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The Effect of *Lycopodium clavatum* Extracts on Apoptosis in SW480 Colorectal Cancer Cells

Rikhil Seth

Irvington High School, 41800 Blacow Rd, Fremont, California 94538, USA; imrikhil@gmail.com
Mentor: Dr. Embler, Dr. Lowengrub, Ms. Wiedemann, Ms. Hussein

ABSTRACT: The extract from *Lycopodium clavatum* (LC), a vascular plant, is traditionally used as a homeopathic medication to aid in digestive, urinary, liver, and neural disorders. Recently, LC has been shown to have potential anti-cancer effects on cervical and colon cancer cells. LC is believed to activate the CASP3 gene and affect the BCL-2 and p53 genes. These genes are involved in inducing apoptosis, regulating apoptosis, and suppressing the proliferation of tumor cells, respectively. Therefore, it was hypothesized that treating SW480 colorectal cancer cells with higher concentrations of LC would increase the expression of the CASP3 and p53 genes while decreasing the expression of the BCL-2 gene, thus resulting in cellular death. The hypothesis was tested by treating SW480 cells with two different concentrations of LC and counting every 24 hours (for three days), measuring cell proliferation and death. In addition, the expressions of CASP3, BCL-2, and p53 were measured with a qPCR test. Overall, the experiment demonstrated that LC could increase the gene expression of the CASP3, BCL-2, and p53 genes. Additionally, both LC concentrations resulted in a similar arrest of population growth, with 0.05 mg/ml of LC being the optimal concentration to induce apoptosis.

KEYWORDS: Biomedical and Health Sciences; Genetics and Molecular Biology of Disease; Cancer Biology; Gene Expression; *Lycopodium clavatum*.

Introduction

Currently, colorectal cancer is the fourth leading cause of cancer death globally. While there is a high survival rate if the cancer is treated early, many patients are diagnosed far too late. The five-year survival rate can drop from 90% to less than 10% if their cancer metastasizes into Stage IV colon cancer at diagnosis.[1] The cell type of colon cancer being studied in this experiment is SW480, a human colorectal adenocarcinoma cancer caused by genetic mutations that can become life-threatening if not addressed early on.[2]

Recently, a homeopathic medication called *Lycopodium clavatum* (LC) has shown anti-cancer effects on HeLa cervical and HCT15 colon cancer cells *in vitro*. In addition, a recent study describes how LC can activate genes to inhibit cellular proliferation and induce apoptosis.[3] It is hypothesized that one of these genes is the CASP3 gene in the Caspase-3 pathway, which dismantles the cell by condensing apoptotic chromatin and fragmenting DNA within the cell.[4] Two other potential target genes are the BCL-2 and p53 genes. The BCL-2 gene is part of the Bcl-2 protein family, which regulates mitochondria in the intrinsic apoptosis pathway responsible for normal embryonic development and preventing cancer.[5] P53 is a tumor suppressor gene with pro-apoptotic functions that stops tumor growth by promoting cell cycle arrest and prevents cells with DNA damage from proliferating.[6] The hypothesis mentioned above could be validated by measuring gene expressions of CASP3, BCL-2, and p53 relative to a control gene (S18) on LC-treated cultures.

Methods

Plating Cultures:

The experiment was conducted by first plating the SW480 colorectal cancer cells in three sets of Petri dishes. The first set was for the control (no LC treatment), and the two other sets were for the 0.05 mg/ml and 0.1 mg/ml LC concentrations, respectively. Each set included three Petri dishes to measure cell proliferation and one to quantify gene expression through a qPCR test.

The readily available *Lycopodium clavatum* extract came in the form of pellets. The prime stock solution was created by crushing one 0.0472-gram pellet (containing 0.443 mg of the active ingredient) and adding it to 8.86 ml of growth media. 10 ml of growth media was pipetted into each Petri dish already traced with the SW480 colorectal cancer culture. To measure the growth of the cells, the stock solution was diluted to a ratio of 1:1000 of stock to growth media; therefore, ten microliters of the stock solution were added to the set labeled 0.05 mg/ml of LC while twenty microliters were added to the set labeled 0.1 mg/ml of LC.

Lifting and Counting Cultures:

Subsequently, the cells were incubated at 37 °C for the next 72 hours. At the end of each 24 hours, the cells in each Petri dish were lifted and counted. Lifting was done by carefully removing the excess growth media and adding the cell dissociation reagent (TrypLE Express) to separate adherent cells from the Petri dish. Then, the mixture was centrifuged to collect the lifted cells at the bottom in a condensed pellet. After centri-

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fuging, the reagent was removed, and the cells were returned to the Petri dish with new growth media to sustain the culture. A small sample was taken from this Petri dish and placed on a grid, observed under a microscope, where Trypan Blue was used to distinguish dead cells from living cells.

qPCR Test:

Additionally, a qPCR test was done at the end of day two to measure the gene expression of CASP3, BCL-2, and p53 compared to the control gene, S18. The test was conducted by measuring the RNA levels for each culture and using reverse transcription to convert and amplify the RNA into cDNA. This cDNA was used along with the forward and reverse primers of the genes, iTaq Universal SYBR Green Supermix, nuclease-free water, microfuge tubes, and two 96-well PCR plates to obtain quantitative results of each gene's expression.[7] Tables 1 and 2 depict the layout of the qPCR test on the 96-well PCR plates corresponding with the gene being tested. The plates were placed in a CFX Opus 96 Real-Time PCR System to analyze the wells and provide data and statistical analysis. Finally, MATLAB was used to create and model cellular growth in the different plates, while Microsoft Excel was used to develop bar graphs of percent population increase and gene expression.

Table 1: Layout of the 96-well plate for the qPCR test of CASP3 and S18.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD
B	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD
C	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD
D	LC 0	LC 0	LC 0	LC 0.05	LC 0.05	LC 0.05	LC 0	LC 0	LC 0	LC 0.05	LC 0.05	LC 0.05
E	LC 0.1	LC 0.1	LC 0.1				LC 0.1	LC 0.1	LC 0.1			
F												
G												
H	CASP3						S18					

Table 2: Layout of the 96-well plate for the qPCR test of BCL-2 and p53.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD
B	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD
C	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD	Std 1	Std 1/5	Std 1/25	Std 1/125	Std 1/625	NTD
D	LC 0	LC 0	LC 0	LC 0.05	LC 0.05	LC 0.05	LC 0	LC 0	LC 0	LC 0.05	LC 0.05	LC 0.05
E	LC 0.1	LC 0.1	LC 0.1				LC 0.1	LC 0.1	LC 0.1			
F												
G												
H	Bcl-2						p53					

Results and Discussion

Over the course of this experiment, the SW480 colorectal cancer cultures and the expression of the CASP3, BCL-2, and p53 genes were measured. The cancer culture population and the possible apoptotic effect of LC on the cells will be covered first, and then the gene expression.

Figures 1-3 depict the cell population growth for the three LC concentrations: 0 mg/ml (control), 0.05 mg/ml, and 0.1 mg/ml. Whereas all three graphs show substantial population growth for the first 24-48 hours, the graphs depicting cultures exposed to LC show a consistent decrease in population during the 48-72 hour period. Unfortunately, there is significant variation in individual measurements in Figures 2 and 3, resulting in a curve-fitting model that needs to represent the values better. Finally, Figure 4 illustrates the overall percent growth of the cancer cell population. The percent increase in 0.05 mg/ml of LC is the lowest at 23.46% compared to 52.50% for 0.1 mg/ml of LC and 83.725% for the control.

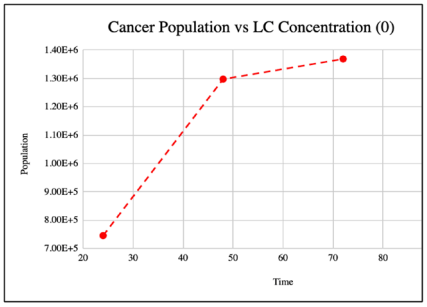

Figure 1: SW480 cell population change over time in 0 mg/ml LC solution.

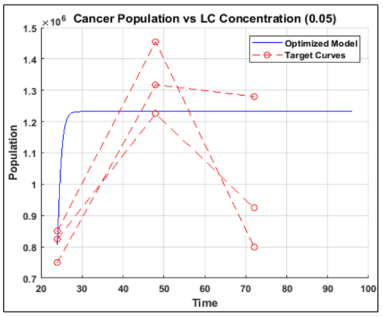

Figure 2: SW480 cell population change over time in 0.05 mg/ml LC solution.

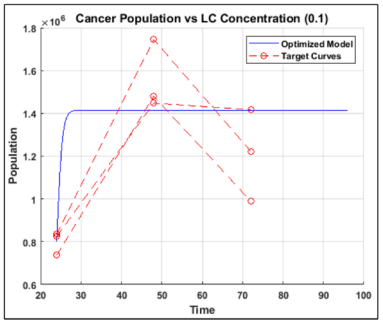

Figure 3: SW480 cell population change over time in 0.1 mg/ml LC solution.

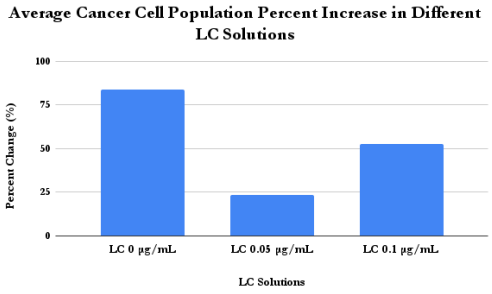

Figure 4: SW480 cell population percent increase over a 24-72 hour period..

According to Figure 5, LC treatment at both 0.05 and 0.1 mg/ml induced both CASP3 and BCL-2 expression, with the intermediate (0.05 mg/ml) treatment resulting in the highest expression of both genes. At the same time, the variability in the qPCR results for p53 implies that no concentration of LC induced higher expression of p53; however, treatment with LC resulted in consistently higher expression of p53 than the con-

trol. In the cancer cell population graphs, there is an increase in cell population until 48 hours, followed by a decrease of varying severity. Although there is a possibility of more genes being involved, the shape of the graph could be justified because BCL-2 is an apoptotic inhibitor when upregulated and is possibly upstream of CASP3 and p53, two apoptotic inducers.

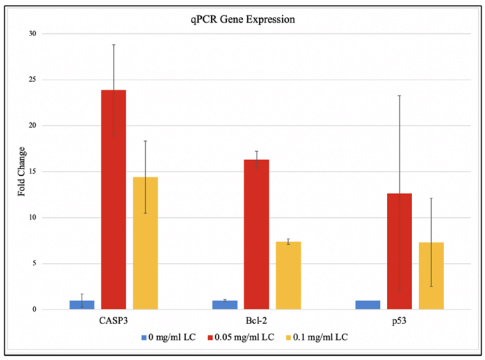

Figure 5: The expression of CASP3, BCL-2, and p53 genes in various LC concentrations relative to the control S18 gene.

Another aspect to cover is the percent change between the different concentrations. Both populations exposed to LC had a lower overall population increase than the control. Although the general trend in the cancer cell population was similar between the cultures exposed to LC, the population treated with 0.05 mg/ml of LC had a percent increase less than the population with 0.1 mg/ml of LC. The qPCR results corroborate this data since all three genes were expressed the most with the 0.05 concentration of LC. This indicates that both the cell counting, and the qPCR support a non-monotonic nature of the LC dosage, where a concentration of 0.05 mg/ml is the most potent.

Conclusion

Based on the observations and measurements in the experiment, we can conclude that treating SW480 with LC increases the expression of the CASP3, BCL-2, and p53 genes. These observations also justify the sudden death depicted in the cell cultures after 48 hours (Figures 2 and 3). Expanding the time frame would be crucial for future studies to display LC's long-term effects on cancer cells. Additionally, lengthening the time frame would help explain and understand the unexpected population dynamics in cancer cultures treated with LC. Experiments can also be conducted to measure the cytotoxicity of *Lycopodium clavatum* (LC) on standard human or mouse cell lines to determine the possibility of the drug as a future pharmaceutical treatment. Based on this experiment, it is known that the relationship of concentration of LC to cell death is non-monotonic. Determining the reason why a lower dosage of LC led to higher potency is crucial and can be tested in future experiments. Finally, testing concentrations in smaller intervals around 0.05 mg/ml of LC could be done to find a more precise concentration of LC.

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Author

Rikhil Seth is a junior at Irvington High School. He is interested in cancer biology, especially cardiopulmonary diseases, and has worked on various research projects using macrophages, VOCs, and gene expression to elucidate his findings. He hopes to work as an oncologist in the future.

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