

The Use of Fisetin in an *in Vitro* Model of the Diabetic Eye

Neha Vazarkar

Mentors: Mrs. Donna Leonardi, Dr. German Sabio

Acknowledgement

I did not work with other scientists or students other than my research mentors, Mrs. Donna Leonardi and Dr. German Sabio. I was the sole writer of my research paper. My mentors' roles were to provide guidance and support during my research process, and they did not direct my research process. I was responsible for the inception of my research idea and I formulated my own hypothesis based on the scientific literature I read. I designed my procedures and experiments, carried out all of my experiments, recorded and analyzed data, and formulated my own conclusions as part of my research project.

ABSTRACT

Age-related Macular Degeneration (AMD) is the most common cause of vision loss in the United States, affecting over 10 million Americans. AMD impacts the retina, with an accumulation of unresolved oxidative stress as a by-product of metabolic processes. Diabetic complications such as Diabetic Nephropathy have been shown to have a strong association with AMD, and diabetic patients are three times as likely to develop AMD. While the connection between diabetes and AMD has been explored in previous research studies, little research has been done on the impact of the AGE-RAGE signaling pathway, a common thread in diabetic complications, in AMD. The AGE-RAGE pathway in diabetic complications has been shown to lead to an increase in oxidative stress and inflammation as a result of the production of advanced glycation end products (AGEs) and is irreversible. The production of AGEs results in a positive feedback loop through the RAGE receptor and oxidative stress, which exacerbates its negative impact on cells. The AGE-RAGE pathway is also associated with VEGF, TNF- α , and IL-1 β , which result in inflammatory responses. This research examined the effect of the AGE-RAGE pathway in AMD on oxidative stress and the impact of the antioxidant Fisetin in reducing this oxidative stress. Fisetin is an antioxidant which is commonly found in fruits and vegetables such as cucumbers, strawberries, and persimmons. This interdisciplinary study identified common Differentially Expressed Genes (DEGs) in Fisetin-treated and AMD datasets by using GEO datasets GSE5258 and GSE103060. These DEGs were used to suggest the enrichment of pathways and protein-protein interactions using online tools. Subsequently, an *in vitro* model was made, using 661w murine photoreceptor cells which were treated with concentrations of fructose to model the diabetic microenvironment. This *in vitro* model was also treated with Fisetin. AGE-RAGE, VEGF, IL-1 β , and TNF- α were studied *in vitro* through ELISAs. These ELISAs suggest a decreased secretion of TNF- α , IL-1 β , and VEGF when cells were treated with higher concentrations of Fisetin, but an increased expression of the AGE-RAGE pathway and ROS. This research suggests that the AGE-RAGE pathway is connected to ROS production through oxidative stress, and TNF- α , IL-1 β , and VEGF through inflammatory mechanisms. This paper suggests that Fisetin has anti-inflammatory properties which result in the potential to be considered as an adjuvant therapy in lower concentrations for patients with AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is the most common cause of vision loss in the United States, affecting over 10 million Americans. AMD is a slow-progressing, age-related disease characterized by the deterioration of the macula. The macula is located in the center of the retina and controls eye focus (Ehrlich et al., 2008). This results in difficulty sending clear, accurate images to the brain through the optic nerve, affecting a person's ability to perform everyday tasks such as reading, writing, driving, and seeing the finer details of objects. As aging progresses, structural and blood flow changes can predispose patients to age-related macular degeneration.

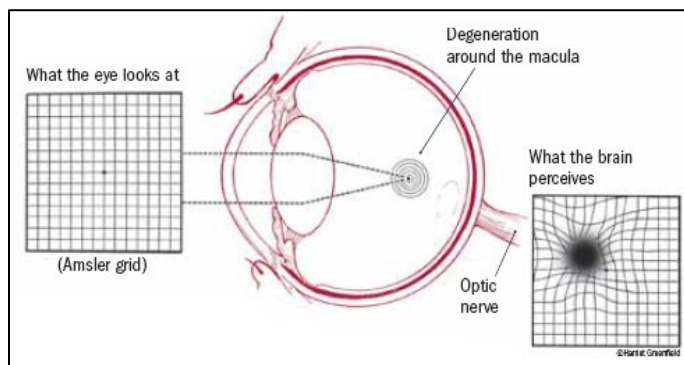


Diagram 1. The Effect of Macular Degeneration.

Image adapted from Harvard Health Publishing.

There are two forms of AMD: dry AMD and Choroidal Neovascularization (wet) AMD. While dry AMD is much more common, the effects of wet AMD can be much more severe. The term Choroidal

Neovascularization (CNV) refers to the “wet” form of age-related macular degeneration. Patients with wet macular degeneration have new blood vessels which originate from the choroid and break through the Bruch's membrane, severely affecting the retina. Wet AMD generally progresses faster in patients compared to dry AMD and is the form modelled in this research (Gwairi et al., 2016).

At the cellular level, AMD includes damage to the Retinal Pigment Epithelium (RPE), which is a thin layer of cells located between photoreceptor cells and the Bruch's membrane and serves many important functions in the retina. The RPE provides nutrients to photoreceptor cells and recycles debris in its lysosomes (Ehrlich et al., 2008). As these cells begin to age, the debris which should be recycled and cleaned from the retina by the RPE becomes accumulated in the cells instead, causing damage and oxidative stress (Ao et al., 2018).

Additionally, cells in the RPE perform functions such as protecting other cells in the retina from oxidative damage, ensuring ionic homeostasis, and synthesizing and releasing growth factors (Ao et al. 2018). As the RPE cells age, they are subject to a decrease in melanin production and begin to absorb the debris from incomplete degradation of rod and cone membranes. Some of this debris is lipofuscins and fatty acids, which are exposed to light and therefore more susceptible to the increased production of reactive oxygen species (Ehrlich et al. 2008).

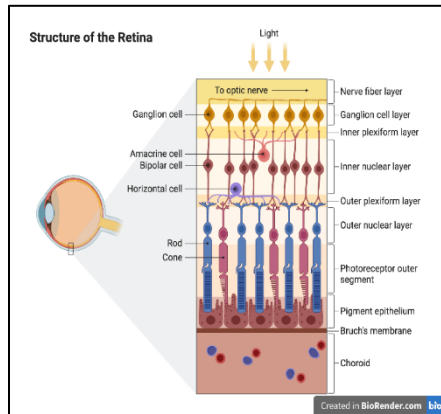


Diagram 2. *The Structure of the retina.* Image adapted from Biorender.com

Photoreceptor cells serve important functions in human vision and are also damaged by AMD. Rod photoreceptor cells allow humans to see at low light levels and have peripheral vision. Cone photoreceptor cells allow humans to see at bright light levels and support color and central vision. The macula, the most afflicted region of the eye by macular degeneration, contains more cone photoreceptor cells than the rest of the retina. This underscores the

importance of preventing and slowing the development of age-related macular degeneration.

Additionally, it brings to light concern regarding the health of the photoreceptor cells and the oxidative stress within the cells and in their microenvironment (Gwairi et al., 2016).

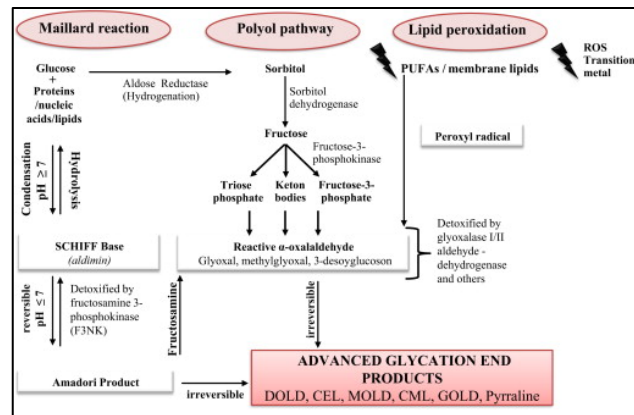
In the later stages of dry AMD, which can take months or years to occur, Geographic Atrophy (GA) can occur in patients. Geographic Atrophy is irreversible and causes a loss of central vision. This leads to a significant reduction in the photoreceptor cells in the macula and an atrophy of RPE cells located within the GA areas. The atrophy can expand over time. Dry AMD is considered to be a risk factor for wet AMD, with 65% of dry AMD patients being susceptible to developing wet AMD (Gwairi et al., 2016).

As a whole, the retina is a very energy-consuming part of the human body and it relies on excessive mitochondria to continue oxidative phosphorylation and aerobic glycolysis (Ozawa 2020). Reactive Oxygen Species (ROS) are unstable molecules created as a result of energy metabolism. ROS molecules have an uneven number of electrons, making them highly reactive and potentially disruptive to other molecules, although some act as signaling molecules. ROS is a normal result of active metabolic processes and can usually be recovered by antioxidants and other repair systems present in the eye. However, when ROS levels exceed the ability of the antioxidants and repair systems, they can create a damaging microenvironment for the retina. (Pole et. al, 2016). In the retina, this is most significant in the RPE and photoreceptor cells.

Many patients with diabetes and diabetic complications are more susceptible to macular degeneration, specifically wet AMD. Hyperglycemia is well known to be associated with increased inflammation and oxidative stress. One of the pathways which leads to increased inflammation and oxidative stress in diabetes and its related complications is the AGE-RAGE signaling pathway.

Diagram 3. The AGE-RAGE Pathway. Image adapted from Ott et al. from “Role of Advanced Glycation End Products in Cellular Signaling.”

The AGE-RAGE signaling pathway consists of advanced glycation end products and their receptor. The pathway results in the non-enzymatic modification of macromolecules in cells such as proteins, lipids, and nucleic



acids. Instead of enzymes, macromolecules are modified by saccharides through a process known as the Maillard Reaction. This process results in the formation of unstable Schiff bases which are precursors of AGEs (Ott et al., 2014). A significant cause of the production of AGEs is oxidative stress. Furthermore, the continued production of AGEs due to positive feedback exacerbates the oxidative stress in a cell.

Other key pathways involved in the production of ROS and inflammation, which facilitate the degeneration of the macula, are signal-transduction pathways such as the $\text{NF-}\kappa\text{B}$, $\text{IL-1}\beta$, and $\text{TNF-}\alpha$ pathways. The $\text{NF-}\kappa\text{B}$ pathway has been shown to be involved in diabetic vascular complications and can be activated by high glucose or Advanced Glycation End Products. An increase in the expression of this pathway leads to proinflammatory processes within the cell. While some of these are critical to regular cell functioning, the increase in expression can lead to negative impacts on cells. In retinal pericyte cells, an increase in expression of the $\text{NF-}\kappa\text{B}$ pathway led to an increase in apoptosis (Romeo et al., 2002).

$\text{TNF-}\alpha$ is another proinflammatory cytokine that can cause harm in the retina when overexpressed. The pathway has been shown to be involved in retinal degeneration, including AMD (Mirshahi et al., 2012). $\text{TNF-}\alpha$ leads to an increase in the VEGF (Vascular Endothelial Growth Factor) within the retina, which can further neovascularization through the creation of irregular retinal blood vessels in the choroid. Pharmaceutical products blocking the increased production of $\text{TNF-}\alpha$ were successful in reducing $\text{TNF-}\alpha$ expression levels, but have been shown to increase intraocular inflammation (Mirshahi et al., 2012). In the diabetic retina, $\text{IL-1}\beta$ is a pro inflammatory cytokine which is also overexpressed and can trigger and amplify inflammation in the cell (Liu et al., 2012). The overexpression of pro-inflammatory pathways in the diabetic retina underscores the importance of finding chemicals which have the potential to reduce oxidative stress in the retinal microenvironment.

Antioxidants are expected to have therapeutic benefits to persons suffering from AMD as they neutralize free radicals, or pro-oxidants, in cells (Jabbehdari & Handa, 2020). Fisetin is an antioxidant found in cucumbers, strawberries, and persimmons. It has been shown to significantly decrease the

number of pro-oxidants and increase the number of antioxidants in D-galactose-induced rat cells, which cause aging (Singh et al. 2018).

Currently, the treatments for macular degeneration include Avastin and Lucentis, though both drugs can have side effects of blindness. Fisetin, as an antioxidant that is found in fruits and vegetables, has the potential to alleviate symptoms in patients with AMD and diabetes by targeting the AGE-RAGE signaling pathway.

There is minimal research which reports the connections between Fisetin, age-related macular degeneration, oxidative stress, and inflammation. Through this *in silico* and *in vitro* interdisciplinary research, these connections were further investigated. Overall, the research question of this study is: What is the potential impact of Fisetin on the photoreceptor cells of the retina in high fructose conditions?

The researcher's hypothesis is that Fisetin will be beneficial in reducing gene expression and protein expression of pro-oxidant and inflammatory molecules in an *in vitro* model of photoreceptor cells within a high fructose microenvironment. This microenvironment is often found in the diabetic patient and can lead to the development of wet macular degeneration.

MATERIALS AND METHODS

In silico Methods

Gene Expression Omnibus (GEO)

Two relevant datasets were identified through the Gene Expression Omnibus (GEO). (1) GSE5258: Connectivity Map Dataset with two PC3 samples treated with Fisetin and two PC3 control samples. (2) GSE103060: Expression of angiogenic and inflammatory factors in CNV-derived RPE contained 8 samples of late-stage choroidal neovascularization (CNV) membrane-derived retinal pigment epithelium (RPE) from 83-year-old males and 84-year-old females, 4 samples of primary human RPE cells, and 4 samples of the ARPE-19 human RPE cell line. The CNV-membrane derived RPE samples represented exudative macular degeneration and inflammation. They were derived through a surgical extraction. The human RPE and ARPE-19 samples were used as controls. Both datasets used expression profiling by array.

Gene Pathway Analysis

The common Differentially Expressed Genes (DEGs) determined from the two datasets (DEG was designated by an adjusted “p” less than 0.05 and absolute value log₂ FC greater than 1) were then exported to Enrichr (version March 29, 2021) and relevant pathway visualizations were produced through Appyter (version BokehJS 2.3.2) and then ranked by combined score. Pathways considered to be statistically significant were those with a combined score of less than 0.05.

Prediction of Protein-Protein Interactions

The common DEGs were entered into String.dB (2021 version) to determine potential protein-protein interactions. Genes (nodes) were represented as circles. Edges, or lines between the genes, indicate interactions between two nodes which can be inferred from previously known data. A broad interaction network was generated from the DEGs and colors were assigned to different pathways to represent the expression of those pathways in the nodes. Direct (physical) and indirect (functional) interactions were obtained and visualized.

In vitro methods

Thawing Cells

A vial of 661w cells were removed from the -150°C freezer and thawed at room temperature for three minutes. The cell suspension was then transferred into a 15 mL centrifuge tube and 8 mL of Dulbecco's modified Eagle medium (DMEM) was added. The tube was centrifuged at 1000 rpm for 8 minutes. Excess media was discarded, and cells were resuspended in 11 mL of DMEM (10569044, ThermoFisher, Waltham, MA) along with 10% fetal bovine serum (97068-085, Avantor, Radnor, PA) and 1% penicillin-streptomycin (15140163, ThermoFisher, Waltham, MA), then transferred to a T75 flask.

Cell Culture

661w photoreceptor cells were cultured DMEM with 10% fetal bovine serum (97068-085, Avantor, Radnor, PA) and 1% penicillin-streptomycin (15140163, ThermoFisher, Waltham, MA). 661w cells were obtained as a gift of their creator Dr. Al-Ubaidi from the Univ. of Houston over 10 years ago. This cell line was created in 2004 in his lab. The cells are considered biosafety level 1 as a result of the mechanism of immortalization. Cells were maintained in T75 flasks and incubated at 37°C, 5% CO₂, and 100% humidity. When cells were observed to be 80% confluent, cells were trypsinized with 0.25% trypsin EDTA (25200114, ThermoFisher, Waltham, MA).

Preparation of Reagents and Treatments

Fisetin (15246, Cayman Chemical Company, Ann Arbor, MI): 661w cells were treated with the following concentrations of Fisetin: 6.25 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM, with DMSO serving as the control. A stock solution was prepared at 10 mM by reconstituting 2.862363 mg in 1 mL of DMSO (100x). Cells treated with solvent (DMSO) were used as a control. All stock solutions were made 100 times more concentrated to achieve the appropriate concentrations in cell culture.

Fructose (1288290, Frey Scientific Inc., Nashua, NH): 661w cells were treated with the following concentrations of Fisetin: 6.25 mM, 12.5 mM, 25 mM, 50 mM, and 100 mM, with sterile water serving as the control. A stock solution was prepared at 10 Molar by reconstituting 1806 mg in 1 mL of sterile water (100x). Concentrations of fructose were sterilized by filtering using a syringe. Cells treated with solvent (sterile water) were used as a control. All stock solutions were made 100 times more concentrated to achieve the appropriate concentrations in cell culture.

Trypan Blue Exclusion Viability Assay

Cell viability was determined through the trypan blue exclusion using a Vi-CELL XR Viability Analyzer (Beckman Coulter, Brea, CA).

MTS Assay for Cell Proliferation

Cells were plated at 0.15×10^6 viable cells per mL in a 96 well cell culture plate. After 24 hours, cells were treated with pre-prepared Fisetin concentrations ranging from 0 μ M (DMSO) - 100 μ M or pre-prepared Fructose concentrations ranging from 0 mM (sterile water) – 100 mM. These concentrations refer to the final concentrations that the cells were exposed to. After an additional 48 hours, MTS based CellTiter® 96 Aqueous One Solution Reagent (G3582, Promega, Madison, WI) was added at 15 μ L per well and incubated for 45 minutes. Proliferation was then measured in each well using ELx808 Biotek at 490 nm (BioTek, Winooski, VT).

Preparation of Lysates

5 mL of 661w cells were transferred to T25 flasks with 0.2×10^6 viable cells/ mL and incubated for 24 hours. After incubation, old media was replaced with 3 mL of new DMEM media and 10 μ L of a 100 mM concentration of Fructose was administered in each flask along with 10 μ L of concentrations of Fisetin ranging from 0 μ M (DMSO) – 100 μ M. Cells were incubated for an additional 48 hours. During the lysing procedure, phosphate buffer saline (PBS) (10010023, Gibco, Waltham, Massachusetts, USA), 1X lysis buffer (9803S, Cell Signaling Technology, Danvers, MA, USA), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were kept on ice. Media (supernatant) was poured into 15 mL centrifuge tubes. Cells were trypsinized then centrifuged at 1200 rpm for 7 minutes to form a pellet. Cell pellets were resuspended with 1 mL of PBS, centrifuged in a microcentrifuge tube, and subsequently centrifuged again at 1000 rpm for 7 minutes. PBS was poured out and 1 mL of lysis buffer (containing 1% protease inhibitor cocktail) was added at a concentration of 1×10^6 cells/mL. Microcentrifuge tubes were placed on ice for ten minutes. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. Clear supernatant was decanted into microcentrifuge tubes which were chilled on ice. Lysates and supernatants obtained were stored in the -80°C until ready for use.

Indirect Enzyme-Linked Immunosorbent Assays (ELISAs)

Indirect ELISAs were used to determine the secretion of AGE-RAGE, VEGF, IL-1 β , and TNF- α . Antibodies of these proteins were used (1:1000). Lysates and supernatants followed the same ELISA protocol. Lysates and supernatants were thawed at room temperature. Lysates or supernatants were plated at 100 microliters per well in a 96 well ELISA plate and sealed with a plastic film. After 2 to 3 hours, liquid in the plate was decanted and 300 μ L of BSA Blocking Buffer was administered to each well for 10 min. BSA Buffer was decanted and 100 μ L of the primary antibody was added in each well for one hour and incubated at room temperature. The primary antibodies used in this research were VEGF (ab9570,

Cambridge, UK), IL-1 β (ab2105, Cambridge, UK), TNF- α (P300A, ThermoFisher, Waltham, MA), and AGE-RAGE (AB9890, MilliporeSigma, Burlington, MA). Primary antibody was removed, plates were washed 3 times with a wash buffer, and 100 μ L of Goat AntiRabbit Secondary Antibody (31460, Invitrogen) was added in each well for one hour and the plate was incubated at room temperature. After washing with a wash buffer, 100 μ L ABTS Substrate Solution (KPL) was added to each well for ten minutes. The plate was read at 405 nm in the ELx808Biotek.

ROS-GloTM H2O2 Assay

Assay was performed on living cells. ROS production was analyzed using a ROS-Glo luminescence assay (G8820, Promega, Madison, WI). Cell count was determined through the Trypan Blue Exclusion Viability Assay. Cells were plated at 20,000 cells per well in 60 μ L of Dulbecco's modified Eagle medium (DMEM) and incubated for 24 hours at 37°C in a CO2 incubator. After 1 hour of incubation, 10 μ L of a pre-prepared 100 mM concentration of Fructose was added to each well along with 10 μ L of pre-prepared Fisetin concentrations ranging from 0 μ M (DMSO) - 100 μ M. After 18 hours of incubation in treatments, H2O2 Substrate Dilution Buffer was placed on ice to chill. H2O2 substrate was diluted from 10 mM to 125 μ M in the H2O2 Substrate Dilution Buffer. 1.0 mL of H2O2 Substrate dilution buffer was prepared with 12.5 μ L of Substrate solution. 20 μ L of this solution was added to each well to achieve a total well volume of 100 μ L and substrate concentration of 25 μ M in each well. Cells were incubated at 37°C in a CO2 incubator while the substrate solution was administered for 6 hours. 100 μ L of ROS-GloTM Detection Solution was added to each well. The plate was incubated for 20 minutes at room temperature, and the luminescence was measured using a plate reader (SynergyHT, BioTek).

Figures

Diagrams created by the author were created using BioRender unless otherwise noted. Column graphs were created using Excel. Figures such as Volcano plots and Enrichr diagrams were created using Appyter.

Statistical Significance

Adjusted p value of <0.05.

Biological Relevance

Log fold change values >1 or <-1 signified biological relevance. A positive log fold change value above 1 denoted upregulated genes and a negative log fold change value below -1 denoted downregulated genes.

Statistical Analysis

Assays were repeated two or more times and “n” is greater than or equal to 5. Data analysis was performed by the author and graphs were created using Microsoft Excel.

RESULTS AND DISCUSSION

I. Differentially Expressed Genes (DEGs) from Gene Expression Omnibus (GEO) datasets

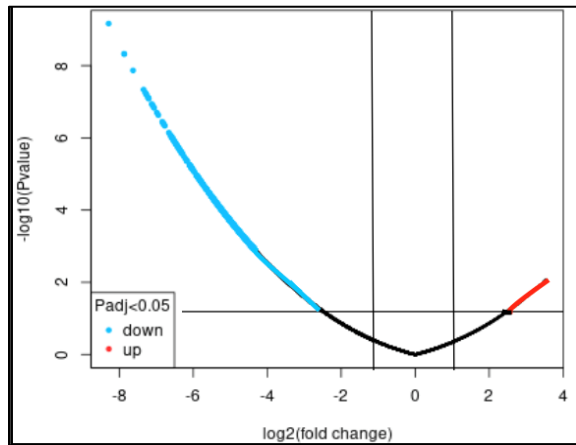


Figure 1. Differentially Expressed Genes (DEGs) in GSE5258 (Fisetin vs Control): Connectivity Map Dataset. This dataset compared 2 untreated, control samples of PC3 cells to 2 samples of Fisetin-treated PC3 cells. Each point on the plot represents a DEG which was either upregulated or downregulated after a treatment of Fisetin. Statistically significant genes were those with a logFC value either greater than 1 or less than -1, and adj. P value less than 0.05. Red genes represent DEGs which are upregulated based on a logFC value greater than 1. Blue genes represent DEGs downregulated based on a logFC value less than -1. Black genes are those with no significant difference in the Fisetin treatment versus the control. Data suggests there are more downregulated genes than upregulated genes in the Fisetin-treated samples.

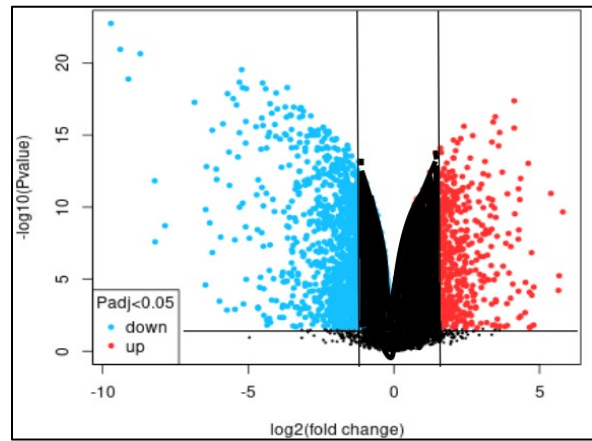


Figure 2. Differentially Expressed Genes (DEGs) in GSE103060 (CNV vs Control): Expression of Angiogenic and Inflammatory Factors in CNV-Derived RPE. This dataset compared 8 samples of CNV-derived RPE cells from 83- and 84-year-old males and females to ARPE and HRPE cell samples, which served as controls. Each point in the plot represents a DEG which is either downregulated or upregulated in CNV-derived RPE cells in patients with exudative macular degeneration. Choroidal Neovascularization is the formation of new blood vessels that break through the Bruch's membrane in patients with exudative macular degeneration and cause vision loss. Statistically significant genes were those with a logFC value either greater than 1 or less than -1 and an adj. P value less than 0.05. Red genes represent DEGs which are upregulated based on a logFC value greater than 1. Blue genes represent DEGs which are downregulated based on a logFC value less than -1. Black genes are those with no significant difference between the CNV patients and the control.

II. Common Differentially Expressed Genes between Patients with Macular Degeneration and Fisetin-treated Datasets

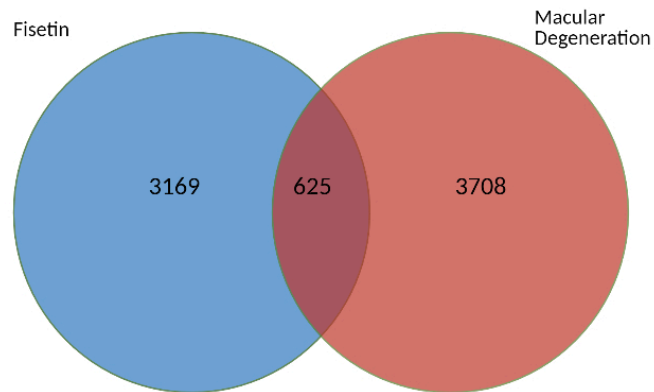


Figure 3. Venn Diagram of Downregulated DEGs in Fisetin-treated and Upregulated DEGs in AMD. Figure 3 represents a Venn diagram illustrating the overlap of downregulated genes in GSE5258 (Fisetin treated) and upregulated genes in GSE103060 (patients with exudative macular degeneration). There were 3169 genes differentially expressed and downregulated in Fisetin-treated PC3. There were 3708 genes differentially expressed and upregulated in CNV-derived RPE cells with macular degeneration. The Venn diagram of these resulted in 625 common DEGs. Figure created by author using Biorender.com.

III. Common Differentially Expressed Genes (DEGs) Demonstrate Enriched Pathways and Protein-Protein Interactions

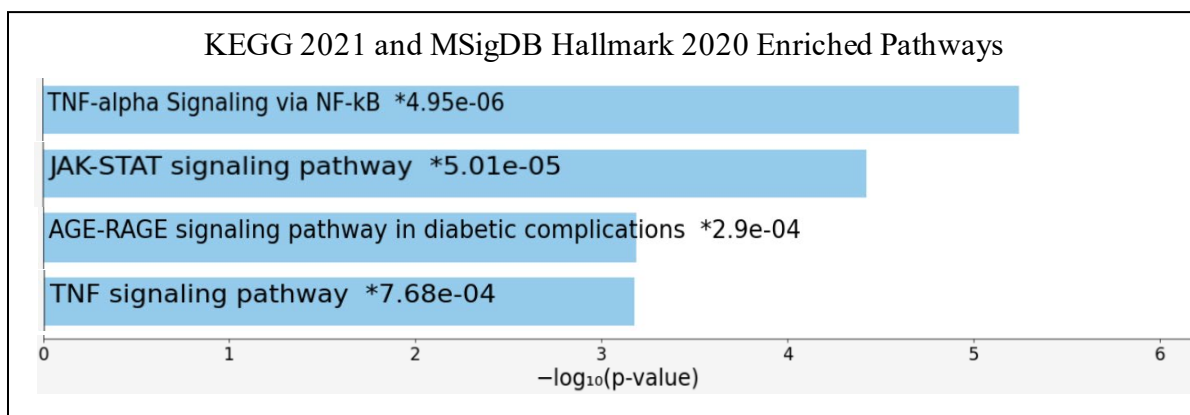


Figure 4. Enriched Pathways in the Common Differentially Expressed Genes (DEGs). Enrichr is an online tool which performs enrichment analysis by inferring knowledge about the genes in the entered set of genes by comparing it to previous gene sets, which consist of genes with similar function with prior biological knowledge. Each of the prior gene sets is denoted by an enriched term. Genes from the gene set shown above were derived from KEGG 2021 and MSigDB Hallmark 2020. Enrichment analysis was performed on the DEGs which were downregulated in Fisetin-treated and upregulated in patients with Macular Degeneration. The chosen enriched pathways were TNF- α signaling via NF- κ B, JAK-STAT signaling pathway, AGE-RAGE signaling pathway in diabetic complications and the TNF- α signaling pathway. Each chosen pathway was statistically significant.

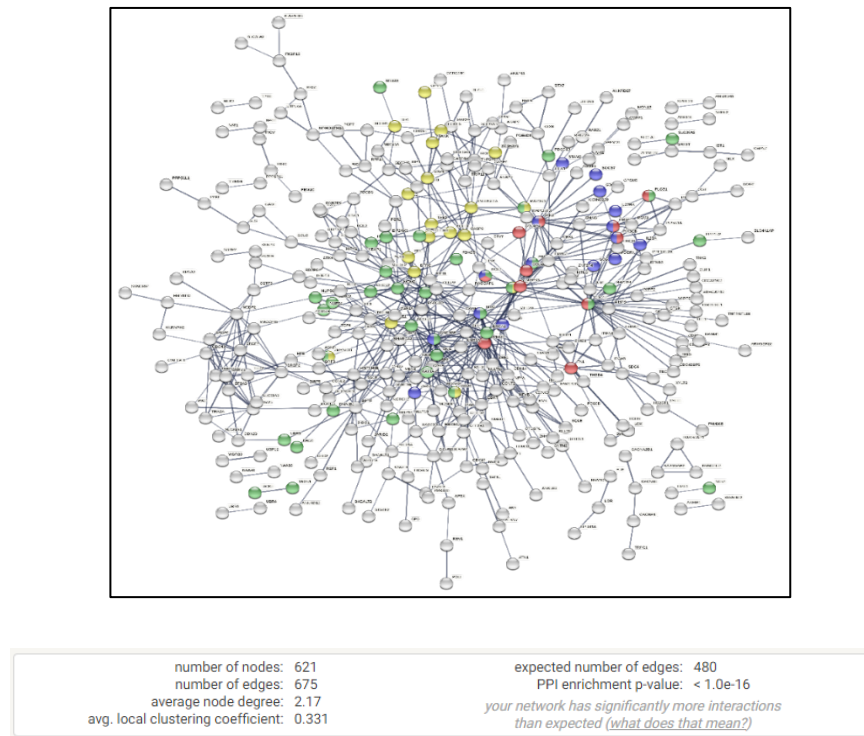


Figure 5. Protein-Protein Interactions in the Common Differentially Expressed Genes (DEGs). Using the online program String.dB, a broad interaction network was generated from the DEGs downregulated in GSE5258 (Fisetin treated) and upregulated in GSE103060 (patients with exudative macular degeneration). Genes are represented as circles, known as nodes. Edges, or lines between the genes, indicate interactions between two nodes which can be inferred from previously known data. Genes entered into String.dB generated 646 nodes and 436 edges. Within the network, colors were assigned to different genes to represent the expression of those genes. Blue nodes represent the JAK-STAT pathway. Red nodes represent the AGE-RAGE signaling pathway in diabetic complications. Green nodes represent the regulation of NF- κ B signaling. Yellow nodes represent the regulation of response to stress. Nodes shaded in more than one color represent genes which are involved in more than one of the above processes or pathways. The data suggests that there are common genes which are shared between two or more of the aforementioned pathways.

IV. The Relationship between Cell Proliferation and Treatments of Fructose and Fisetin *in vitro*

The preamble part of the *in vitro* research investigation confirms the proliferation and survival of cells under fructose treatments. Survival of the 661w photoreceptor cells under high fructose levels is critical to the diabetic microenvironment and macular degeneration model tested *in vitro*.

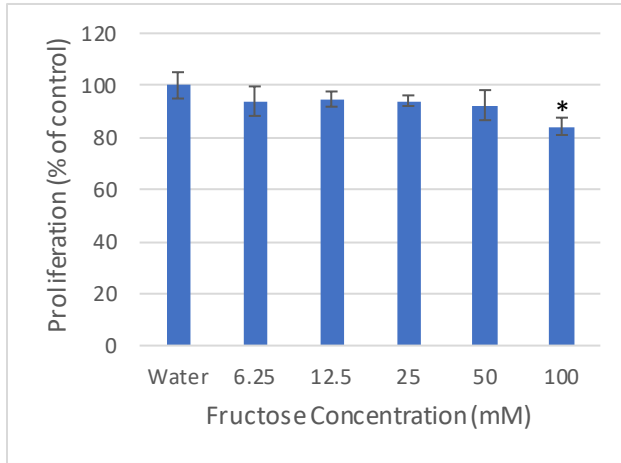


Figure 6. Effects of Fructose Concentrations on Cell Proliferation. 661w photoreceptor cells were grown in media and plated. Cells were treated with fructose concentrations ranging from 6.25 mM – 100 mM with sterile water used as a control. Cell proliferation was measured using an MTS assay. The data suggests that there is a negative correlation between increased concentrations of fructose and cell proliferation. Bars are means $\pm$ STDEV (n=5). Asterisks denote * $p < 0.05$.

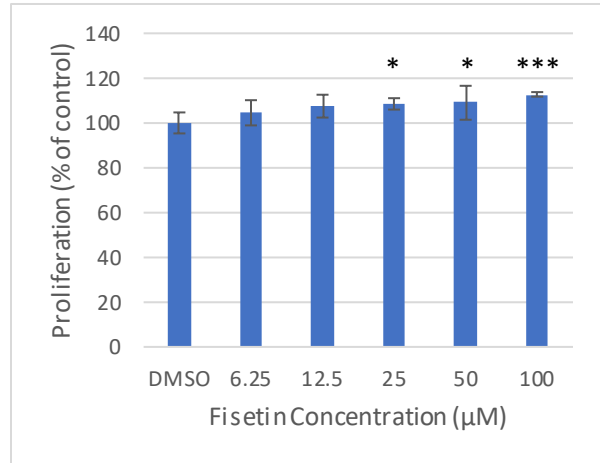


Figure 7. Effects of Fisetin Concentrations on Cell Proliferation. 661w photoreceptor cells were grown in media and plated. Cells were treated with Fisetin concentrations ranging from 6.25 μM – 100 μM with DMSO used as a control. Cell proliferation was measured using an MTS assay. The data suggests that there is a positive correlation between increased concentrations of Fisetin and cell proliferation. Bars are means $\pm$ STDEV (n=5). Asterisks denote * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The data suggests the 100 mM fructose concentration led to lower cell proliferation and proliferation and was significant based on a p value < 0.05 (Figure 6). The 100 mM fructose concentration significantly affected the proliferation of 661w cells, as would be suspected in creating a model of macular degeneration, so this concentration was chosen for later experiments. Next, 661w cells were treated with Fisetin to study whether Fisetin, a known antioxidant, could lead to higher cell proliferation

values. Fisetin treatment resulted in an increase in proliferation at the highest concentrations, 25 μM to 100 μM , as shown in Figure 7.

V. The Relationship between Combination Treatments of Fructose and Fisetin on VEGF secretion in an in vitro model of Macular Degeneration

The cell proliferation results suggested a negative relationship between cell proliferation and higher concentrations of fructose, so the highest concentration of fructose was chosen to best model cells which were in a diabetic microenvironment modelling the pathology of macular degeneration. Cells were placed into T25 flasks for 24 hours and then each flask was treated with the 100 mM concentration of Fructose and a concentration between 6.25 – 100 μM of Fisetin, with DMSO serving as the control. Cell culture supernatants as well as cell culture lysates were used for indirect ELISAs, each calculated as “% of control.” Supernatant was used to measure the VEGF secretion of the 661w cells. The results are shown below in Figure 8.

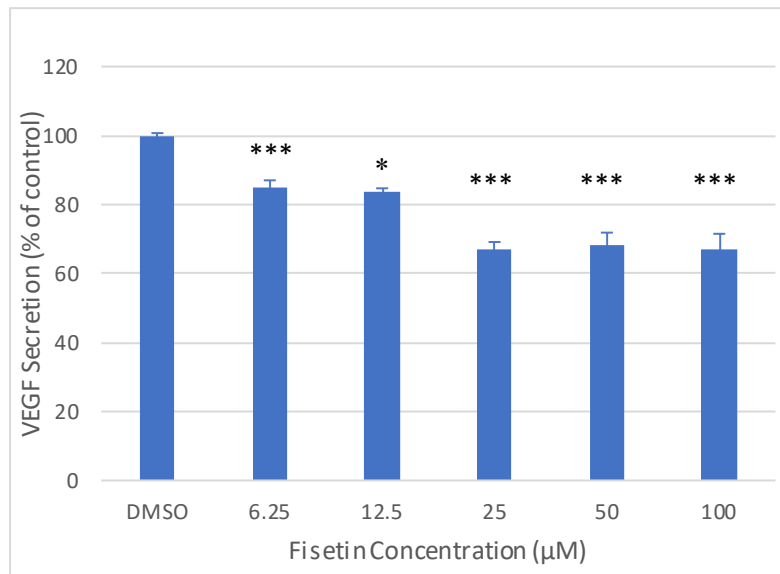


Figure 8. Effects of Fisetin on VEGF Secretion. Vascular Endothelial Growth Factor (VEGF) protein secretion was measured through ELISA using the supernatant of 661w cells which were treated with 100 mM of fructose and a treatment of Fisetin concentrations ranging from 0 μM (DMSO) – 100 μM . The data suggests higher concentrations of Fisetin led to a decrease in the secretion of Vascular Endothelial Growth Factor in 661w cells. This suggests a negative relationship between concentrations of Fisetin and VEGF secretion. Bars are means $\pm$ STDEV (n=5). Asterisks represent * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

VEGF secretion in 661w cells was measured since VEGF is an angiogenic protein strongly correlated to AMD by promoting endothelial cell proliferation. Angiogenesis refers to the formation of new blood vessels, which occurs in the choroid of the eye. In the diabetic eye, angiogenesis is a key factor in neovascular age-related macular degeneration (wet AMD) (Bressler, 2009). The highest levels of secretion of VEGF were demonstrated in the 661w cells which were treated with 100 mM of fructose and DMSO, which served as the control. However, in the cells supplemented with the Fisetin treatment, VEGF secretion significantly decreased, with a p value less than 0.001 for the 6.25 μ M, 25 μ M, 50 μ M, and 100 μ M concentrations. This suggests a decrease in angiogenic conditions in the retinal microenvironment as seen in Figure 8.

VI. The Relationship between Combination Treatments of Fructose and Fisetin, IL-1 and TNF- α secretion in vitro

Increased inflammatory cytokine secretion is associated with increased levels of oxidative stress and inflammation. The treatment of Fisetin concentrations between 6.25 μ M and 100 μ M, with DMSO as a control, suggest that Fisetin has the potential to decrease inflammatory cytokine secretion in 661w cells. The cytokines TNF- α and IL-1 β were tested as these cytokines are part of the NF- κ B and TNF- α pathways which were found to be statistically significant in the *in silico* Enrichr and String.dB analyses of the common DEGs. The results of the TNF- α and IL-1 β indirect ELISAs are shown below in Figures 9 and 10 respectively.

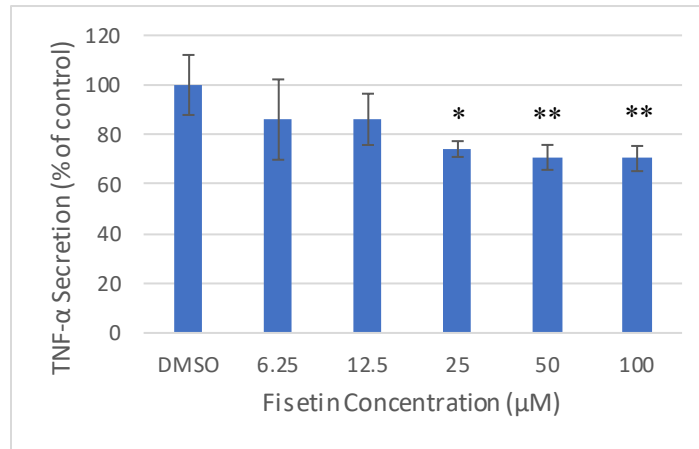


Figure 9. Effects of Fisetin on TNF- α Secretion in vitro. Tumor Necrosis Factor Alpha (TNF- α) protein secretion was measured through ELISA using the supernatant of 661w cells which were treated with 100 mM of fructose and a treatment of Fisetin concentrations ranging from 0 μ M (DMSO)– 100 μ M. The data suggests higher concentrations of Fisetin led to a decrease in the secretion of Tumor Necrosis Factor Alpha in 661w cells. This suggests a negative relationship between concentrations of Fisetin and TNF- α secretion. Bars are means $\pm$ STDEV (n=5). Asterisks represent * p<0.05, ** p<0.01, *** p<0.001.

TNF- α and IL-1 β are inflammatory cytokines that play a role furthering inflammation in the retina. TNF- α , as an inflammatory mediator, can stimulate Retinal Pigment Epithelial cells (RPE) to secrete more VEGF and other interleukins, including IL-4, IL-8, and IL-10 (Tan et al., 2020). This further promotes neovascularization in the retina. Figures 8 and 9 suggest a decrease in TNF- α coupled with a decrease in VEGF. In the eye, the Retinal Pigment Epithelial (RPE) cells serve a key role in providing nutrients to photoreceptor cells, suggesting that the damage of RPE cells caused by the overexpression of these pathways in AMD would lead to lower photoreceptor cell survival.

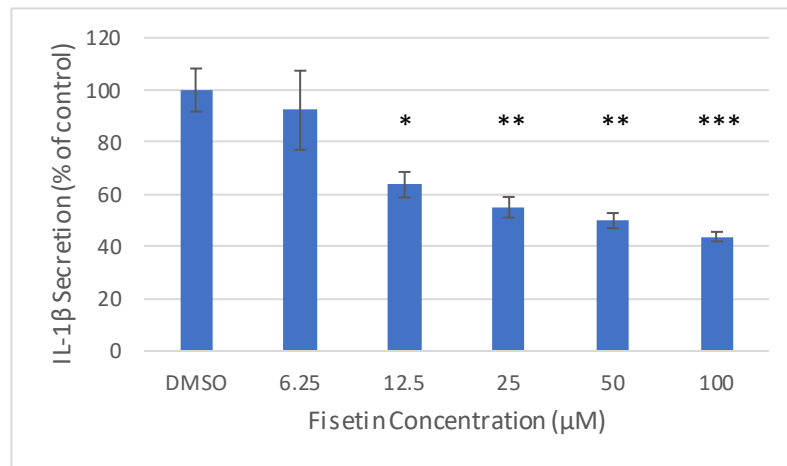


Figure 10. Effects of Fisetin on IL-1 β Secretion in vitro. Interleukin 1 (IL-1 β) protein secretion was measured through ELISA using the supernatant of 661w cells which were treated with 100 mM of fructose and a treatment of Fisetin concentrations ranging from 0 μ M (DMSO) – 100 μ M. The data suggests higher concentrations of Fisetin led to a decrease in the secretion of Interleukin-1 β in 661w cells. This suggests a negative relationship between concentrations of Fisetin and IL-1 β secretion. Bars are means $\pm$ STDEV (n=5). Asterisks represent * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IL-1 β is another inflammatory mediator which has been shown in the literature to increase under high glucose concentrations. Activated through the NF- κ B pathway, IL-1 β has been shown to increase inflammation in the diabetic eye (Kowluru and Odenbach, 2004). The increased secretion of IL-1 β in RPE cells can stimulate the release of VEGF and other inflammatory cytokines (Tan et al., 2020). Figures 8 and 10 suggest a decrease in IL-1 β coupled with a decrease in VEGF.

Figures 9 and 10 suggest Fisetin has a positive impact on 661w photoreceptor cells under high fructose conditions through a reduction of TNF- α and IL-1 β secretion compared to the control. Data suggests that a 100 μ M concentration of Fisetin, which was statistically significant in both figures, is most effective at decreasing secretion compared to the control.

VII. The Relationship between Combination Treatments of Fructose and Fisetin on Reactive Oxygen Species (ROS) and the AGE-RAGE pathway in the in vitro model of the Diabetic Eye

Reactive Oxygen Species (ROS) are unstable, highly reactive molecules that can create a damaging environment in cells when present in excessive levels (Pole et al., 2016). Under homeostatic conditions, they serve important functions in cell signaling and redox regulation. ROS is present at high levels in the retina as a by-product of metabolic processes.

An ROS assay was conducted to measure the presence of ROS in 661w cells. It was suspected that the antioxidant Fisetin would decrease ROS levels in 661w photoreceptor cells compared to the control. The results of the ROS assay are shown below in Figure 11.

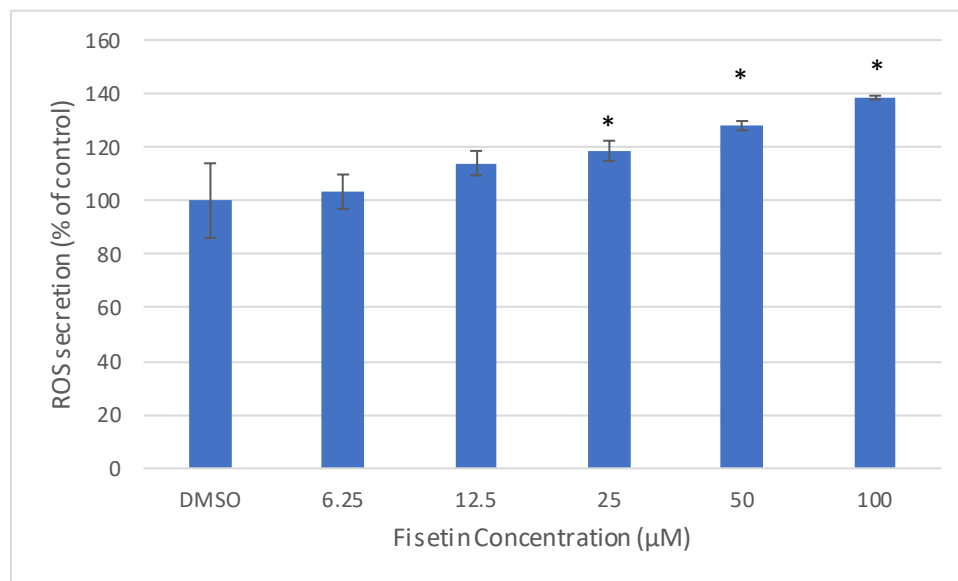


Figure 11. Effects of Fisetin on H₂O₂ Reactive Oxygen Species (ROS) secretion. Reactive Oxygen Species production was measured through assay. 661w cells were plated at 20,000 cells per well in a 96-well plate in 60 μL of DMEM medium. Cells were treated with a 100 mM concentration of fructose and a concentration of Fisetin ranging from 6.25 μM to 100 μM with DMSO serving as the control. The plate was incubated for 18 hours at 37 °C in 5% CO₂ with the concentrations administered, then 20 μL H₂O₂ Substrate Dilution Buffer (125 μM H₂O₂) was added. After two hours of room-temperature incubation, 100 μL of ROS-GLo™ Detection Solution was added to each well. The plate was incubated at room temperature for 20 minutes and then the luminescence of each well was measured. The data suggests higher concentrations of Fisetin led to an increase in the secretion of ROS in 661w cells compared to the control. This suggests a negative relationship between concentrations of Fisetin and ROS secretion. Bars are means $\pm$ STDEV (n=5). Asterisks represent * p<0.05.

Advanced Glycation End-Products (AGEs) and the Receptor for Advanced Glycation End Products (RAGE) are part of the AGE-RAGE pathway in diabetic complications. The pathway results in the non-enzymatic modification of key macromolecules in cells such as proteins, lipids, and nucleic acids. This modification is known as the Maillard Reaction. It results in the formation of unstable Schiff bases which are precursors of AGEs. A significant cause of the production of AGEs is oxidative stress (Ott et al., 2014). Furthermore, the continued production of AGEs due to a positive feedback loop with RAGE and ROS exacerbates the oxidative stress in a cell. In the literature, Fisetin has not been used as a potential treatment to reduce the formation of Advanced Glycation End Products in a murine cell line. The results of the AGE-RAGE Indirect ELISA are shown below in Figure 11 and warrant further research.

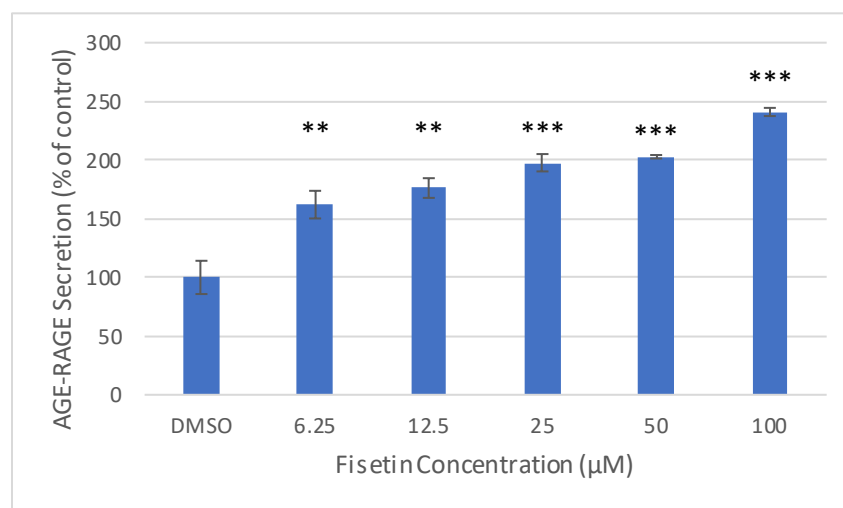


Figure 12. Effects of Fisetin on Advanced Glycation End Products (AGE) secretion. Advanced Glycation End Products (AGE) protein expression was measured through ELISA using the lysates of 661w cells which were treated with 100 mM of fructose and a treatment of Fisetin concentrations ranging from 0 μM (DMSO)– 100 μM (lysed at 1×10^6 cells/mL). The data suggests concentrations of Fisetin led to an increase in the secretion of AGEs in 661w cells compared to the control. This suggests a negative relationship between concentrations of Fisetin and AGE secretion. Bars are means $\pm$ STDEV (n=5). Asterisks represent * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Overall, *in silico* results suggest an upregulation of the TNF- α , IL-1 β , VEGF, and AGE-RAGE pathways in patients with macular degeneration, and a downregulation of those pathways in Fisetin-treated samples. In the *in vitro* results, 661w cells suggested a decrease of TNF- α , IL-1 β , and VEGF under high fructose conditions and increasing concentrations of Fisetin, while AGE-RAGE and ROS expression within the cells increased.

CONCLUSION

Experiment	Assay	Significant Results	Proposed Implications of Treatment
Cell Proliferation (Fructose)	MTS Assay	↓ (100 mM)	High cellular levels of fructose resulted in decreased proliferation.
Cell Proliferation (Fisetin)	MTS Assay	↑ (25 μ M – 100 μ M)	High cellular levels of Fisetin resulted in increased proliferation.
Vascular Endothelial Growth Factor (VEGF)	ELISA	↓ (6.25 μ M – 100 μ M)	A decrease in angiogenesis in the retinal microenvironment.
IL-1 β (Interleukin – 1 Beta)	ELISA	↓ (25 μ M – 100 μ M)	A decrease in IL1 β secretion and inflammation.
TNF- α (Tumor Necrosis Factor Alpha)	ELISA	↓ (12.5 μ M – 100 μ M)	A decrease in TNF- α secretion and inflammation.
AGE-RAGE pathway	ELISA	↑ (6.25 μ M – 100 μ M)	An increase in AGEs production in cells.
ROS	Assay	↑ (25 μ M – 100 μ M)	An increase in oxidative stress in the retinal microenvironment.

Table 1: A summary of in vitro results in this research paper. Significant results refer to treatments which were statistically significant.

Age-related macular degeneration is a disease with the potential to significantly affect patients' lives. Fisetin treatments at specific concentrations suggested a statistically significant decrease in the

secretion of key proinflammatory pathways such as TNF- α , IL-1 β , and VEGF. However, the AGE-RAGE pathway, which was suggested to be downregulated in the *in silico* data from the GEO datasets, increased expression in the *in vitro* results as concentrations of Fisetin treatments increased. ROS followed a similar downwards trend and increased in expression as concentrations increased *in vitro* compared to the control.

The discrepancy between the *in silico* and *in vitro* results, based on the opposite effects on the AGE-RAGE pathway in the 661w photoreceptor cells, could suggest a connection to an idea discussed in the literature which is known as “antioxidative stress.” Poljsak et al. claim that while antioxidants can be beneficial to cells in lower concentrations, higher concentrations of antioxidants can result in harmful effects for cells, since antioxidants cannot differentiate between harmful oxidative stress and oxidative stress which is key to the homeostatic functioning of the cell. TNF- α , IL-1 β , and VEGF are all key pathways associated with regular cellular functioning and can be helpful to the cell in lower levels of secretion. However, in a disease such as AMD, these pathways are overexpressed at levels which can be dangerous to the cell, and this research suggests that Fisetin can be used as a treatment to alleviate this overexpression in 661w photoreceptor cells with high fructose microenvironments.

In the GSE5258 dataset which provided the gene expression for control and Fisetin treated samples, PC3 cell samples were treated with 10 μ M of Fisetin. This is higher than just one of the concentrations which were used to treat 661w cells in the *in vitro* part of this study. The ELISA results of 6.25 μ M concentration of Fisetin used in this research study suggests no statistically significant difference in expression of the AGE-RAGE pathway compared to the control. However, the treatment of PC3 cells with 10 μ M concentration of Fisetin used *in silico* suggests a statistically significant reduction in the expression of the AGE-RAGE pathway. While the increased *in vitro* expression of AGE-RAGE in higher concentrations of Fisetin warrants further study, it is possible that the *in silico* data reflects lower concentrations of Fisetin. The concentrations above 10 μ M *in vitro* could have resulted in “antioxidative stress” in cells which were already treated with high fructose concentrations. Discrepancies could also arise from the different cell types which were used, underscoring the difference in cellular environments between PC3 (human prostate cancer cells) and 661w (murine photoreceptor cells). Additionally, inconsistencies in results could be attributed to the fact that PC3 cells used in the GSE5258 dataset were not supplemented with any other chemicals, while the 661w cells used in the *in vitro* part of this study were also treated with 100 mM of fructose.

The simultaneous decrease in secretion of TNF- α , IL-1 β , and VEGF and increase in expression of AGE-RAGE and ROS could be explained through separate subsections within the AGE-RAGE pathway.

The following pathway diagram, shown in diagram 4, illustrates the AGE-RAGE signaling pathway in diabetic complications and suggests how each section of the pathways could be connected to the AGE-RAGE pathway in 661w photoreceptor cells with some pathway modulation downstream of RAGE activation and just upstream of inflammation.

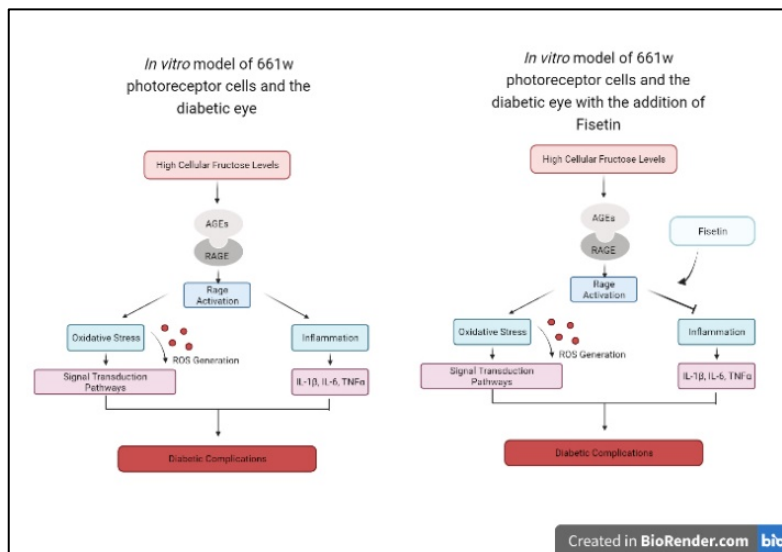


Diagram 4. A Chart of the AGE-RAGE Pathway in 661w photoreceptor cells. Created by author in Biorender.com

The flow chart shown in diagram 10 suggests two mechanisms which AGE-RAGE could work through: inflammation and oxidative stress. An increase in oxidative stress and inflammation in cells suggests an exacerbation of diabetic complications. In the *in vitro*

research conducted, an increase in AGE-RAGE expression as a result of increasing concentrations of Fisetin could have affected the oxidative stress part of the pathway, meaning an increase in ROS levels. However, it suggests that this increase in the expression of the AGE-RAGE pathway did not result in a similar increase in the inflammation portion of the pathway suggesting unique downstream modulation of RAGE activation. In fact, the molecules under inflammation were shown to reduce *in vitro* with the addition of increasing concentrations of Fisetin. This is shown by Diagram 4. Thus, these results warrant further investigation.

It is important to note that a reduction in the pro-inflammatory pathways discussed in this paper, TNF- α , IL-1 β , and VEGF, is not always good for cells as these molecules may impart normal function and intercellular and intracellular signaling. Thus, the statistically significant reduction of these pathways at higher concentrations of Fisetin, such as 100 μ M, warrants future research into the cellular effects of this phenomenon. While increased TNF- α and IL-1 β secretion can suggest increases in the ROS levels in cells, this ROS can be beneficial to cells. ROS levels in cells help in cell growth and migration, modulation of intracellular modulation, activating transcription factors and cellular pathways such as the ones studied in this paper, and activating protein kinases. Therefore, it is important to continue studying the treatment of Fisetin in 661w cells which serve as an *in vitro* model of the photoreceptor's involvement in macular degeneration in high fructose environments.

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