

Effect of Aflatoxin B₁ from *Aspergillus flavus* on MDA-MB-231 Human Breast Cancer Cells



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ABSTRACT

Aflatoxin is a known carcinogen, expelled from naturally occurring fungi such as *Aspergillus flavus*. In this experiment, different concentrations of aflatoxin were placed in samples of MDA-MB-231 human breast cancer cells and observed for five days. Four samples of MDA-MB-231 human breast cancer cells were prepared: a Control (no aflatoxin), 10 $\mu\text{mol/g}$ aflatoxin B₁, 50 $\mu\text{mol/g}$ aflatoxin B₁, and 100 $\mu\text{mol/g}$ aflatoxin B₁. The change in cell number was determined by cell counting. The results showed that aflatoxin B₁ in a 100 $\mu\text{mol/g}$ concentration terminated the breast cancer cells; while the other concentrations decreased the number of breast cancer cells in comparison to the Control. In addition, the morphology of all samples was examined, with pronounced differences between the treated and untreated samples.

Introduction

Aflatoxin is a carcinogen from naturally occurring fungi such as *Aspergillus flavus*. The fungus growth is more prevalent in tropical climates where it tends to grow on food such as corn, nuts and rice. This poses a danger to humans as much of the developing world depends on such food. The mass consumption of carcinogen-tainted food can result in widespread cancer epidemics. This contamination has been linked to liver cancer epidemics in the said third world countries. Indeed, this is what led scientists to classify aflatoxin as a carcinogen.

Cancer is defined as an uncontrolled proliferation of cells that are no longer responsive to growth regulating signals, and tend to spread to other parts of the body through direct contiguous extension, blood stream, or the lymphatics. Breast cancer is the second most common cancer world wide, and ranks fifth (after lung, stomach, liver, and colorectal) as a cause of death

(Kamangar *et al.* 2006). Breast cancer is a malignant tumour on the breast, affecting its tissue, and eventually spreading to the lymph nodes which grant the tumour access to the rest of the body. The molecular triggers of breast cancer are unclear, but abnormal oestrogen levels are said to be related. The Susan G. Komen Foundation estimated that 182,460 cases of breast cancer in women would be reported in 2008 alone.

Methods to halt cancer growth and eliminate tumours include radiotherapy, chemotherapy, and surgery; all are, however, tied to extremely risky side effects. Scientists have been working for a long time to find less invasive and dangerous methods to attack this increasingly prevalent disease and in this experiment, the effects of Aflatoxin on breast cancer cells will be examined.

Materials and Methods

Risk Assessment

All procedures are to be handled in a biochemistry

laboratory at the University of Puerto Rico Medical Campus. Advanced and hazardous laboratory procedures are to be handled by trained laboratory aides and investigators are to serve as observers. A qualified scientist will be present at all times. Appropriate laboratory attire will be worn at all times, and proper disposal will be made, following the regulations of the University of Puerto Rico Medical Campus. All procedures involving the MDA-MB-231 human breast cancer cells will be performed under the chemical hood to avoid contamination.

Preparing the MDA-MB-231 Human Breast Cancer Cell Samples

The cells used for this experiment were harvested in 2007 and preserved in a sub-zero refrigerator at a temperature of -143°C . They were removed and placed in room-temperature water to unfreeze and placed in a $100\text{-}\mu\text{L}$ plastic flask with $10\ \mu\text{L}$ of medium to prepare a stock solution of cells. The old medium was discarded after the cells were obtained. A trypsin solution was prepared to clean the cell plates, with 2mL of 25% of trypsin and 3mL of PBS. The flasks were washed with $1\ \text{mL}$ of PBS 10% buffer each. 2mL of the trypsin solution was added to one flask, and then the flask's contents were transferred to a second flask. 2.5mL of the second flask's contents were then transferred to the first one. Both flasks were incubated. A microtube was prepared with $450\ \mu\text{L}$ of PBS to later dilute the cells for counting.

After incubating for 10 minutes, the cell flasks were removed. About 10mL of 2% medium was put in each flask and mixed. The 10mL of medium was transferred from the first flask to the second, now with 20mL of medium in the second flask. 1mL of cells was placed into a new microtube. Then, from that microtube, $50\ \mu\text{L}$ of cells was placed in the PBS solution of the first microtube prepared for counting.

Counting the Cells

About $20\ \mu\text{L}$ from the cell microtube was placed in the counting chamber. One quadrant of the counting chamber was counted. According to the number of cells counted, the amount of cells and medium needed per well was determined for the experiment. To determine this, the total number of cells was divided by (the number of sections counted in a quadrant), then multiplied by 10 (the mL of PBS 10% buffer used for dilution), then multiplied by 10^4 (a standard number used when determining cell number). The result was $2.75 \times 10^5 / \text{mL}$; this is the number of cells per well, round the number off to 2.6

$\times 10^6$. This number was then multiplied by 70 (number of wells to be prepared, although only 50 wells will be used). Result- 14×10^5 . This number was divided by 2.75×10^5 (use the original number of cells to assure precision) to determine the amount of cells in mL per well. The result was 5.09mL which was to be subtracted from 70mL (the total volume/number of wells to be prepared) to determine the amount of medium. The result: $64.9\ \text{mL}$.

The solution was prepared in a new flask using the amounts determined. The amounts were estimated using 70 wells, or $70\ \text{mL}$, because of the percentage error during the preparation of the cell solution. This error is due to the formation of air bubbles in the solution when preparing the wells. Using the new flask with the cell solution, the cell plates were prepared. About $1\ \text{mL}$ of solution was placed in each well- 60 wells in total. $5.09\ \text{mL}$ of 10% serum medium was placed in the second flask of cells, where the $5.09\ \text{mL}$ of cells were taken from. About $10\ \text{mL}$ from the second cell flask was taken, and placed in the first (empty) cell flasks so that there were two $10\ \text{mL}$ cell flasks and one cell solution flask. The newly prepared cell plates and the three cell flasks were incubated.

Preparation of the Aflatoxin B₁ Concentrations

About $0.936\ \text{mg}$ of pure aflatoxin B₁ was weighed. It was provided by Sigma Aldrich and placed in a $75\ \text{mm}$ test tube. About 1mL of DMSO was added to the tube. The tube was placed in the Vortex diluter to dissolve the aflatoxin B₁ in the DMSO; one more mL of DMSO was added and vortexing was repeated. The solution was $1,000\ \mu\text{mol/g}$ of aflatoxin.

The tube was placed in a heated bath of water for a few minutes until the water reached 37°C . About $1\ \text{mL}$ of DMSO was added and the tube was vortexed. Four 45mL tubes were obtained and labelled: one each for the Control, $10\ \mu\text{mol/g}$ of aflatoxin B₁, $50\ \mu\text{mol/g}$ of aflatoxin B₁, and $100\ \mu\text{mol/g}$ of aflatoxin B₁. The amount of 2% serum medium and aflatoxin B₁ needed in each concentration sample was determined to add up to a total $15,000\ \mu\text{L}$ of solution. For the $10\ \mu\text{mol/g}$ of aflatoxin B₁, $14,850\ \mu\text{L}$ of medium and $150\ \mu\text{L}$ of aflatoxin B₁ were needed; for the $50\ \mu\text{mol/g}$ sample, $14,250\ \mu\text{L}$ of medium and $750\ \mu\text{L}$ of aflatoxin were needed; and for the $100\ \mu\text{mol/g}$, $13,500\ \mu\text{L}$ of medium and $1,500\ \mu\text{L}$ of aflatoxin were needed.

Then 18mL of the 2% serum medium was added to the Control tube, and 14mL to the other three tubes. From the Control, $850\ \mu\text{L}$ of medium was taken

and placed in the 10 $\mu\text{mol/g}$ tube, and 250 μM for the 50 $\mu\text{mol/g}$ tube; 500 μL of medium was taken from the 100 $\mu\text{mol/g}$ tube. The amounts of aflatoxin determined for each concentration were placed in the corresponding tubes. Tubes were sealed and mixed.

Adding Aflatoxin B₁ to the MBA-MD-231 Human Breast Cancer Cells

The cell well plates were removed from the incubator and cells were checked. The old medium was discarded, and 1 mL of PBS 10% buffer added to each well. The PBS was discarded and the process was repeated. The well plates were labelled to indicate the concentration to be added in each well. Counting was to take place over five days, and for each day there were three samples of each concentration counted, for a total of 12 wells per day (three Control wells, three 10 $\mu\text{mol/g}$ of aflatoxin B₁ wells, three 50 $\mu\text{mol/g}$ of aflatoxin B₁ wells, and three 100 $\mu\text{mol/g}$ of aflatoxin B₁ wells). 1 mL of the concentration solution corresponding to each well was added. Wells were incubated.

Counting the MDA-MB-231 Human Breast Cancer Cells

Twenty four hours after preparing the cell plates, one plate was removed from the incubator. Cells were checked, old medium was discarded from the wells to be counted and 200 μL of trypsin was added to each and discarded. After this, 200 μL of trypsin was re-added to the wells, and the cell plate was reincubated for 10 to 15 minutes. The cells were removed from the incubator, and 300 μL of PBS 10% buffer was added to each well to be counted. Twelve microtubes were taken, and labelled according to the sample to be placed in it and the number of the sample: C₁, C₂, C₃, 10₁, 10₂, 10₃, 50₁, 50₂, 50₃, 100₁, 100₂, and 100₃. Each of the well's contents was placed in the corresponding microtubule.

The Control₁ sample was placed in the counting chamber. One quadrant of the two-quadrant counting chamber was counted. The Control₂ sample was placed in the other quadrant, count, and recorded. The chamber was cleaned with distilled water and dried between samples. This process was repeated with all 12 of the cell samples. After counting all the samples, the cells were destroyed according to laboratory protocols. To obtain the number of cells, an average was taken for each treatment.

Cell Morphology Study

Before counting the cells each day, the wells (with

samples) were observed under a microscope. Each individual sample was photographed as evidence for the morphological study. The photos taken were meticulously examined and the changes in shape and size of the cells recorded. Cell pictures of the 10 $\mu\text{mol/g}$, 50 $\mu\text{mol/g}$, and 100 $\mu\text{mol/g}$ of aflatoxin B₁ samples were compared to the Control sample to take note of the changes due to carcinogen exposure. The most important cell changes to be seen were apoptosis (programmed cell death) and change in the nuclear structure of the cells.

Statistics

T-tests were used to determine statistical significance of the cell numbers between the Control and the samples and between the different samples.

Observations

Table 1 – Cell count: All samples

	Day 1	Day 2	Day 3	Day 4	Day 5
Control	8	9	35	53	64
10 $\mu\text{mol/g}$	11	6	11	19	13
50 $\mu\text{mol/g}$	8	4	3	2	5
100 $\mu\text{mol/g}$	0	1	0	0	0

Statistical Analysis

Table 2 – T - test results

Samples tested	P-value	Null-Hypothesis (no difference)
Control and 10 $\mu\text{mol/g}$	0.0949	Fail to reject
Control and 50 $\mu\text{mol/g}$	0.0323	Reject
Control and 100 $\mu\text{mol/g}$	0.0179	Reject
10 $\mu\text{mol/g}$ and 50 $\mu\text{mol/g}$	0.0117	Reject
10 $\mu\text{mol/g}$ and 100 $\mu\text{mol/g}$	0.0050	Reject
50 $\mu\text{mol/g}$ and 100 $\mu\text{mol/g}$	0.0039	Reject

The T-test analysis between the Control and 10 $\mu\text{mol/g}$ samples had a P value of 0.0949, which is not statistically significant. On the other hand, the Control with the 50 $\mu\text{mol/g}$ of aflatoxin B₁, the Control with the 100 $\mu\text{mol/g}$ of aflatoxin B₁, the 10 $\mu\text{mol/g}$ of aflatoxin B₁ with the 50 $\mu\text{mol/g}$ of aflatoxin B₁, the 10 $\mu\text{mol/g}$ of aflatoxin B₁ and the 100 $\mu\text{mol/g}$ of aflatoxin B₁, and the 50 $\mu\text{mol/g}$ of aflatoxin B₁ and the 100 $\mu\text{mol/g}$ of aflatoxin B₁ were all statistically significant.

Discussion of Results

Current modalities of breast cancer treatment are surgical excision, chemotherapy, and radiotherapy. Carcinogens are not used as a way to halt cancer growth. This study was designed to test the effects of different concentrations of aflatoxin B₁ from the *Aspergillus flavu* on MDA-MB-231 human breast

cancer cell stocks. All treatments were compared to a Control stock of MDA-MB-231 human breast cancer cells to determine the change in the numbers of cells. Also, pictures were taken of each sample on each day of counting to observe the morphological changes of the cells and the cell death process of the MDA-MB-231 human breast cancer cells.

The Control sample exhibited normal growth, as shown in Figure 1. It began with a lag stage during the first two days of observation, having an average cell count of 8 on the first day and 9 on the second. On the third day of observation, the cell number rose to an average cell count of 35. The cell count kept rising steadily on the other days of counting; the fourth day of counting results in an average of 53 cells, and the counting period for the Control sample culminated in 64 cells. The morphology observed for the Control cells was that expected for living human breast cancer cells. The cells were of an elongated shape, with two antennae on each end. They began forming webs, but then separated to allow room for reproduction. A few cells died during the observation period, but that is to be expected. These cells were the minority, and they became round circles with no center.

The 10 $\mu\text{mol/g}$ of aflatoxin B₁ sample observed a rise-and-fall trend, as shown in Figure 1. The counting began with an average of 11 cells, but went down to six on the second day of counting, then rose once again to 11 on day three. On day four, the number rose to an average of 19 cells, but lowered

to 13 on the last day. This was not enough to reject significance. Morphologically, cell death could be observed from the second day onwards. Living cells exhibited their expected shape and size, but the number was less than that observed in the Control. Cell death could also be more clearly observed. Due to the toxin treatment, the cell death can be classified as necrosis, a cell death due to a foreign toxin.

However, the 50 $\mu\text{mol/g}$ aflatoxin B₁ sample showed significant change [Figure 1]. The average cell number continued to decrease to the last day, when the cell number rose from an average of two to five cells. On the other hand, the cell growth trend had showed a gradual reduction of cell numbers up to the fifth day, when the cell number rose once again. But compared to the Control sample, the number of cells in this concentration of aflatoxin B₁ is significantly lower than that observed in the Control sample.

In the 100 $\mu\text{mol/g}$ of aflatoxin B₁- treated cells, no cells were seen on any of the days of counting, with the exception of the second day when one cell was counted. This cell count proves that the 100 $\mu\text{mol/g}$ concentration had an immediate effect on the MDA-MB-231 human breast cancer cells, killing them instantly after the carcinogen treatment. The cells died a necrotic death from the high concentration of aflatoxin B₁, and the cells in the other concentrations also suffered from the effects of the toxin. Morphologically, the cells died immediately, and from the first day of counting all the cells that could be seen were small, empty circles- meaning they

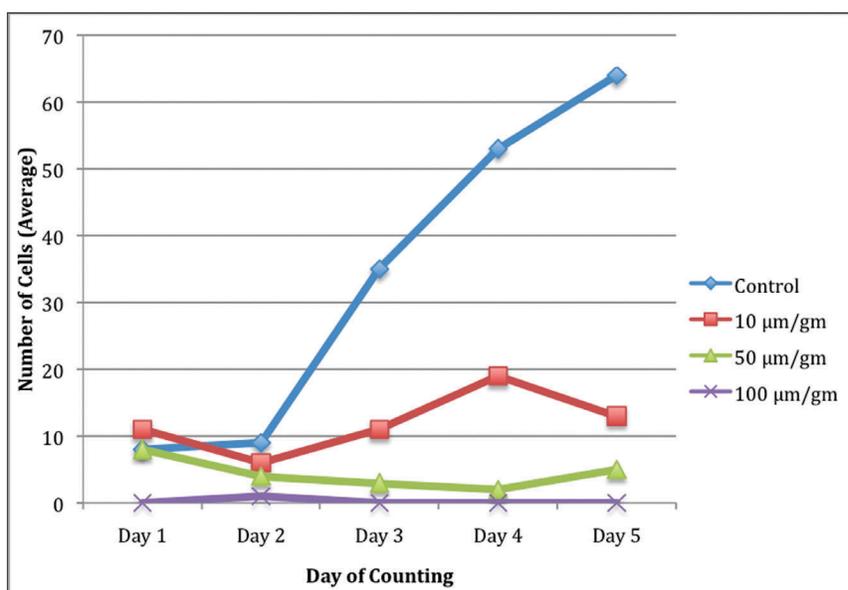


Figure 1: Cell Count: All Samples

were dead. As the days of counting passed, fewer dead cells could be seen, and dying cells were the only ones visible.

Conclusion

The treatment of cells with the carcinogen has proved to be successful, eradicating the breast cancer cells especially in the highest concentrations of aflatoxin B₁ solution. Further investigation and precision of the treatment process, along with a risk assessment of the subsequent carcinogenicity of aflatoxin will be necessary to investigate the potential of using aflatoxin B₁ in the treatment of breast cancer.

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References

1. International Agency for Research on Cancer (IARC). "Aflatoxins (IARC Summary and Evaluation, Volume 56, 1993)." IPCS INCHEM. Available from: <http://www.inchem.org/documents/iarc/vol56/09-afl.html>. [last cited on 2008 Dec 12].
2. NIEHS. "Aflatoxin and Liver Cancer." National Institute of Environmental Health Sciences (NIEHS). Available from: <http://www.niehs.nih.gov/health/impacts/aflatoxin.cfm>. [last cited on 2008 Dec 20].
3. CBWInfo.com. "Aflatoxins: essential data." CBWInfo home. Available from <http://www.cbwinfo.com/Biological/Toxins/aflatoxins.html>. [last cited on 2009 Jan 12].
4. "Cancer - Prevalence, Types of cancer, Growth and spread of cancer, Causes of cancer." Internet RFC/FYI/STD/BCP Archives. Available from: <http://www.faqs.org/nutrition/Ca-De/Cancer.html>. [last cited on 2009 Jan 20].
5. Cornell University Department of Animal Science. Available from: <http://www.ansci.cornell.edu/plants/toxicagents/aflatoxin/aflatoxin.html>. [last cited on 2008 Aug 14]. [last retrieved on 2008 Jan 28].
6. BreastCancer.org - Breast Cancer Treatment Information and Pictures. (n.d.). Available from: <http://www.breastcancer.org/>. [last retrived on 2009 Jan 28].
7. Susan G. Komen for the Cure. (n.d.). Available from <http://www.komen.org/default.asp>. [last retrived on 2009 Jan 28].
8. Breast Cancer Treatment - National Cancer Institute. (n.d.). Available from: <http://www.cancer.gov/cancertopics/pdq/treatment/breast/patient>. [last retrieved on 2009 Jan 28].
9. Aflatoxin. (n.d.). Available from: <http://www.aflatoxin.info/aflatoxin.asp>. [last retrived on 2009 Jan 28].
10. GraphPad Software, Inc.. "GraphPad QuickCalcs: t test calculator." GraphPad Software. Scientific graphing, curve fitting (nonlinear regression) and statistics. <http://www.graphpad.com/quickcalcs/ttest1.cfm>. [last cited on 2009 Jan 20].

About the Author

Ana Victoria Colón is a high school senior from San Juan, Puerto Rico. She aspires to become either the first Puerto Rican woman to serve as an ambassador to the United Nations or work at the World Health Organization. Her interests include international relations, oncology, languages, history, writing, and film. Having been raised by two doctors, an interest in science has always been a significant part of her life. However, she does not want to limit herself to only one area of study, and hopes to incorporate all of her interests into a viable career.