ANTIOXIDANT AND ANTI-INFLAMATORY EFFECTS AND MECHANISMS OF GREEN TEA IN VITRO IN VASCULAR EPITHELIAL CELLS

A Senior Scholars Thesis

by

ABIDA HASAN

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Nutritional Sciences

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Approved by:

Research Advisor: Susanne Talcott
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Antioxidant and Anti-Inflammatory Effects and Mechanisms of Green Tea in vitro in Vascular Epithelial Cells. (April 2009)

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In the human body there are free radicals present which have one or many unpaired or lone electrons. These electrons are reactive and have the capability to cause damage to tissues if left in their unstable state. In order to counteract these free radicals there are antioxidants which seek out the free radicals and bind to them, creating a stable state and preventing the formation of a reactive oxygen species.

Antioxidants are made by the body's natural biological systems as well as acquired through the diet. Ideally these two groups, antioxidants and reactive oxygen species (ROS), are kept in a balance amongst the body's biological systems but when the balance is skewed and there are ROS in excess antioxidants, oxidative stress occurs. This stress can cause severe damage to body tissues.

For this experiment it is essential to note what oxidative stress means for vascular epithelial cells. The oxidative stress on the lipids in the vascular cell walls leads to a

form of heart disease called atherosclerosis or hardening of the arteries. It is also important to note that there is evidence to suggest that adequate amounts of antioxidants from the diet have been found to slow this disease process down and in some cases actually prevented it.

In the United States cardiovascular disease is the number one killer for both men and women, thus the research in this field of study is vital. One of the dietary choices that have decreased the oxidative stress is the consumption of plant phenols specifically through tea.

ACKNOWLEDGMENTS

I would like to acknowledge my parents who have taught me that hard work, determination, and perseverance always pay off in the end. I would also like to thank Dr. Susanne Talcott who gave me the encouragement to pursue this challenge and take on such a tremendous responsibility. I would also like to acknowledge Alex Halgood, another Undergraduate Research Scholar, who has been through this adventure with me and helped me with my first research excursion, time and time again. This project is also for all of the other students who have ever felt over their heads but still came out successful.

NOMENCLATURE

DCFH	Dichlorofluorescin Diacetate
DMSO	Dimethyl Sulfoxide
EC	(-)-Epicatechin
ECG	(-) - Epicatechin-3-gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
FBS	Fetal Bovine Serum
GA	Gallic Acid
GAE/L	Gallic Acid Equivalents
ppm	Parts per Million
ROS	Reactive Oxygen Species
RPM	Rotations per Minute

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CHAPTER I

INTRODUCTION

Tea is the world's most consumed drink. It is rich in flavanoids or plant metabolites which are thought to be the reason for the health benefits that plants have. Through several studies it has been found that the risk of having a heart attack for people who had one cup of black tea a day is decreased by 56% compared to those who did not drink any tea. It has also been noted that the consumption of green tea is inversely proportional to coronary heart disease in Japanese women. It not clear what the role is of tea and the decrease risk of disease-causing oxidative stress but it is known that antioxidant enzymes in the body do increase their activity when tea catechins and polyphenols are involved. Though there is evidence to suggest the benefits of tea, they have also been found to be short-lived. The reason for this is thought to be due to the fact that tea catechins have partial absorption as well as prompt metabolism and excretion (Higdon and Frei 2003). The experiment being conducted for this research topic is focusing on green tea.

This thesis follows the style of the journal Oecologia..

Green tea characteristics

Green tea is created by the drying and steaming the leaves of the *Camellia sinensis* plant this processing is also classified as the non-fermented type of tea. The unique property of green tea is the polyphenols present, specifically flavonoids, that contain four key catechins: (-)-epigallocatechin-3-gallate, (-)-epigallocatechin, (-) - epicatechin-3-gallate, and (-)-epicatechin. These catechins are metabolized quickly; EGCG is disposed of through the bile and EGC and EC are released through the urine as well as through the bile (Cabrera et al. 2006). It has been found that it is not the tea itself that determines the health benefits; it is actually the quantity ingested and the bioavailability of these components. In addition to this, there is also gallic acid as well as additional polyphenol components such as carotenoids, vitamin C, and other phytochemicals present in green tea. Green tea's antioxidant effects are speculated to be associated with its "inhibition of the redox-sensitive transcription factors, inhibition of "pro-oxidant" enzymes, and it's induction of antioxidant enzymes" (Cabrera et al. 2006). Though much is known about the health benefits of introducing tea into a diet regimen there is still much research to conduct to understand how they work. With this experiment, the results will add some insight into how the mechanism of absorption and the bioavailability of tea phenolics works- an area still unknown to scientists. Studies have also revealed there are still several unanswered questions regarding tea consumption, oxidative stress, and cardiovascular disease development (Halliwell 1996). This experiment is also important to the world of nutrition due to the conflicting evidence of whether the benefits of tea on endothelial cells is due to the antioxidant

components of tea phenols or other unknown beneficial components of tea (Higdon and Frei 2003).

CHAPTER II

METHODS

Preparing to seed CCD-18 cells for experiment

CCD-18 cells were colonized on Petri dishes for 48 hours before they could be seeded into the 96 well and 24 well assay plates for the experiments. Initially the existing medium was removed from the Petri dish. The plate was then washed with 10mL of buffer solution in order to remove any old medium and dead cells. This medium was also then aspirated out and 1mL of trypsin was added to detach the cells from the walls of the growth plate. The trypsinated cells were placed in the incubator for 5 minutes at 37 degrees Celsius. Once this time period was over 10mL of buffer solution with 10% FBS was added to deactivate the trypsination process. This mixture was then homogenated with an auto pipette and moved to a 15mL falcon tube. The falcon tube was then placed in the centrifuge at a rate of 1000 RPM for 2 minutes. After this process was complete, a pellet was visible at the bottom of the falcon tube-these were the cells and the fluid on top of the pellet was called the supernatant. The supernatant was then removed from the falcon tube and 10mL of medium was added in its place. The pellet was then resuspended in the mixture with the medium.

Cell counting

50µL of the resuspended solution was added to a cuvette containing 10mL of isotonic solution. The cuvette was then inverted to mix the solution and then placed into the cell counting machine. This was done twice. The average of both readings is what was used in the experimental calculations. Once the average number of cells was acquired it was multiplied by the dilution factor of 400 thus equaling the number of cells in 1mL. The goal for the ROS fluorescence part of the experiment was to seed 5,000 cells per wel1 of the experimental solution and 15,000 cells per well for the cell proliferation part of the experiment.

Seeding CCD-18 cells

ROS fluorescence

The adjusted volume of cell solution and medium was placed in a basin to allow each of the 96 wells to have 5,000 cells. This combination of solutions was then homogenated with the auto pipette. Then, using a multichannel pipette, each well of a 96 wells on the assay plate were filled with 100µL of the cell solution. The assay plate was then observed under a microscope to verify that the CCD-18 cells were evenly distributed throughout the plate as well as if they were correctly placed in the wells. The assay plate was then incubated for 24 hours (Meng et al. 2008).

Cell proliferation

The adjusted volume of cell solution and medium was placed in a basin to allow each of the 24 wells to have 15,000 cells. This combination of solutions was then homogenated with the auto pipette. Using a 1000µL pipet, 500µL of the cell solution was placed in all 24 wells in the assay plate. The assay plate was then observed under a microscope to verify that the CCD-18 cells were evenly distributed throughout the plate as well as if they were correctly placed in the wells. The assay plate was then incubated for 24 hours. (Zheng et al. 2002)

Tea extract's stock solutions

The CCD-18 cells were tested by at 5 different concentrations of green tea extract. These concentrations were 1 mg GAE/L, 2 mg GAE/L, 4 mg GAE/L, 8 mg GAE/L and 16 mg GAE/L. In order to create these concentrations the stock solution (20,000 ppm) was diluted in DMSO. From this stock solution the dilution of different aliquots were used to achieve a final concentration 1-16 mg GAE/L.

Treating CCD-18 cells

ROS fluorescence

Before beginning this part of the experiment the cells were observed under the microscope to confirm that they had attached to the bottom of the wells and were viable for experimentation. Once that was accomplished, the existing medium was removed

from each well and replaced with fresh medium containing the extract at different concentrations.

On the plate, columns 2-6 had increasing concentrations (ex. column 2- 1mg GAE/L, column 3- 2 mg GAE/L, etc.) while column 1 was the designated blank- no cells were seeded in this column. This plate was incubated for another 24 hours to allow the extracts to effect the cells.

After 24 hours the wells were washed with 100µL of buffer solution in order to remove any old medium and dead cells, the buffer was then aspirated out of each well. Next, a 200µM solution of hydrogen peroxide (H_2O_2) was added to each well and incubated for 2 hours. Once this time was over, the hydrogen peroxide was aspirated out and the cells were rewashed with 100µL of buffer solution, this was then aspirated out. After this, the cells were treated with a 10 µM concentration of DCFH (a dye for fluorescent identification) and incubated for 15 minutes. The assay plate was then placed in the ROS fluorescence device and read for 30 minutes at 15 minute intervals (0 time, 15 minutes, and 30 minutes) (Meng et al. 2008).

Cell proliferation

In the 24 assay plate, each well had 15,000 cells seeded. Before beginning this part of the experiment the cells were observed under the microscope to confirm that they had attached to the bottom of the wells and were viable for experimentation. Prior to adding

the tea extracts, one row of cells in the assay plate was designated time zero. For these 3 wells the existing medium was removed from each well and washed with 500µL of buffer solution, aspirate. Next, 200µL of trypsin were added to the 3 zero time wells and incubated for 7 minutes. Once this time period was over 900µL of buffer solution with 10% FBS was added to the 3 wells deactivate the trypsination process. This mixture was then homogenated with an auto pipette and placed in a cuvette containing 10mL of isotonic solution. The cuvette was then inverted to mix the solution and then placed into the cell counting machine. Once the zero time results were acquired, the other wells were treated with their respective tea extract concentrations and incubated for 72 hours.

After the 72 hours were complete, the remaining wells were treated the same way the time zero wells were treated. These results are below. (Zheng et al. 2002)

CHAPTER III

RESULTS

Figure 1 shows the results obtained from the experimental and control runs for my ROS fluorescence experiment. As can be seen in figure 1, as the concentration of green tea extract increased the fluorescence decreased.

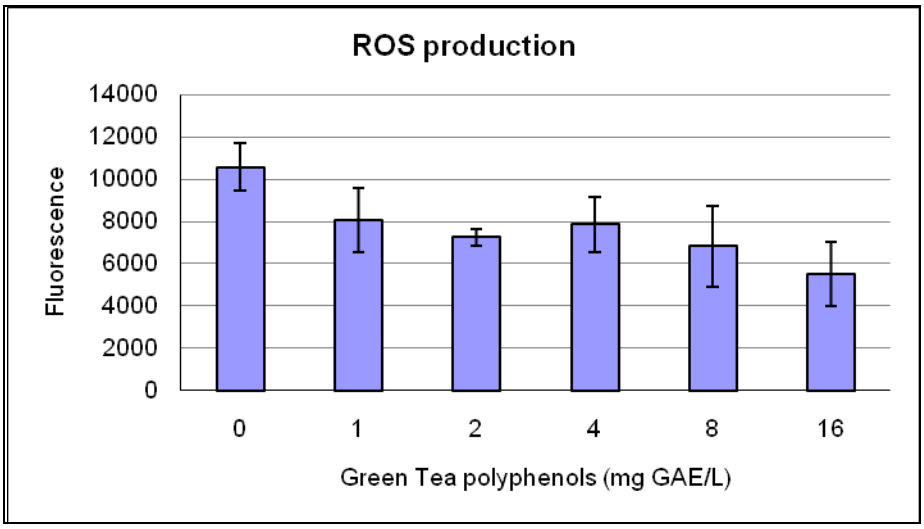

Figure 1: ROS Fluorescence Production with Varying Concentrations

The ROS fluorescence results illustrate there are more cells present in the wells with the higher concentrations of green tea extract in comparison with the wells with lower concentrations of extract. Thus confirming that the more green tea extract present the more protected cells are from oxidative stress.

Figure 2 demonstrates that more cell growth was present at higher concentrations of green tea extract than at lower concentrations. However since the cell line being tested, CCD-18, is a colon cancer cell line these are not the ideal results. This suggests that the cell proliferation portion of the experiment will have to be repeated in order to attain more agreeable results.

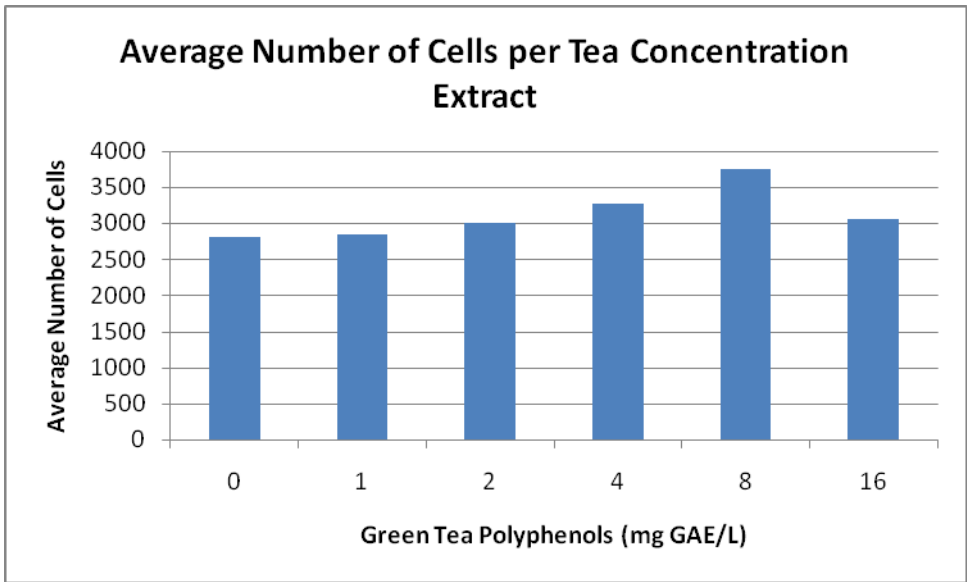

Figure 2: Cell Proliferation Data (Average Number of Cells per Well)

CHAPTER IV

SUMMARY AND CONCLUSIONS

In conclusion, the ROS fluorescence portion of the experiment illustrates that an increase in green tea concentration protects the cells from oxidative stress in vascular epithelial cells thus decreasing the number of cells damaged in the experiment. This is seen through the dose-dependent ROS fluorescence portion of the experiment proving that an increase in green tea extract concentration aids the cells in avoiding the oxidative stress within the cellular environment, thus green tea in certain concentrations can act as an effective antioxidant. The cell proliferation data obtained does not give the results expected and therefore must be repeated.

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