely to die prematurely than people without spinal cord injuries due to neurological and physical deficits caused by these SCIs (WHO, 2013). Accordingly, neuropathic/neurodegenerative diseases -- diseases that degenerate neurons structure and function in the brain -- such as Alzheimer disease, Parkinson's disease, and Huntington's disease account for more than 6.4 million cases in the United States (Harvard, 2018). Although attempts have been made to uncover the causes of these diseases and address the effects of SCIs - such as surgery to treat nerve roots and allow them to heal - they have merely been effective.

In the study, neurons will be discussed and their corresponding structures, why neurons fail to regenerate and exhibit axon and neurite outgrowth after SCI and/or through neuropathic disabilities, the research previously conducted corresponding to neuron growth and outgrowth, and ultimately the role laminin and Sildenafil citrate plays in terms of neuronal growth, neurite outgrowth, and potential synapse formation.

RESEARCH QUESTION AND HYPOTHESIS

Research Question : what is the The Effect of Laminin Concentration and Sildenafil Citrate on the Outgrowth and Elongation of Neurites and Effective Formation of Synaptic Structures Among Sympathetic Neurons

Hypothesis*:* Increasing the concentration of Laminin and adding Sildenafil Citrate will promote the formation of neurites among sympathetic neurons and incense the number of synapse formations among them

PROPOSED PROCEDURES

The Pheochromocytoma (PC12) cells will be kept in a growth medium, Dulbecco's Modification of Eagle's Medium (DMEM), in order to allow the survival and growth of the cells. To the DMEM (1L), 75 mL of horse serum (horse blood), 75 mL of fetal bovine serum (bovine fetus blood), and 10 mL of the antibiotic Penicillin-Streptomycin will be added (all obtained from ThermoFisher Scientific). Horse serum, fetal bovine serum, and the antibiotic Penicillin-Streptomycin will be used because they support in-vitro antibody response in the cell culture against any bacterium or foreign substances (Levy, 1980). The final medium will be sterilized with a 250 mL Nalgene™ Rapid-Flow™ Sterile Disposable Filter Unit (Fisher Scientific), in order to filter and remove any bacterium or unwanted microorganisms from the medium - with the ultimate goal of preventing contamination in the final cell culture. The PC12 cells will be previously stored at -80° C in 0.1% Dimethyl Sulfoxide (DMSO). DMSO will be used to increase the viscosity of cell membranes, which allows the accommodation of ice crystals to form without damaging the cells' membrane when the cells are to be stored at cold temperatures. This will ultimately allow the cells to revitalize once thawed out to room temperature.

The PC12 cells will be left out to be thawed and then centrifuged at approximately 1000 revolutions per minute (RPM) for 10 minutes in order to separate the DMSO from the cells themselves. Following, once all the DMSO is removed and the cells are isolated, they will be placed along with 20 mL of the aforementioned Dulbecco's Modification of Eagle's Medium (DMEM) solution in addition to 100 ng/mL of nerve growth factor - in order to differentiate the PC12 cells into neurons - in a 175 mL tissue culture flask. The cells in medium will then then incubated for approximately 24 hours at 37°C in a ~5% CO_2 incubator in order to allow the cells to grow. In order to maintain research accuracy the cells will need to be confirmed for confluence. In consideration of the studies effect in cell replication at a fast rate, the

standard confluence will be maintained between 20-80%. At 80% or higher, the study would experience misleading data given the likelihood of cell degeneration. After 24 hours the PC12 cells will be split with 5 mL of trypsin that will let simmer for 10 minutes. The original tissue culture flask will then be divided evenly (~10 ml of cells + solution in two new flasks) to maintain cell confluence. These two new flasks will then be kept incubated at 37°C in a ~5% CO_2 incubator for approximately three days.

The PC12 cells will be released from the cell culture flask by agitation, run through a low speed centrifugation in order to discard the old modified medium, and resuspended in a medium consisting *only* of DMEM. This medium of cells will then be seeded in 16-millimeter diameter wells that holds approximately 1.0×10^4 cells per 500 µl DMEM solution in each well, this becomes significant once the number of synapse-like formations in each well are statistically measured and analyzed - the number of cells in each well will be expected to remain relatively constant in order to compare them to one another. Each of the wells will then be coated with 0 ng/mL (control), 0.1 ng/mL, 1 ng/mL and 10 ng/mL of laminin respectively (obtained from Fisher Scientific), in addition to .5 mL of water, as seen in *figure 3* below, with three consecutive trials. The water will act as an activator of the laminin; since laminin is very fatty and concentrated, the water will denature the laminin compounds and open up its biological domains -- easily allowing it to bind to the neurons' membranes.

The cells will then be seeded into a different set of a 24-well tissue cluster plate containing 16-mm diameter well that will be either coated with 10 ng of laminin or left untreated using blank medium or medium containing 100 ng/ml of NGF. The PC12 cells will then be treated with 50 uM Sildenafil Citrate (SC) and observed for neurite outgrowth. The cells in each well will then be observed under an Axiovert S100 Inverted Binocular Microscope at a total magnification of 320X in order to visualize the neurites. ANOVA analysis of variance test and Tukeys HSD test will be conducted in order to analyze the difference in cultures.

Risks :

There are no prominent risks within the completion of this experiment. No hazardous chemicals, activities, or devices will be used. However, glove wear and hood use for chemicals will still be maintained.

Data Analysis

To analyze, a one way analysis of variance test will be conducted, with a significance level of .05, to determine whether there is significant variance in the number of synapse formations among the cells coated with different concentrations of Laminin. A Tukey's HSD test will then be conducted in order to determine where the significance, if any, lay among the different concentrations. A simple morphometric analysis will be used in order to determine whether there was an increase in the length of the neurites among the cells coated with and without Sildenafil.

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NO ADDENDUM EXISTS.