TED ANKARA COLLEGE FOUNDATION HIGH SCHOOL

# Investigating the effects of different doses of resveratrol(10μM, 100μM, 1000μM) on the change in the number of the rat mesenchymal stem cells exposed to 25 μM of cisplatin, measured by the real time cell analyzer over 48 hours period

**Biology Extended Essay**


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Word count : 3998

**Abstract**

Antioxidants are molecules that inhibit the damaging effects of free radicals which are highly reactive chemicals that are produced when the weak bonds split in a molecule.[1]Cisplatin, on the other hand, is a highly cytotoxic chemotherapeutic agent nearly used for 30 years as an anticancer drug. It inhibits the cell proliferation due to its molecular structure.[2]

The purpose of this extended essay is to evaluate the effects of resveratrol, an antioxidant mainly produced by plants such as red grape and Japanese knotweed, to the cytotoxicity caused by cisplatin by using rat (*Rattus norvegicus*) mesenchymal stem cells.

A controlled experiment was carried out with rat mesenchymal stem cells in which the resveratrol concentration was the independent variable and the number of the stem cells given 25μM of cisplatin was the dependent variable.

A possible change in the number of the cells would be the indicator of ant cytotoxic effect of resveratrol. Therefore the research question is "How does the number of mesenchymal rat stem cells exposed 25 μM of cisplatin change in different resveratrol concentrations (10μM, 100μM and 1000μM) measured by the real time cell analyzer over 48 hours period?"

The hypothesis states that the cytotoxic effect of cisplatin which is a chemotherapeutic agent used in cancer treatments can be reversed by adding different concentrations of resveratrol.

The results indicated that moderate doses of resveratrol, approximately 10μM, decreases cytotoxicity due to cisplatin and results in higher rates of reproduction. As seen in the graph, high doses (100μM and 1000μM), on the other hand, have a significant short term effect causing cell index to increase at first. However, their overall effects are not as effective as 10μM dose.

Word Count: 286

[1]http://www.cancer.gov/cancertopics/factsheet/prevention/antioxidants(Retrieved on July 19, 2014)
[2]http://www.3dchem.com/molecules.asp?ID=214(Retrieved on July 19,2014)

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**Introduction**

I have always been interested in molecular biology and tried to investigate how things work in molecular level. Ever since my childhood I tried to understand scientific publications and documentaries with my limited knowledge, I didn't understand most of them but I didn't give up and the things that I learn in school and my independent curiosity to read informative publications soon allowed me to understand a lot more.

While I was watching a documentary about human body, I learned about some effective antioxidants and their beneficial effects. They seemed to me as potential solutions to the unfavorable results of aging process and I wanted to learn more about them. I encountered with the term French paradox while I was making research and after reading related articles such as "The French paradox: lessons for other countries" and "Resveratrol: French paradox revisited", I learned that resveratrol, an antioxidant found in grape seed and therefore in red wine may be the reason why French people are healthy although they have a diet high in carbohydrate and fat. As the action mechanism was not fully understood and as there were many controversies around this topic, it captured my attention and I got more focused on it.

As the extent of the research expanded, I found out that it is so beneficial for decreasing the oxidative stress and therefore have some ant cytotoxic effects. After learning all these information, I wondered how that molecule can affect the cytotoxic effects of some medications in organisms. As it is an important process in medical sciences to develop more target based treatments, the potential effect could be significant in some areas such as cytotoxic chemotherapy.

Resveratrol is a phenol compound produced naturally by several plants, mostly by *Vitis vinifera* (wine grape), in response to UV light, injury or attack by pathogens such as bacteria or fungi. Some clinical trials proved that in the direct interaction with skin and gastrointestinal

tumors, resveratrol acts as an anticancer agent and according to some animal studies resveratrol has antidiabetic and neuroprotective effects.[3]

Some studies also illustrated that resveratrol can induce apoptosis in some leukemia cells and plays a suppressor role on carcinogenesis initiation and progression since it has been shown as apoptosis-inducing agent in cancer cell line.[4]

After I talked to Dr. Ahmet Yeşilyurt who is a qualified expert in molecular biology and dealing with hereditary diseases, we reached to a conclusion that an experiment to test the effects of resveratrol to cytotoxicity can be carried out. I read related publications from PubMed and we reached to make an experiment design.

The effect was decided to be tested on stem cells as these cells affect the body significantly. Stem cells are undifferentiated biological cells that can differentiate into other specialized cells and can divide to produce more stem cells.[5] Therefore they are important cells that are widely used in cellular treatments. They take part nearly in all metabolic actions and act as building blocks to refresh the body and to get rid of aged, not fully functioning parts. So a possible effect on stem cells would also have an important effect on organism.

In order to test the anticytotoxic effect of resveratrol, a highly cytotoxic chemotherapeutic drug that inhibits the cell proliferation, cisplatin, was to be added to the stem cells. So, the change in the number according to the changing concentrations of resveratrol added would be the sign of the anticytotoxic effect of resveratrol.

Cisplatin has a molecular structure in which chloride ligands tend to change places with water molecules to form a highly reactive molecule.[6] The resulting molecule binds to DNA

[3]http://www.prohealth.com/library/showarticle.cfm?libid=14933(Retrieved on July 26, 2014)
[4]http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2690394 (Retrieved on July 26, 2014)
[5]http://www.stempeutics.com/about_stem_cells.html (Retrieved on July 26, 2014)
[6]http://www.rsc.org/education/eic/issues/2012January/ruthenium-compounds-anticancer-agents.asp (Retrieved on July 26, 2014)

from guanine bases, combines with them and activates DNA repair mechanisms. The cell understands that it is not possible to repair the DNA and activates apoptosis.[7]

For the experiment to be carried out, it was decided to take the stem cells from a rat's inguinal region. The tissue was going to be taken from other experiments that isolated this tissue. After the stem cells were isolated from the other cells found in adipose tissue, a solution of resveratrol should have been made for an interaction to occur.

The methods of isolating the stem cells, the doses of resveratrol to be used and all the materials that were going to be used to achieve a clear result came to a conclusion with the supervision of Dr. Ahmet Yeşilyurt and related experimental articles such as "Resveratrol Exerts Dosage and Duration Dependent Effect on Human Mesenchymal Stem Cell Development" and "Resveratrol protects bone marrow mesenchymal stem cell derived chondrocytes cultured on chitosan-gelatin scaffolds from the inhibitory effect of interleukin-1β".

My research question became clear along with the certainty in the method. My research question in this study is **"How does the number of mesenchymal rat stem cells exposed 25 µM of cisplatin change in different resveratrol concentrations (10µM, 100µM, and 1000µM) measured by the real time cell analyzer over 48 hours period?"**

[7]http://cheminfo2010.wikispaces.com/MLivingsFinalProject(Retrieved on July 26, 2014)

**Hypothesis**

Antioxidants are molecules that inhibit the damaging effect of free radicals. They have anticytotoxic effects and are used in many dietary supplements to be preventive against many diseases such as cancer and cardiovascular diseases[8]. Resveratrol is an antioxidant produced naturally by several plants in response to injury or attack by pathogens such as bacteria or fungi. It is thought to have antidiabetic and neuroprotective effects but the action mechanism is not fully understood.[9]

Stem cells on the other hand are undifferentiated biological cells that can turn into other cells. They are so important for biological actions to take place properly. They have the same biological contents with other cells and they are prone to chemicals too.

Cisplatin, which is a chemotherapeutic agent, has significant cytotoxic effects. It pulls the cell to apoptosis and inhibits cell proliferations by binding to DNA. It is used to treat cancer because of this effect.

In order to test the anticytotoxic effect of resveratrol on the cisplatin, a controlled experiment is carried out in which the resveratrol concentration will be the independent variable and the number of the stem cells given 25μM of cisplatin will be the dependent variable.

The hypothesis states that the **cytotoxic effect of cisplatin which is a chemotherapeutic agent used in cancer treatments can be reversed in rat mesenchymal stem cells by adding different concentrations of resveratrol.**

[8]http://www.ncbi.nlm.nih.gov/pubmed/21261638(Retrieved on July 26, 2014)
[9]http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2835915(Retrieved on July 26, 2014)

**Method Development and Planning**

To test the research question "How does the number of mesenchymal rat stem cells exposed 25 µM of cisplatin change in different resveratrol concentrations (10µM, 100µM, and 1000µM) measured by the real time cell analyzer over 48 hours period?", resveratrol solutions with concentrations 10µM, 100µM, 1000µM should be added to identical mesenchymal stem cells of rat (*Rattus norvegicus).*

In order to get a meaningful result, the experiment had to be carried out with much attention and the designing process should have been clear. As a non-professional, I got help from experienced professionals who have theoretical and practical experience on stem cell isolation. I had to use a laboratory equipped with essential products to get results and therefore I obtained permission from Dr. Ahmet Yeşilyurt to use the laboratories of ADACELL which is a medical institution specialized in genetics and stem cells. After I told my ideas and found out that the experiment could be done. I focused on the method design. I shared my ideas and reached to have a clear method.

First, the stem cells had to be taken either from an embryo or a stem cell niche present in an animal or human. Because of the requirements about rats to be used in laboratory, the stem cells had to be taken from another experiment that also uses stem cell containing tissues. Fortunately, they allowed me to use these stem cell niche containing tissues taken from rats.

After the adipose tissue is taken out, the stem cells must be separated from other cells present in the tissue. The following process must involve certain methods to isolate the stem cells. There are many ways to isolate the stem cells, but for this experiment, the techniques stated in the articles "Adipose-derived stem cells: isolation, expansion and differentiation" and "Isolation, culturing and characterization of rat adipose tissue derived mesenchymal stem cells: a simple technique" were decided to be applied. The standard techniques are explained in the

appendices section. (Appendix 1) The standard procedures that were used during the experiment are stated in the appendix. The process basically depends on the adherent properties of stem cells as these cells tend to bind to the bottom of the plates. The media in which the stem cells are put was decided to be the Dulbecco's Modified Eagle Medium Solution (DMEM) as this media is mostly used in mammalian cell culture experiments for its protective effects from burning, freezing or undesired reactions with other compounds and it also supports the growth of a broad spectrum cells.[10]

After the tissue culture mixture is prepared (Appendix 2), stem cells are remained in this medium and they proliferate in high rates. After the passage process is applied to sustain the higher growth rate by decreasing stress due to the decreasing space (Appendix 3), the proliferated cells that are found in the bottom of the plates are removed without harming the cell structure.

Cells are then put into a flow cytometer to separate the stem cells from other cells. The flow cytometer separates the cells according to their cells surface markers which are specific to the cell type. In the experiment the cells that contain CD11b/c CD29, CD44 and CD45 markers are collected as these markers are used to distinguish stem cells other cell types. By this way, one can have the ability to work only with stem cells without including other cells which have different structural and chemical properties. As using the flow cytometer requires special education, I got help from professionals working there.

In order to find the optimum concentrations of cisplatin and resveratrol to be used in the experiment, I read related articles such as "Resveratrol exerts dosage and duration dependent effect on human mesenchymal stem cell development" and "Effect of cisplatin, topotecan,

[10]http://www.biochrom.de/en/products/cell-culture-media/dmem/(Retrieved on July 26, 2014 )

daunorubicin and hydroxyurea on human mesenchymal stem cells". The amounts became clear after applying several doses that were stated in the articles and observing their short term effects. The doses of resveratrol to create effective differences among groups and create a significant result were decided to be 10μM, 100 μM, and 1000μM. Cisplatin dose was decided to be 25 μM after applying Giemsa Staining and seeing the effects of cisplatin to the structure of the cells. (Appendix 4)

I used an organic solvent, DMSO, to make solutions of resveratrol as resveratrol is a solid compound at room temperature and a medium that is not harmful for the cells and suitable to dissolve enough resveratrol is required. Cisplatin is liquid and it could be given directly and there was no need to find any solvents. In the experiment, the optimum number of cells to be used was decided to be 8000. The cells that were stained with trypan blue were decided to be counted by an automated cell counter. By this way the mortality could be seen and the number of cells could be estimated. (Appendix 5)

In order to understand the change in the number of the cells, cells were decided to be put in a real time cell analyzer as this tool basically works by the current change at the bottom of the wells in which the cells are found. During the proliferation process, cells cover more space in time and current beside the wells change. The data collected at decided time intervals give a relative perspective of change in the number of the cells.

In order to avoid any systematic and random error, the experiment had to be carried out with much attention. Therefore I got help from professionals and made my experiment under the supervision of them. I watched professionals applying the similar methods in order to gain experience and knowledge about the techniques.

I expect that the proliferation rates and therefore the number of the cells will be different in different resveratrol concentrations.

**Materials:**

- Inguinal region tissue of a wistar rat(*Rattus norvegicus*)
- An incubator set at 37.0℃ and 5.0% $CO_2$ concentration
- An inverted microscope
- A flow cytometer
- A centrifuge
- -86℃ Ultra-Low Temperature Freezer
- A real time cell analyzer
- A computer automated cell counter
- 8×T25 flasks
- 5×lancet
- 4× 24 well cell culture plates special for the real time cell analyzer
- 2×micropipette
- 10×100 mL syringes
- 15 grams of pure resveratrol
- 2×500 cc Dulbecco's Modified Eagle Medium Solution (DMEM)
- 2×100 cc Bovine serum
- 1×100 cc L-Glutamine
- 1×100 cc Penicillin-Streptomycin-Amphotericin B solution
- 1×10cc Phosphate Buffer Saline solution
- 1×10cc Trypsin solution
- 1×100 cc DMSO solution
- 1×100 cc Trypan blue solution
- 200 mL of deionized water

**Method:**

- Take the adipose tissue and divide it into pieces by using a lancet until each piece becomes 1 $mm^3$.
- Put the pieces into a T25 flask which is containing 10-15 mL of DMEM solution.
- Remain the T25 flask in the incubator in $CO_2$ concentration of 5% for 15 minutes.
- Take the flask out of the incubator and look it under a microscope. If the cells seem to be glued to the bottom of the flask, add tissue culture mixture.(Appendix 2)
- After 3 days, look at the flask under a microscope to see the confluency. If the cells are 80% or more confluent passage the cells to increase the area for growth and decrease the stress due to the space.(Appendix 3)
- After the passage process, classify the cells according to the cell surface markers in a flow cytometer.
- Put the cells into an automated cell counter to check out the mortality and the number.(Appendix 7)
- The optimal portion for this experiment was decided to be 8000 cells after some trials to get meaningful data. Calculate the volume of the sample you add to the cell counter and note the number of the cells given by the device. By constructing a direct proportion between the number of the cells and the volume take the required volume of the solution to get 8000 cells.
- Carefully locate the cells into the pits of the real time cell analyser with a pipette.
- Add 10µM, 100 µM, and 1000µM concentrations of resveratrol solution and 25 µM of cisplatin solution with a pipette until the total volume becomes 200 µl in a single pit.
- Do not add resveratrol to a single pit in order to make it a control group for data comparison.

- Remain the groups in the Real Time Cell Analyzer for 48 hours. This device must be connected to a computer while working in order to transfer the data.
- After 48 hours, take the cell index data, in order to understand the relative change in the number of the cells, from the software that the Real Time Cell Analyzer is connected to.
- Repeat the same process for 4 more times.

**Data Collection and Processing**

| Concentration of Resveratrol in the wells (±0.5 μM) | Trials | Volume of total mixture in each well (±0.05 μL) | Concentration of cisplatin (±0.5 μM) | Pressure of the room (±0.5 hPa) | Temperature of the room (±0.05 ℃) | Type of Incubator | Conditions of the Incubator | Cell Index Values According to the Time(±0.00005) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Initial Value | 12th hour | 24th hour | 36th hour | 48th hour |
| 10 | 1 | 200.00 | 25.0 | 1067.0 | 25.00 | Tissue Culture Incubator | 37 ℃ 5% $CO_2$ | 0,0032 | 0,0030 | 0,1865 | 0,3427 | 0,6582 |
| | 2 | | | | | | | 0,0030 | 0,0033 | 0,1863 | 0,3425 | 0,6580 |
| | 3 | | | | | | | 0,0028 | 0,0030 | 0,1865 | 0,3426 | 0,6581 |
| | 4 | | | | | | | 0,0027 | 0,0027 | 0,1868 | 0,3431 | 0,6582 |
| | 5 | | | | | | | 0,0035 | 0,0031 | 0,1864 | 0,3426 | 0,6579 |
| 100 | 1 | 200.00 | 25.0 | 1067.0 | 25.00 | Tissue Culture Incubator | 37 ℃ 5% $CO_2$ | 0,0031 | 0,0026 | 0,2019 | 0,3098 | 0,3572 |
| | 2 | | | | | | | 0,0031 | 0,0025 | 0,2018 | 0,3099 | 0,3574 |
| | 3 | | | | | | | 0,0030 | 0,0029 | 0,2020 | 0,3099 | 0,3571 |
| | 4 | | | | | | | 0,0032 | 0,0026 | 0,2019 | 0,3097 | 0,3573 |
| | 5 | | | | | | | 0,0029 | 0,0025 | 0,2019 | 0,3098 | 0,3572 |
| 1000 | 1 | 200.00 | 25.0 | 1067.0 | 25.00 | Tissue Culture Incubator | 37 ℃ 5% $CO_2$ | 0,0033 | 0,0014 | 0,0418 | 0,0347 | 0,0361 |
| | 2 | | | | | | | 0,0030 | 0,0013 | 0,0415 | 0,0346 | 0,036 |
| | 3 | | | | | | | 0,0034 | 0,0012 | 0,0417 | 0,0344 | 0,0363 |
| | 4 | | | | | | | 0,0035 | 0,0015 | 0,0415 | 0,0349 | 0,0361 |
| | 5 | | | | | | | 0,0029 | 0,0014 | 0,0419 | 0,0347 | 0,036 |
| 0 (control group) | 1 | 200.00 | 25.0 | 1067.0 | 25.00 | Tissue Culture Incubator | 37 ℃ 5% $CO_2$ | 0,0032 | 0,0032 | 0,0029 | 0,0033 | 0,0032 |
| | 2 | | | | | | | 0,0030 | 0,0030 | 0,0032 | 0,0032 | 0,0030 |
| | 3 | | | | | | | 0,0033 | 0,0031 | 0,0029 | 0,0031 | 0,0031 |
| | 4 | | | | | | | 0,0032 | 0,0033 | 0,0031 | 0,0035 | 0,0031 |
| | 5 | | | | | | | 0,0034 | 0,0032 | 0,0027 | 0,0032 | 0,0034 |

**Table 1:** The concentrations of resveratrol, volume of total mixture in the wells, the concentration of cisplatin, the temperature and the pressure in the room, the conditions of the incubator and the cell index values obtained according to the trials and taken at different stages during the experiment are illustrated with appropriate units and uncertainties. As cell index is a dimensionless parameter, it hasn't got a unit.

| Concentration of Resveratrol (μM±0.5) | Trials | Cell Index(±0.00005) | | | | |
|---|---|---|---|---|---|---|
| | | Initial Value | 12th hour | 24th hour | 36th hour | 48th hour |
| 10.0 | 1 | 0,0032 | 0,0030 | 0,1865 | 0,3427 | 0,6582 |
| | 2 | 0,0030 | 0,0033 | 0,1863 | 0,3425 | 0,6580 |
| | 3 | 0,0028 | 0,003 | 0,1865 | 0,3426 | 0,6581 |
| | 4 | 0,0027 | 0,0027 | 0,1868 | 0,3431 | 0,6582 |
| | 5 | 0,0035 | 0,0031 | 0,1864 | 0,3426 | 0,6579 |
| 100.0 | 1 | 0,0031 | 0,0026 | 0,2019 | 0,3098 | 0,3572 |
| | 2 | 0,0031 | 0,0025 | 0,2018 | 0,3099 | 0,3574 |
| | 3 | 0,0030 | 0,0029 | 0,202 | 0,3099 | 0,3571 |
| | 4 | 0,0032 | 0,0026 | 0,2019 | 0,3097 | 0,3573 |
| | 5 | 0,0029 | 0,0025 | 0,2019 | 0,3098 | 0,3572 |
| 1000.0 | 1 | 0,0033 | 0,0014 | 0,0418 | 0,0347 | 0,0361 |
| | 2 | 0,0030 | 0,0013 | 0,0415 | 0,0346 | 0,0360 |
| | 3 | 0,0034 | 0,0012 | 0,0417 | 0,0344 | 0,0363 |
| | 4 | 0,0035 | 0,0015 | 0,0415 | 0,0349 | 0,0361 |
| | 5 | 0,0029 | 0,0014 | 0,0419 | 0,0347 | 0,0360 |
| 0(control group) | 1 | 0,0032 | 0,0032 | 0,0029 | 0,0033 | 0,0032 |
| | 2 | 0,0030 | 0,0030 | 0,0032 | 0,0032 | 0,0030 |
| | 3 | 0,0033 | 0,0031 | 0,0029 | 0,0031 | 0,0031 |
| | 4 | 0,0032 | 0,0033 | 0,0031 | 0,0035 | 0,0031 |
| | 5 | 0,0034 | 0,0032 | 0,0027 | 0,0032 | 0,0034 |

**Table 2:** shows the cell index values according to the concentration of resveratrol in the well, trial number and time passed during the experiment. Resveratrol concentration is in micromolar and has an uncertainty value of 0.5 micromolars. As cell index is a dimensionless parameter it has not got a unit but the uncertainty is ±0.00005.

| Concentration of Resveratrol (μM±0.5) | Cell Index(±0.0025) | | | | |
|---|---|---|---|---|---|
| | Initial Value | 12th hour | 24th hour | 36th hour | 48th hour |
| 10.0 | 0.0030 | 0.0030 | 0.1865 | 0.3427 | 0.6581 |
| 100.0 | 0.0031 | 0.0026 | 0.2019 | 0.3098 | 0.3572 |
| 1000.0 | 0.0032 | 0.0014 | 0.0419 | 0.0347 | 0.0361 |
| 0(control group) | 0.0032 | 0.0032 | 0.0030 | 0.0033 | 0.0032 |

**Table 3:** shows the average cell index values obtained according to the trials. As each concentration value had 5 trials, the uncertainties are added up and gave an uncertainty value of 0.0025

**Anova: Single Factor**

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Column 1 | 5 | 3,2904 | 0,65808 | 1,7E-08 |
| Column 2 | 5 | 1,7862 | 0,35724 | 1,3E-08 |
| Column 3 | 5 | 0,1805 | 0,0361 | 1,5E-08 |
| Column 4 | 5 | 0,0158 | 0,00316 | 2,3E-08 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 1,419841 | 3 | 0,47328 | 27840015 | 6,06E-54 | 3,238872 |
| Within Groups | 2,72E-07 | 16 | 1,7E-08 | | | |
| Total | 1,419841 | 19 | | | | |

**Table 4:** The Anova: Single Factor results obtained from the data in Table 2. The P value being numerically less than the alpha value of 0.05 expresses the truth of the hypothesis.

| Concentration of Resveratrol (µM±0.1) | For | Cell Index | | | | |
|---|---|---|---|---|---|---|
| | | Initial Value | 12th hour | 24th hour | 36th hour | 48th hour |
| 10 | Mean | 0,003 | 0,0030 | 0,1865 | 0,3427 | 0,6580 |
| | Median | 0,003 | 0,003 | 0,1865 | 0,3426 | 0,6581 |
| | SD | 0,0003 | 0,0002 | 0,0002 | 0,0002 | 0,0001 |
| | SE | 0,0001 | 1E-04 | 8E-05 | 0,0001 | 6E-05 |
| 100 | Mean | 0,0031 | 0,0026 | 0,2019 | 0,3098 | 0,3572 |
| | Median | 0,0031 | 0,0026 | 0,2019 | 0,3098 | 0,3572 |
| | SD | 0,0001 | 0,0001 | 6E-05 | 7E-05 | 0,0001 |
| | SE | 5E-05 | 7E-05 | 3E-05 | 4E-05 | 5E-05 |
| 1000 | Mean | 0,0032 | 0,0014 | 0,0417 | 0,0347 | 0,0361 |
| | Median | 0,0033 | 0,0014 | 0,0417 | 0,0347 | 0,0361 |
| | SD | 0,0002 | 0,0001 | 0,0002 | 0,0002 | 0,0001 |
| | SE | 0,0001 | 5E-05 | 8E-05 | 8E-05 | 5E-05 |
| 0(control group) | Mean | 0,0032 | 0,0032 | 0,003 | 0,0033 | 0,0032 |
| | Median | 0,0032 | 0,0032 | 0,0029 | 0,0032 | 0,0031 |
| | SD | 0,0001 | 0,0001 | 0,0002 | 0,0001 | 0,0001 |
| | SE | 7E-05 | 5E-05 | 9E-05 | 7E-05 | 7E-05 |

**Table 5:** shows mean, median, standard deviation and standard error values for resveratrol concentrations of 0, 10, 100 and 1000 µM according to the time passed.



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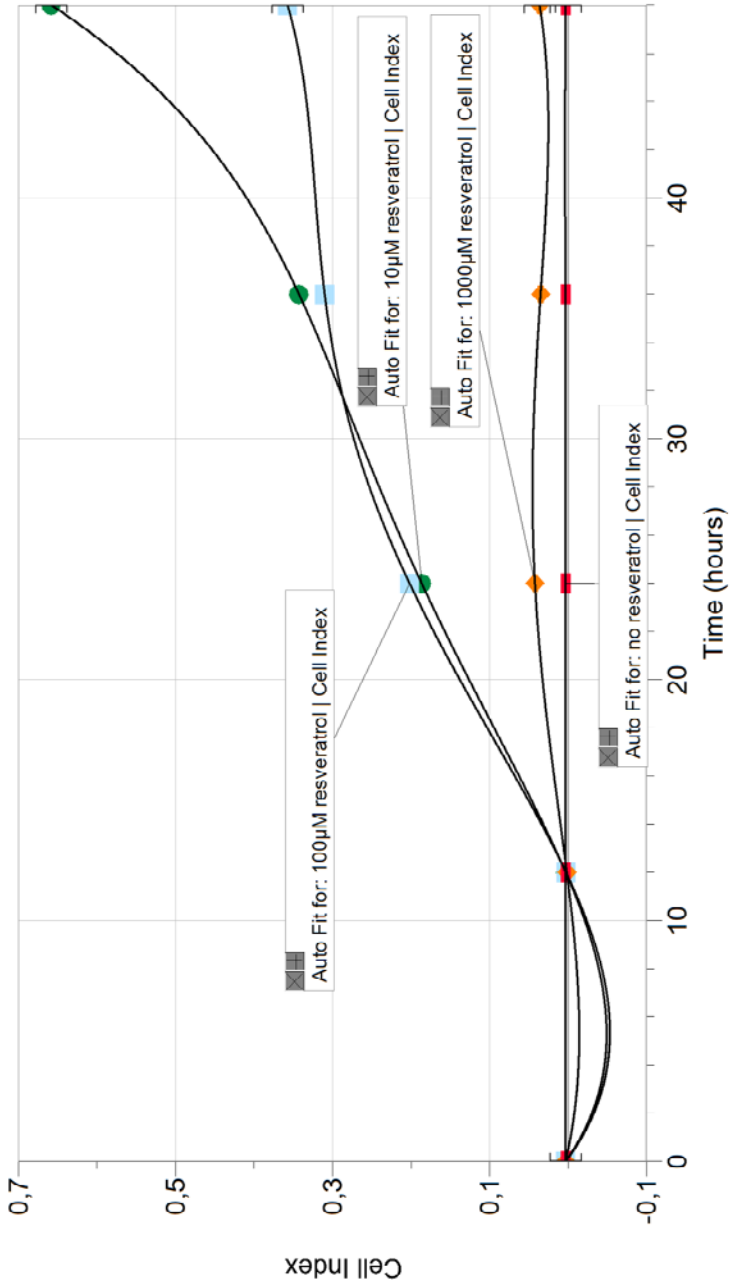



**Graph 1:** Shows the change in the cell index according to the resveratrol concentrations of 10µM, 100µM, 1000µM and 0µM. Standard error is less than 0.01 so error bars are neglected.

- for 10 µM resveratrol concentration
- for 100 µM resveratrol concentration
- for 1000 µM resveratrol concentration
- for no resveratrol containing wells

**Discussion**

The aim of this research was to investigate the effect of resveratrol against cytotoxicity caused by cisplatin. 10µM, 100µM, 1000µM concentrations of resveratrol were prepared and given to adipose tissue derived rat mesenchymal stem cells which were exposed to 25µM of cisplatin. The hypothesis states that the cytotoxic effect of cisplatin which is a chemotherapeutic agent used in cancer treatments can be reversed by adding different concentrations of resveratrol.

The stem cells were isolated from adipose tissue of a rat (*Rattus norvegicus*).An automated cell counter was used to find the number of cells and proper amount of solution containing 8000 cells was taken by constructing a direct proportion between volume and the number of the cell. The cells are then put into the wells of a real time cell analyzer with resveratrol dissolved in the DMSO and cisplatin given directly at the same time. The temperature and the pressure of the room, which were 25.0℃ and 1067 hPa respectively, were constant during the experiment. The concentration of cisplatin was 25µM in all wells. The incubator was set to 37℃ and 5% $CO_2$ concentration. The total volume of the mixture in the wells containing DMEM, resveratrol dissolved in DMSO, cisplatin and stem cells add up to 200.00 µL in a single well.

It was seen that number of the cells, cell index, was highest at 1000µM till $24^{th}$ hour but decreased gradually till $48^{th}$ hour. However, the cells in the wells containing 10µM and 100µM of resveratrol remained in low levels with small fluctuations until the $24^{th}$ hour. After that they increased in number. At the end of the $48^{th}$ hour, 10µM containing wells had the highest number of cells followed by 100µM and 1000µM.

The results indicate that the anticytotoxic effect of resveratrol against cisplatin is decreased at most in approximately 10µM concentration. The higher doses decrease the

cytotoxicity better at first as it can be deduced from the higher proliferation rates but the effect is short termed.

It can be seen that the data supports the hypothesis as proliferation rate and the number of the cells increase in the concentration of 10µM but decrease in the concentrations of 100µM, 1000µM as resveratrol is beneficial to decrease cytotoxicity due to cisplatin in low amounts and increase the proliferation rate but has negative impacts in higher amounts by inducing apoptosis due to its molecular properties.

The standard deviation and standard deviation values are comparably small when compared to the data. These small values indicate that the values are accurate and precise. The error gets bigger from 1000µM to 10µM. Also the P- value of $6.06\times10^{-54}$obtained from the ANOVA statistical analysis proves the hypothesis as it is smaller than 0.05.

As a summary, it can be said that **cytotoxic effect of cisplatin which is a chemotherapeutic agent used in cancer treatments can be reversed in rat mesenchymal stem cells by adding optimum concentrations of resveratrol, approximately 10µM, but this anticytotoxic effect decreases in higher concentrations it is said in the hypothesis.**

Although the data proved that there were significant changes in the effect of different resveratrol concentrations, there were some error sources affecting the results.

- There are uncertainties in the tools such as micropipette, the real time cell analyzer and cell counter. Therefore cell index and the number of the cells contain errors.
- In order to take the preferred number of cells, it is assumed that there is an exact direct proportion between volume and the number of the cells. However, this is not a 100% flawless method as it's not certain that cells are distributed homogeneously.

- There may be contamination caused by microorganisms which may affect the change in the number of cells.
- Some cells may be damaged during the centrifuge process and processes that include syringes due to the pressure.

In order to minimize the errors,

- The number of trials can be increased
- Several measurements could have been done with the cell counter and the average could be taken as the number of the cells.
- The experiment could have been repeated with cells belonging to different individuals. This would decrease a potential source of error due to the damaged cells. Also, it could be understood that whether the dose differs much from individual to individual.

**Conclusion**

My research question in this study was "How does the number of mesenchymal rat stem cells exposed 25 μM of cisplatin change in different resveratrol concentrations (10μM, 100μM, and 1000μM) measured by the real time cell analyzer over 48 hours period?" According to the results of the experiment, there are significant differences between the effects of resveratrol concentration to the anticytotoxic effects of cisplatin.

The anticytotoxic effects are best reduced in an optimal concentration of 10μM of resveratrol. Other doses including 100μM and 1000μM are highly effective to reduce the anticytotoxic effect at first as it can be determined by the high proliferation rates in the number of the cells. Although there were some errors in the experiment, the overall data is enough to show the change. The method can be improved to get more accurate and precise data yet the experiment was successful.

The experiment strengthen the idea that resveratrol, as an antioxidant, can be used to develop more target based treatments involving cisplatin. As cisplatin is highly harmful to the body by causing side effects such as nephrotoxicity (kidney damage), neurotoxicity (nerve damage) and ototoxicity (hearing loss), resveratrol can be used to inhibit these effects.[11] However, further investigations must be done to be definite about the effects of resveratrol on humans.

Like other drugs whose lead compounds were taken by nature, resveratrol could be one of them. The results are convincing to guide our minds to find the solutions in nature. To conclude, resveratrol holds great opportunities for developments in medicine and human health.

[11]http://ar.iiarjournals.org/content/33/10/4183.full(Retrieved on October 4, 2014)

**Further Investigations**

After the experiment and the results, it is clear that resveratrol decreases the cytotoxic effect of cisplatin. However, the experiment was designed on the rat cells. This raises new questions. These questions involve “How does different cell types are affected from the resveratrol?” or “Does resveratrol shows the same effects in the human and rat cells?”

Also even if these questions are answered, there are many steps to take. The possible side effects must be observed, optimum dosages must be measured and the molecule must be tested on every type of cell and on people from different ages.

## Appendices

### Appendix 1: Method for stem cell isolation

- The adipose tissue is put into several T25 flasks which contains 10-15 mL of DMEM solution.
- These T25 flasks are remained in the incubator with a $CO_2$ concentration of 5% for 15 minutes.
- A tissue culture mixture is added to the medium.
- Every 3 days the mixture is refreshed and after 15 days, first cell passage is done.
- The cells are then classified according to the cell surface markers. (CD11b/c CD29, CD44 and CD45) in a flow cytometer.
- After the stem cells are isolated, the whole sample is put into a automated cell counter. The mortality and the number of the cells are observed. The optimal portion is taken according to the volume and cell number.

Dulbecco's Modified Eagle's medium (DMEM). DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components.[12] DMEM is used in a wide range of mammalian cell culture applications.[13]


**Figure 1:** The type of DMEM solution used

Dimethyl sulfoxide (DMSO), another compound used in the experiment to dissolve resveratrol, is an organosulfur compound. This colorless liquid is an important polar solvent that dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water.[14]

[12]http://www.sigmaaldrich.com/life-science/cell-culture/classical-media-salts/dmem.html(Retrieved on October 4, 2014)
[13]http://www.lonza.com/products-services/bio-research/cell-culture-products/classical-media/dmem.aspx(Retrieved on October 4, 2014)
[14]http://www.viscopedia.com/viscosity-tables/substances/dimethyl-sulfoxide(Retrieved on October 4, 2014)

**Appendix 2: Making the cell culture**

- 20% bovine serum
- 2% L-Glutamine
- 1%Penicilin-Streptomycin-Amphotericin solution
- 77% DMEM solution

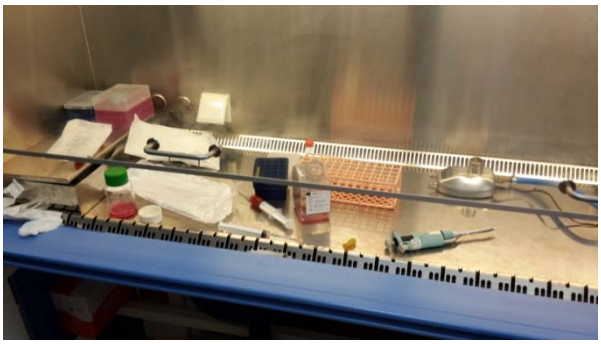
Containing mixture is made by using micropipettes.


**Figure 2:** The type of fetal bovine serum used in the experiment.

Bovine serum is the blood fraction remaining after the natural coagulation of blood, followed by centrifugation to remove any remaining red blood cells.[15]

Glutamine, or L-glutamine, is an amino acid [illegible]nic acid. Glutamine plays a role in the health of the immune system, digestive tract, and muscle cells, as well as other bodily functions. It appears to serve as a fuel for the cells that line the intestines.[16]

Penicillin-Streptomycin-Amphotericin B Solution is used to reduce the chance of microbial contamination while propagating primary cells or continuous cell lines in culture.[17]



**Figures 3 and 4:** A picture of mine preparing the solution and a picture of the lab that I worked in.

[15]http://www.pan-biotech.com/en/bovine-sera.html(Retrieved on October 4, 2014 )
[16]http://www.med.nyu.edu/content?ChunkIID=21749(Retrieved on October 4, 2014 )
[17]http://www.lgcstandards-atcc.org/products/all/PCS-999-002.aspx?geo_country=tr(Retrieved on October 4, 2014 )

**Appendix 3: Cell passage**

- For the cell passage, the mixture containing T25 flask is submerged into a waterbath adjusted to 37℃.
- 2 cc of Phosphate Buffer Saline is added to the T25 flask.
- The saline solution is then removed by a pipette and 2 cc of trypsin is added to the flask.
- The cells are observed under a microscope to confirm that the cells are visibly detached from the surface.
- The flask is put into a centrifuge and centrifuged under 1200 RPM for 10 minutes
- Supernatant part is removed and DMEM is added.
- The centrifuge is repeated

This process basically forms a favorable, suitable condition for cell growth.

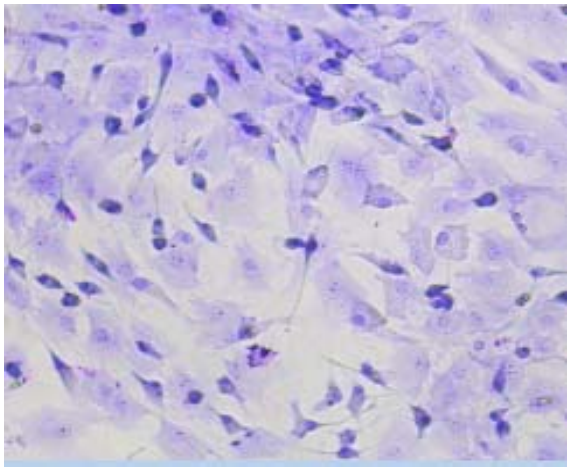
Figures 5 and 6: The centrifuge and the inverted microscope used in the experiment.

**Appendix 4: Giemsa staining method**

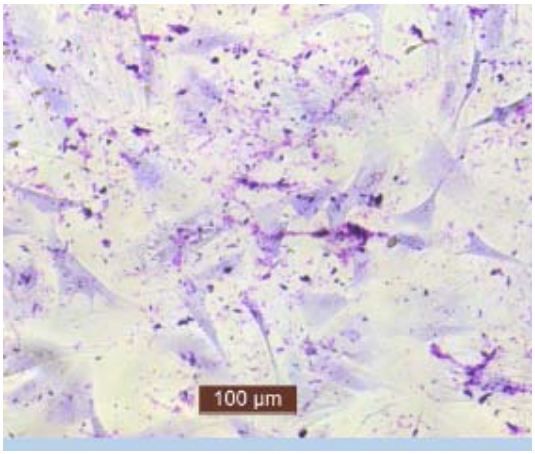
In order to understand the effect of cisplatin, Giemsa staining method was also applied.

A flask only containing stem cells and another one containing stem cells exposed to 25 µM of cisplatin is taken. The media in the 70-80 percent confluence T25 flasks are taken out and 10 cc of phosphate buffered saline solution is added to the flasks.

After 2 minutes, the solution is taken out with a pipette and 5% Giemsa stain is added. Giemsa stain is taken out after 5 minutes and 100% ethyl alcohol is added. After 2 minutes the alcohol is also taken out and the cells are observed under the microscope.



Normal Stem Cells




Stem Cells Exposed to Cisplatin

**Figures 7 and 8:** The pictures taken by the microscope used in the experiment. The picture on the left shows the cells without cisplatin. The picture on the right shows the stem cells exposed to cisplatin. It is clearly seen that cisplatin exposed cells are highly damaged. Their cell structures are significantly corrupted.

**Appendix 5: Counting cells by using automated cell counter**

- Trypan blue is added to a portion of the cells by using a micropipette.
- The proportion is then put onto a microscope slide.
- The slide is pinned to the kit that is specific to the tool.
- The device gives a clear magnified image of the cells and the number of the cells present in the slide.
- By using the ratio of the volume and the cell number, the desirable number of cells can be taken


**Figure 9:** The automated cell counter used in the experiment.

**Appendix 6: The statistical analysis**

Statistical relationships in the experiment can be determined by using the Microsoft Excel 2007 program. The following formulas are used to obtain the values in Table 3.

**Mean:**

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

Where;

n is the number of trials (n=5 for this experiment)

$x_i$ is the cell index values obtained for each resveratrol concentration

**Standard Deviation:**

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}$$

Where;

n is the number of trials (for this experiment n=3)

x is the cell index values obtained for each resveratrol concentration

$\bar{x}$ is the mean value of the corresponding group

**Standard Error**

$$\sigma_{\bar{x}} = \frac{\sigma}{\sqrt{n}}$$

Where;

$\sigma$ is the standard deviation of the corresponding group

n is the number of trials

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