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Article

Testing the effects of resveratrol, apigenin, and glucosamine to effectively reduce prostate cancer cell proliferation, migration levels, and increase apoptosis

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SUMMARY

The current five-year survival rate of metastasized prostate cancer is only 30% and occurs in every one in nine men. Researchers have shown that people with a type of dwarfism called Laron's Syndrome are immune to cancer due to their low levels of insulin-like growth factor-1 (IGF-1). For this reason, experimentally modifying the level of IGF-1 could provide better insight into whether lowering the levels of IGF-1 in prostate cancer cell lines (e.g. PC-3) could be an effective treatment to reduce their rates of proliferation and migration and increase apoptosis. We selected three compounds, which researchers have shown decrease IGF-1 levels, to test and combine to determine which is the most promising. We conducted a cell proliferation assay in order to determine the impact of each treatment on cell proliferation. We used a migration assay to measure the migration of the PC-3 cells after each treatment. In this assay, we seeded bone marrow mesenchymal stromatolites cells (BM-MSC) underneath the PC-3 cells to induce migration. Finally, we conducted an apoptosis assay to count the number of cell deaths after treating the PC-3 cells. The compounds we used, glucosamine, apigenin, and resveratrol, can be found naturally in foods or supplements. We found the treatment with a combination of all three compounds to be most effective in decreasing cell proliferation and migration levels in the PC-3 cells. In the cell apoptosis assay, the glucosamine and apigenin combination caused the largest number of cell deaths, but an outlier in this condition resulted in a large margin of error. The combination of all three compounds resulted in the second highest number of cell deaths. With these results and further investigation, a potential new, less costly, and less painful treatment for prostate cancer could be found.

INTRODUCTION

Cancer is a fatal disease that occurs when abnormal cells in the body start to grow and divide uncontrollably, eventually spreading to the surrounding tissues. A healthy cell can turn into a cancerous cell due to a number of mutations (1). Unlike normal cells, cancer cells grow out of control and become invasive. These invasive cells continue to grow because they are less specialized than normal cells and do not have specific functions. They are insensitive to signals that normally end cell growth and apoptosis ---- programmed cell death that the body uses to remove the excess cells (2).

Prostate cancer is one type of cancer in which the cells in the prostate gland start to proliferate uncontrollably. The prostate is small walnut-shaped gland in males that produces seminal fluid that is used to make semen (3). After skin cancer, prostate cancer is the most common cancer amongst men. There were approximately 164,690 new cases of prostate cancer in the United States in 2018. Currently, once prostate cancer metastasizes or spreads, the survival rate drops to 30 percent and becomes extremely difficult to treat as surgery is no longer an option (4).

One of the factors that induces migration for prostate cancer cells is the secretome bone mesenchymal stromal cells. A study by a group of researchers at Tufts Medical Center had shown that when PC-3 prostate cancer cells are co-cultured with bone marrow mesenchymal stromal cells (BM-MSC) migrate towards these BM-MSC cells. This could be because the prostate cancer cells have the ability to promote the overproduction of bone-building cells, or osteoblasts, which then can cause osteoblastic migration (5).

Another factor that affects cancer is Insulin-like growth factor - 1 (IGF-1). IGF-1 is a hormone found in blood naturally (6). It is necessary, especially during neonatal and pubertal growth, for survival and growth of cells, suppressing apoptosis, and promoting cell cycle progression (7). The main job of IGF-1 is to regulate the effects of growth hormone (GH) (8). Upon GH stimulus, the liver works to produce this hormone. IGF-1 then binds to the insulin-like growth factor-1 receptor (IGF-1R). After binding, autophosphorylation activates the IGF-1R which proceeds to phosphorylate the insulin receptor substrate (IRS-1). The activation of the phosphate, phosphoinositide 3-kinase (PI3K) leads to the activation of Akt/PKB protein. The Akt protein then releases the anti-apoptotic protein called the Bcl-2. This P13K/Akt signaling pathway is responsible for controlling and prohibiting cell death (9).

Decreasing IGF-1 levels by a significant and unhealthy amount, growth hormone deficiency can occur. Growth hormone deficiency in adults has some clinical consequences relating to body composition and psychological well being. Growth hormone deficiency can cause an increase in abdominal fat as well as a decrease in muscle mass. These adults can also become depressed and have increased anxiety levels. Low levels of IGF-1 could also lead to many

physical deformities such as short stature. On the other hand, people with low IGF-1 levels have a non susceptibility to cancer (9).

People diagnosed with Laron syndrome have been shown to be resistant to cancer. Laron syndrome is a type of dwarfism that affects 350 people worldwide (10). This disease is a congenital autosomal recessive disorder caused by a mutation in the growth hormone receptor (GHR) gene (6). These mutations diminish hormone-receptor binding and cell signaling. Consequently, there is growth hormone insensitivity; even if the growth hormone were available, the cells would be unable to respond by generating IGF-1 which is responsible for stimulating growth and division. Insensitivity to the growth hormone prevents the growth and division that results in the development of cancerous tumors (9).

The current chemotherapeutic agents and drugs are not only extremely expensive, but painful as well. Some treatments such as radiation can cost up to $25000 while chemotherapy can cost up to $12000 a month. Many chemotherapeutic drugs are not only harmful to cancerous cells, but normal cells as well. Therefore, using chemicals naturally found in ones diet could become a valuable alternative.

In this experiment, we used three treatments, resveratrol, apigenin and glucosamine to see their effects on proliferation levels, migration, and cell death. Resveratrol is a stillbenoid made of mainly the skin of grapes and is a potential dietary compound against several cancers by regulating cell proliferation and apoptosis. A study suggested resveratrol as a potential chemotherapeutic agent when it had successfully suppressed colon cancer cell proliferation and increased apoptosis (11). In another study, resveratrol reduced the growth of prostate cancer. Resveratrol had increased apoptosis in the prostate cancer cells and inhibited wound closures, thus showing that it inhibits the invasiveness of prostate cancer cells (12).

Apigenin is a natural plant flavone found in many fruits and vegetables. It has the ability to regulate the IGF-1 to trigger growth arrest and apoptosis in prostate cancer. A 2012 study demonstrated that Apigenin is able to inhibit cellular proliferation and induce apoptosis in a variety of human cancers, including leukemia and carcinomas of the lung, skin, colon, breast, and prostate without affecting the noncancerous cells. It had the ability to inhibit P13K and Akt activation while continuing to modulate the IGF-1 signaling axis (13).

The final chemical going used in this experiment was glucosamine. Glucosamine is a naturally occurring chemical found in the fluid around the joints in the human body (14). A study showed that this chemical can inhibit the growth of human non-small lung cancer cells and negatively regulate the phosphorylation of Akt and expression of IGF-1R. Glucosamine prohibited tumor growth through reducing IGF-1R signalling and increasing ER-stress. The ER is an important calcium storage organelle; calcium influx and concentrations can effect and trigger apoptosis. Targeting the IGF-1R/Akt pathway with glucosamine could be an effective therapeutic strategy for treating some types of cancers (15).
IGF-1 is a growth hormone that once bonded, activates a series of phosphates, proteins, and processes in the PI3K/Akt signaling pathway that lead to the activation of the Akt protein which eventually result in cell proliferation. We tested three compounds, resveratrol, apigenin, and glucosamine individually and together to test their effects on prostate cancer cell lines.

We hypothesized that treatment with resveratrol, apigenin, and glucosamine would lower levels of migration and proliferation while increasing levels of apoptosis the most.
The purpose of this experiment is to determine which out of resveratrol, apigenin, and glucosamine is the leading treatment in reducing cell proliferation and increasing apoptosis in prostate cancer cells.

RESULTS

We conducted an alamarBlue apoptosis assay to compare the amount of cell deaths between the treatments and the

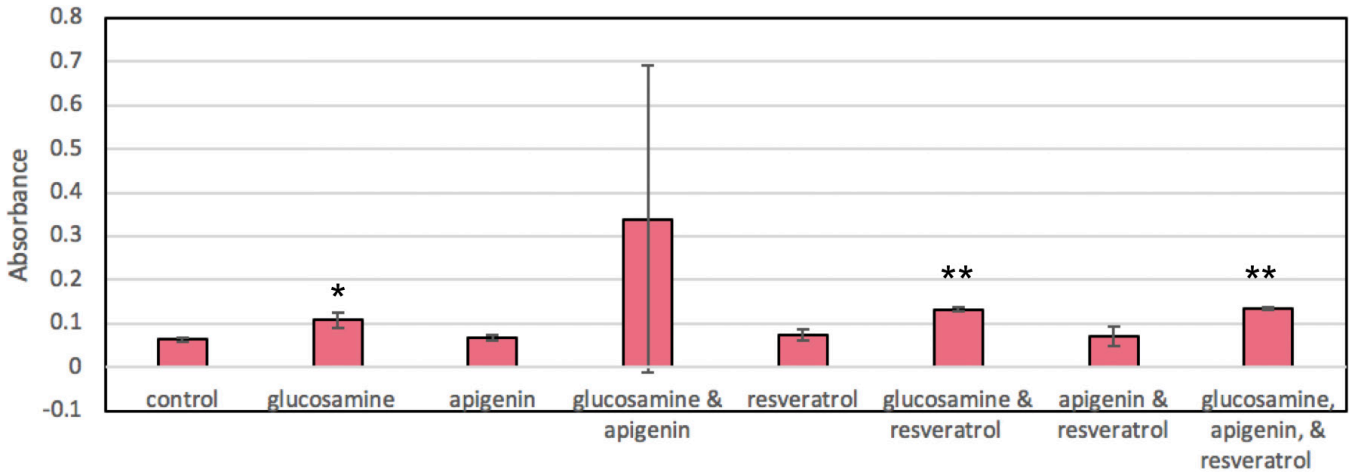

Figure 1: Comparison of cell deaths between the treated PC-3 cells after an apoptosis assay. The treatments with asterisks beside them indicate statistical significance after an ANOVA.

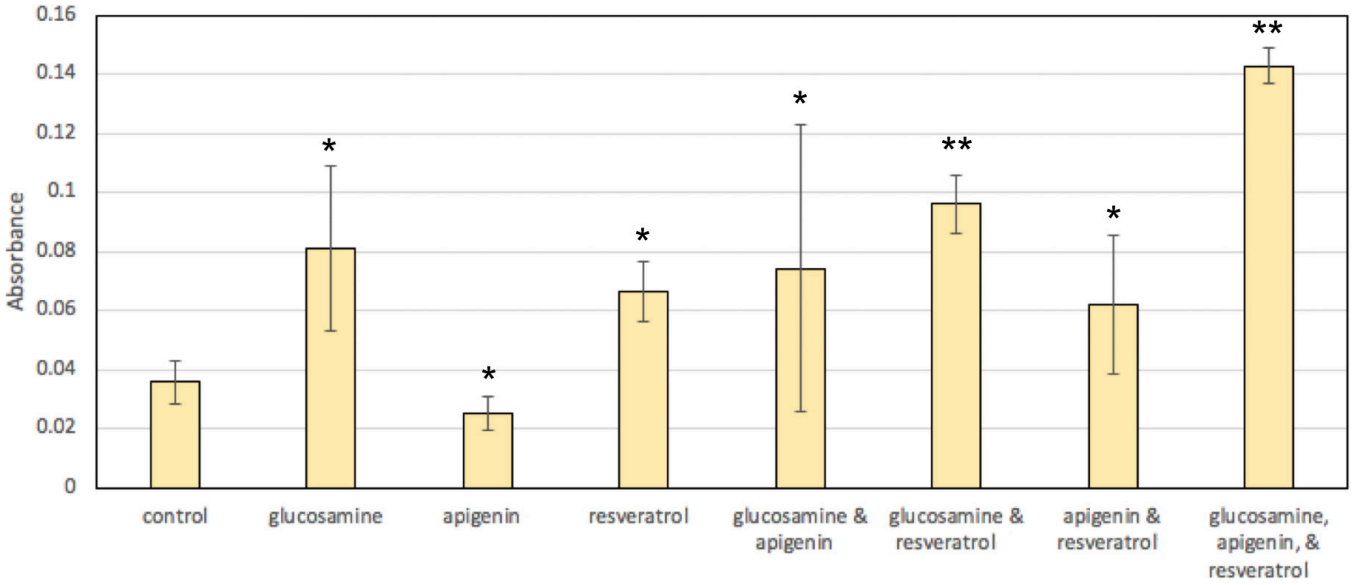

Figure 2: Reduced rate of proliferation of the treated PC-3 cells after a cell proliferation assay. The treatments with asterisks beside them indicate statistical significance after an ANOVA.

control. AlamarBlue undergoes colorimetric change as a result of a cellular metabolic reduction and measures cell viability quantitatively. We used resveratrol at a concentration at 75 micromoles, apigenin at 50 micrograms, and glucosamine at 5 millimoles. After conducting the alamarBlue apoptosis assay, as **Figure 1** had shown that the combination of glucosamine and apigenin had the most cell deaths, containing the highest absorbance amongst all the treatments. The combination group with glucosamine and apigenin contained an outlier, the alamarBlue still being vibrant compared to the other replicates where the alamarBlue solution was a lighter blue and had been more faded. Consequently, that caused an extremely large margin of error. The next most effective treatment had been the combination of all three chemicals: the glucosamine, apigenin, and resveratrol combination, which had a smaller margin of error and is more reliable. Amongst the treatments, the only treatments that showed significant values were glucosamine, glucosamine and resveratrol, and the combination of all three treatments. Glucosamine had a p-value of 0.0209, apigenin only had a p-value of 0.2332, resveratrol only had a p-value of 0.1262, glucosamine and apigenin had a p-value of 0.1532, resveratrol and glucosamine had a p-value of 6.8376E-05, resveratrol and apigenin had a p-value of 0.3075, and the combination of all the treatments had a p-value of 3.8211E-05.

We conducted a proliferation assay to compare the slowed down rates of proliferation with the negative control. The results from the MTT cell proliferation assay had shown that the glucosamine, apigenin, and resveratrol combination was most capable in reducing PC-3 prostate cancer cell proliferation, with a small margin of error as shown in **Figure 2**. All the treatments were significant with glucosamine with a p-value of approximately 0.0485, apigenin with a p-value of approximately 0.0250, resveratrol with a p-value of 0.0493, apigenin and glucosamine with a p-value of 0.0037, resveratrol and glucosamine with a p-value of 0.0007, resveratrol and apigenin with a p-value of 0.0422, and the combination of all three treatments with a p-value of 2.5022E-05.

We conducted a boyden chamber migration assay in order to exhibit the rates of migration before and after treatment. Finally, after the migration assay, the treatment of the combination of all three chemicals had the least number of cells left in the membrane of the inserts exhibiting the fact that it was most successful in being able to reduce the migration of the PC-3 prostate cancer cells as seen in **Figure 3**. In addition, all the ANOVA tests conducted had yielded statistically significant p-values. Glucosamine had shown a p-value of 0.0006, apigenin had shown a p-value of 0.0212, resveratrol had shown a p-value of 0.0003, apigenin and glucosamine had shown a p-value of 0.0005, resveratrol and glucosamine had shown a p-value of 4.1166E-05, resveratrol and apigenin had shown a p-value of 0.0005, the combination of all three treatments had shown a p-value of 0.0002.

DISCUSSION

All the chemicals tested were able to successfully induce apoptosis and inhibit proliferation and migration. However, they were most effective when used in combination, concluding that the combination of these chemicals could lead to a novel, cost effective treatment for prostate cancer in men.

To further investigate, we could test these treatments on other cancer cell lines. Breast cancer is another type of cancer closely linked with IGF-1; therefore, these medications could become a potential treatment. Many cancers such as small intestine cancer, soft tissue cancer, bladder cancer appear following prostate cancer. In addition to prostate cancers, we could test these treatments on second cancers.

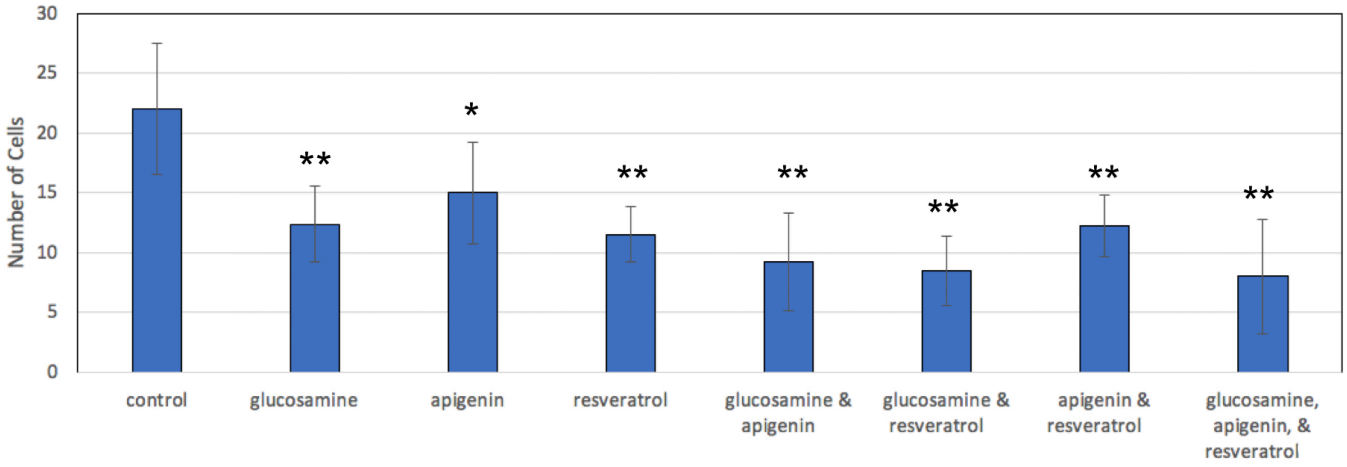

Figure 2: Reduced rate of proliferation of the treated PC-3 cells after a cell proliferation assay. The treatments with asterisks beside them indicate statistical significance after an ANOVA.

With further research, these results could lead to a more efficient and less costly possible treatment than existing treatments for prostate cancer patients.

Additionally, these treatments could treat prostate cancer in animals as well. Prostate cancer appears and develops in many dogs over eight years old. Fifty percent of dogs develop cancer at some point in their lifetime which cannot be treated with cancer. The most effective treatment currently is radiation combined with chemotherapy which only gives them an extension survival time of twenty months. Therefore, these treatments could potentially treat these animals.

We could conduct further research by beginning to use animals as model organisms. Besides humans, dogs are the one of only other species that prostate cancer has significant incidence in. Specifically, the Bernese mountain dog, Irish wolfhound, and Saint Bernard are the breeds most susceptible to fatal tumors. To continue this experiment, each treatment could treat dogs with prostate cancer to test whether the results found in this experiment replicate in a living organism.

Another potential animal test model for further research is immunodeficient mice. These mice can be genetically modified to mimic and develop this human disease. We could give potential treatments to these mice to test which treatment would be most effective in living organisms. Further in vivo testing is essential to see if the cancer cells react the same way they did in vitro before using these drugs on people.

There were multiple limitations that were present in this experiment. In order to minimize human error and show more significant results, we would conduct more treatments. Due to the limited time and budget, this was not possible. Both the PC-3 prostate cancer cells and the HS-5 bone marrow mesenchymal stromal cells (BM-MSC) were susceptible to contamination because of yeast and bacteria. As a result of restricted time we could not conduct more replicates of the assay because of the limited number of cells that could be grown within the set period of time. Another limitation that occurred as a consequence of few numbers of cells, we could not use more cells per well in each assay.

There were various shortcomings during this experiment. When creating the treatments, miscalculations could have occurred in determining how many milliliters we needed to make each treatment when converting from moles. Additionally, we used pipettes, which are not the most accurate or precise form of measurement. A small margin of error could have also occurred when micropipetting the solutions into the wells for experimentation. Moreover, the cell counts can only be an approximation rather than an exact number; each well would have slightly different numbers of cells to start with.

In essence, the results from this study could aid in discovering a treatment for other types of cancer. Specifically, cancers that develop following the prostate cancer such as small intestine cancer, soft tissue cancer, bladder cancer and other second cancers. Other potential cancers that we could treat with the results from this investigation are cancer related to IGF-1 such as colon, pancreas or breast cancers.

MATERIALS AND METHODS

The medium for cell culture was made using RPMI 1640 with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/ Streptomycin (P/S). The treatments were made using dimethyl sulfoxide (DMSO), the medium previously made, resveratrol, apigenin, and glucosamine.

For the cell proliferation assay, we seeded PC-3 cells into a 96-well plate at a density of 5 x 10^2-10^5 cells/well in 10 µl of culture medium with and without compounds. The cells were culture in a CO2 incubator at 37 C for 24-48 hours. We added 10 µl of the MTT reagent to each well using a repeating pipettor. We then mixed the cells gently for one minute on an orbital shaker. We incubated the cells for 3-4 hours at 37 C in a CO2 incubator. After incubation, the formazan produced in the cells appeared as dark crystals in the bottom of the wells. We added 100 µl of crystal dissolving solution to each well, and incubated for 4-18 hours in a 37 C CO2 incubator.

This solution dissolved the formazan crystals and produced a purple solution. We measured the absorbance of each sample at 570 nm using a microplate reader.

Following the cell proliferation assay was an alamarBlue apoptosis assay. We seeded the cells at 5,000 per well in a 96-well plate in 100 µL of medium. We incubated the cells in a 37 C incubator for 24-72 hours. We added 10 µL of the alamarBlue reagent directly to cells. We incubated the cells for 1-4 hours at 37 C incubator and protected from direct light. We monitored the absorbance of alamarBlue at 530 nm.

The last assay conducted was the migration assay. First a homogenous cell suspension of the bone mesenchymal stromal cells was made. Then we seeded 10,000 - 50,000 stromal cells per 24 well in full growth medium. Then a homogenous cell suspension of PC-3 cancer cells was made. We seeded at 5000 - 20,000 cancer cells per 24 well plate in semipermeable trans-well inserts in full growth medium above stromal cells. The 24-well plate for migration assay was set up with the lower chamber containing the stromal cells and the top chamber containing a 200 µl suspension of PC-3 cells in full medium for 24-48 hours to allow for migration. To assess the number of migrated cells, we transferred the membranes to a new 24-well plate and fixed for 10 minutes in a 10% formalin solution. We stained the cells with crystal violet dye to resolve cells on membranes. We submerged them for 5-10 minutes in the staining solution before we washed the membranes in a beaker of water. We dried the membranes before we mounted them onto microscope slides. We quantified the migration by counting 5 representative fields at 10x magnification.

To analyze the data and determine its statistical significance, we conducted ANOVA and Tukey post hoc tests to test the significance.

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