

Eastern Kentucky University

Encompass

Honors Theses

Student Scholarship

Fall 11-10-2022

Examining the Use of Naturally Occurring Free Radical Scavengers Against UV Light Damage in Cancer Prevention

Tanner Justice

Eastern Kentucky University, tanner_justice20@mymail.eku.edu

Follow this and additional works at: https://encompass.eku.edu/honors_theses

Recommended Citation

Justice, Tanner, "Examining the Use of Naturally Occurring Free Radical Scavengers Against UV Light Damage in Cancer Prevention" (2022). *Honors Theses*. 938.

https://encompass.eku.edu/honors_theses/938

This Open Access Thesis is brought to you for free and open access by the Student Scholarship at Encompass. It has been accepted for inclusion in Honors Theses by an authorized administrator of Encompass. For more information, please contact Linda.Sizemore@eku.edu.

EASTERN KENTUCKY UNIVERSITY

Examining the Use of Naturally Occurring Free Radical Scavengers
Against UV Light Damage in Cancer Prevention

Honors Thesis

Submitted

in Partial Fulfillment

of the

Requirements of HON 420

Fall 2022

By

Tanner Justice

Mentor

Associate Professor Dr. Oliver Oakley

Department of Biological Sciences

Examining the Use of Naturally Occurring Free Radical Scavengers
Against UV Light Damage in Cancer Prevention

Tanner Justice

Professor Oliver Oakley, Department of Biological Science

I. Abstract

A strong association has been found between mutated p53 tumor suppressor genes and cancers in humans. Cancers arise from a culmination of genetic and environmental factors, including exposure to ultraviolet (UV) light from the sun. Prolonged exposure to UV has proven negative effects on cell homeostasis. Much of the damage caused by UV light exposure is through generation of free radicals. Zeaxanthin and melatonin are known antioxidant/free radical scavengers which protect cells by neutralizing damage causing free radical molecules. This study was conducted to determine the effectiveness of zeaxanthin and melatonin in preventing the negative effects of UV light on cell viability and cell cycle control. The analyzed parameters of cell health were cell survival and cell cycle staging using flow cytometry. UV light treatments had a negative effect on the cell viability and showed increased numbers of cells in cell cycle arrest. Both treatments with zeaxanthin resulted in increased cell replication and increased cell cycle arrest. However, the control group were the only cells that survived the treatment and all zeaxanthin treated cells died. These preliminary results indicate that zeaxanthin did not provide protection from the UV light damage since there was an increase in cell death cell cycle arrest. The results of the melatonin experiments showed that at the concentrations used no effect was evident. The effects of zeaxanthin and melatonin on preventing free radical damage needs further investigation, as current literature indicates

that treatments using free radical scavenging products reduce potential tumorigenic damage from UV light.

Keywords and Phrases: Cancer; P53; UV light; Free radical scavengers; Zeaxanthin; cell cycle arrest

Table of Contents

I. Abstract..... ii

II. Introduction1

III. Literature Review.....3

IV. Methods and Materials12

 a. Background Information..... 12

 b. Experimental Procedure..... 12

 c. UV Light Treatment..... 14

 d. Zeaxanthin Treatment 14

 e. Melatonin Treatment..... 15

 f. Flow Cytometry used as Analysis Tool 16

 g. P53 ELISA kit used as Analysis Tool..... 16

 h. Cell Cycle test kit used as Analysis Tool..... 17

V. Results19

VI. Discussion.....30

VII. Conclusion.....32

VIII. Limitations32

IX. References.....36

List of Figures

Figure 1. Flow cytometry data from the control set of cells from experiment 1. 21

Figure 2. Flow cytometry data from the UV light treatment set of cells from experiment 1..... 22

Figure 3. Histogram shows the flow cytometry data from the no UV EtOH treatment set of cells from experiment 4. 23

Figure 4. Histogram shows the flow cytometry data from the UV EtOH treatment set of cells from experiment 4 24

Figure 5. Histogram shows the flow cytometry data from the no UV low zeaxanthin treatment set of cells from experiment 4..... 25

Figure 6. Histogram shows the flow cytometry data from the UV low zeaxanthin treatment set of cells from experiment 4..... 26

Figure 7. Histogram shows the flow cytometry data from the no UV high zeaxanthin treatment set of cells from experiment 4..... 27

Figure 8. Histogram shows the flow cytometry data from the UV high zeaxanthin treatment set of cells from experiment 4..... 28

Figure 9. Bar graph of data from no UV vs. UV, each with the groups of control, EtOH, low zeaxanthin, and high treatment groups 29

List of Illustrations

Illustration 1. Image of control group cells viewed at 40X which shows healthy cells covering most of the area being viewed 19

Illustration 2. UV light treated cells viewed at 40X shows many dead cells with some that are alive 20

List of Tables

Table 1. Treatment groups from experiment 1 17

Table 2. Table of treatment groups from experiments 2 and 3 17

Table 3. Table of treatment groups from experiment 4 18

Table 4. Table of treatment groups from experiments 5 and 6. The cell plates were divided into 24 categories as seen below. 18

Table 5. Results from a no UV vs UV experiment that did not include zeaxanthin treatments. Cell number, G0/G1%, S%, and G2/M% data are presented. 22

Table 6. Results from a no UV vs UV experiment that included EtOH. Cell number, G0/G1%, S%, G2/M%, %<G1, and %>G2 data are presented. 24

Table 7. Table of results from a no UV vs UV experiment that included low concentration of zeaxanthin treatments. Cell number, G0/G1%, S%, G2/M%, %<G1, and %>G2 data are presented. 26

Table 8. Shows the results from a no UV vs UV experiment that included high concentration of zeaxanthin treatments. Cell number, G0/G1%, S%, G2/M%, %<G1, and %>G2 data are presented. 28

Table 9. Shows the cell cycle results from the no UV experiment that included control, low concentration treatment of melatonin, and high concentration treatment of melatonin. Cell number percent in the cell cycle analysis, G0/G1%, S%, G2/M%, data are presented. 29

Table 10. Shows the cell cycle results from the UV experiment that included control, low concentration treatment of melatonin, and high concentration treatment of melatonin. Cell number percent in the cell cycle analysis, G0/G1%, S%, G2/M%, data are presented. 30

Acknowledgments

I would like to give my thanks and appreciation to those that helped me in this process. My thanks go out to Dr. Oakley for being my mentor in this. The ECU Honors Program and ECU Biological Sciences Department allowed this to be possible through funding and providing this opportunity. Tamara Justice was a supporter through encouragement and faith. My lab partner Braxton Chafin was crucial as a person to rely on when I couldn't make it to the lab and for information. Dr. Coleman was a resource I could always go to if I needed anything at all. Lastly, I would like to thank everyone else that helped me. This couldn't have been done without you. You know who you are!

II. Introduction

Cancer is unchecked cell division because of damage to the cell's DNA which cannot be fixed by the body's natural systems. Ultraviolet (UV) light has been connected to the development of skin cancers by causing damage to the DNA in cells. UV light can form free radicals in cells which are highly reactive chemical species and are one cause of damage to the cellular processes which are critical to a cell's homeostasis. Long term exposure to UV light has proven negative effects on cell homeostasis including causing damage in cells' DNA, cell cycle arrest, cell death, and potentially leading to cancer. The human body must deal with the damage, and one method the body uses is by upregulating the protein p53 which fixes the damage to the cell's DNA, causes cell cycle arrest, and/or apoptosis. P53 belongs to a class of cellular proteins called tumor suppressors, due to their role in suppressing tumor formation. P53 proteins perform their duty in the nucleus of a cell. P53 will attach to DNA and help determine whether the DNA is damaged and can be repaired, or apoptosis will occur. Normal p53, often referred to as wild type P53 is not mutated and works as it should and has the abilities to induce apoptosis, DNA repair, and cell cycle arrest. Through this method of stopping cells with mutated DNA from replicating, p53 is able to prevent the development of tumors. Use of P53 in the organism's cells result in cell death when the damage done to DNA is too great and this accumulates as causes premature aging. To prevent the use of p53 in cells an antioxidant can be used to block/protect the DNA of cells, thus reducing the amount of p53-induced apoptosis that occurs.

Hence why free radical scavengers not only reduce risks to cancer, but also reduce the aging process. A majority of cancers in humans have been found to have a strong

association with the presence of mutated, non-functional, p53 tumor suppressor genes. In these experiments, UV light was used as a free radical to damage cells, resulting in increased levels of p53, and cell survival. Free radical scavengers, such as zeaxanthin, protect cells from damage by neutralizing damage causing free radical molecules. Zeaxanthin and melatonin are antioxidants and free radical scavengers. Some dietary sources of zeaxanthin are spinach, other leafy greens, and egg yolks. Melatonin is produced by the pineal gland in humans. This study was conducted to determine if zeaxanthin can counteract the negative effects on cell viability and possible mutation of p53 protein caused by UV light damage with the aim to elucidate the mechanism through which zeaxanthin and melatonin provide protection to cells. The parameters of cell health that were analyzed for this experiment were cell count(viability), stage of cell cycle, and cell cycle arrest). The hypotheses for this experiment are that free radical scavenger treatment concurrent with UV light treatment would result in a decreased p53 levels, the cell viability will not be reduced more than the UV light treatment alone, accompanied with reduced cell cycle arrest; the UV light treatment alone is expected to have increased levels of p53 expression, reduced cell viability, and increased cell cycle arrest; the zeaxanthin treated cells will have similar or increased cell viability than the control group, the same or reduced p53 expression than the control group, and a reduced number of cells in cell cycle arrest. The results of these experiments indicate that UV light treatments had increased cell replication and increased cells in cell cycle arrest. The low and high concentrations of zeaxanthin had a negative effect on the cell viability and had increased cell cycle arrest. When testing for p53, level the treatments concentrations were kept the same, the control cells viability but the EtOH, low, and high zeaxanthin treated

cells died, so no p53 data was able to be gathered. Using only the cell viability and cell cycle data obtained in these studies, we determined that zeaxanthin was not able to provide protection from the UV light damage since there was a further increase in cell death and cell cycle arrest. The results of the melatonin experiments showed that at the concentrations used no effect was evident. This is an emerging area of research for supplements that may lead to the elucidation of many vital facts when it comes to cancer prevention that involves UV light and this may give motivation to investigate the use of other supplements as cancer preventatives. This is important for the area of cancer research with regards to free radical scavengers because it shows that it may not be a feasible avenue for future research.

III. Literature Review

P53 literature

In “How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression” the author communicated about the p53 pathway and how it contributes to tumor suppression. This article states how mutated p53 proteins appear in around 50% of human cancers, how the level of p53 protein in unstressed cells are low because otherwise it is broken down by proteasomes, PUMA and (less so but still) NOXA are directly activated through transcription initiated by p53 to induce apoptosis. Also, it talks about how p53 is able to activate cell cycle arrest, cellular senescence, coordination of various DNA damage repair pathways, metabolic adaptations, and apoptotic cell death (Aubrey et al. 2017). This article details how mice without p53 do not spontaneously develop tumors. The loss of p53 and other associated proteins did not

lead to spontaneous tumor development in mice. Evidence from tests done on mice that did not have all mediator for inducing p53 initiated apoptosis, showed that there should not have been cell senescence or that G1/S cell cycle arrest which did not lead to spontaneous development of any tumors. The mice were treated with gamma radiation that did not have p53 proteins and were checked to see whether oncogenesis would occur spontaneously. This may be indicative of a system in which p53-induced apoptosis is important for a stressed state but less so for non-stressed cell survival. This source talks about how p53 works and how it may not work. This includes loss of wild-type function, gain of function, and dominant negative effects from mutant type p53 on wild-type p53. This was useful in understanding whether the absence of p53 is an indicator of oncogenesis or if the malfunction of p53 and the proteins in the p53 pathway are the main cause of loss of p53-induced apoptosis (Aubrey et al. 2017).

“Pharmacological activation of p53 in cancer cells” is a literature review about the p53 pathway and how there have been multiple drugs developed to correct p53 when it is mutated to lose function. It is unknown if the reactivated p53 molecules are able to restore all of the multifaceted biological functions if the cell is not killed in the process of p53 activation. The article illustrated the process and the concepts behind how these drugs were developed while also exploring questions around p53 reactivation. Examples of therapeutic p53-based approaches are questions such as “whether the distinct biological effects regulated by specific post-transnationally modified p53 can effectively be restored by refolding mutant p53...[and] whether reactivation of mutant p53 has similar consequences in cells carrying gain-of-function and loss-of-function p53 mutants” (Current Pharmaceutical Design, Volume 17, Number 6, 2011, pp. 631-639(9)) are raised

and answered in this article. The conclusions of this literature review are that there can be multiple ways to correct non-functional mutated p53 and preventing cancer including a possible vaccine and looking at early genetic testing. Examples of drugs that are able to restore some function to the mutated p53 protein, what each drug does and explanations of the processes of development for all of them are provided. This information can be used in the future to produce ways of mimicking these mechanisms in experiments. Through looking into how other people have thought about the p53 reactivation process which is supportive of other pieces of literature (Athar et al. 2011).

Flow cytometry and the process of how to use flow cytometry specifically on p16 and p21 was talked about in “Comparison of senescence progression in mesenchymal cells from human umbilical cord walls measured by immunofluorescence and flow cytometry of p16 and p21” this information can be applied to p53. This article tells how to measure the amount of cell death in a certain type of cell using the measurement of immunofluorescence and flow cytometry. The authors of the article isolated mesenchymal cells and used a process of immunofluorescence to detect the presence of the p16 protein in the sample. The result of the source’s experiment was that they were able to identify the cells that were at risk of reaching a point in which they are no longer able to replicate, and they found the alternative monitoring processes to the standard that can be used instead. Mesenchymal cells from umbilical cords and the different processes on it to find its results. One strength of the source is that it is clear in explaining how and why each process was used. This is useful as an in-depth guide to the types of proteins and flow cytometry data analysis for the results of this experiment (Silva et al. 2020).

“Mutant p53 gain-of-function in cancer” reports information regarding p53, the process of gain-of-function in p53, and the contribution that it may make to oncogenesis. This article gives information about gain-of-function in p53 through reviewing many articles that had to do with the topic of p53 and other related genes gaining functions. The result of the sources literature review is that regarding cancer research one of the more promising concepts is a molecule that is able to cause mutant p53 to regain wild type activity which may have a dual effect: reinstate the wild type p53 function as a tumor suppressor and to rid the cell of mutant p53 gain of function. This source uses evidence and examples from other literature to support their explanations of p53 gain of function mutations. The article gives a great amount of information on the topic of p53 and more specifically the gain of function mutation (Oren and Rotter 2010).

“Genetic Networks Lead and Follow Tumor Development: MicroRNA Regulation of Cell Cycle and Apoptosis in the p53 Pathways” gives insight regarding how the p53 protein is able to use microRNA in its role in protecting the genetic sequence. This article explains how they believe that p53 uses microRNA to induce cell-cycle regulation and apoptosis. The authors used other evidence and sources that had information about several types of microRNA and p53. The conclusion of this source’s literature review is that there is convincing evidence that p53 uses microRNA for tumor prevention through inducing apoptosis and that these microRNA may be useful for future cancer therapies. This source gives more insight into how the process of p53 protecting cells from mutating into potentially cancerous cells (Otsuka and Ochiya 2014).

This experiment done by Tavana et al. had many aspects in common with the experiment that we had conducted including the use of UV light as a source of free

radicals. The intended goal of the other experiment was to induce p53 protein production (Tavana et al. 2010).

The authors of “Absence of p53-dependent apoptosis leads to UV radiation hypersensitivity, enhanced immunosuppression and cellular senescence” talk about the ability of different cells to respond to UV damage, in the absence of p53 to induce apoptosis in damaged cells. This article studied a hypothesis regarding the relationship between absence of p53 apoptosis, cell death, and UV damage which is greatly important to this experiment. This experiment was done with different cell cultures that either did or did not have the ability to induce p53-mediated apoptosis, and they investigated whether the cells would respond well (die via apoptosis or repair the DNA damage) or whether the cells would respond poorly (mutate and become cancerous). According to the author the study showed that cells without p53-mediated apoptosis are more susceptible to ultraviolet radiation damage and thus are more likely to undergo cellular senescence. The source uses the different pieces of data to show how the mice cells responded to the UV light and describes how they were able to determine such information (Tavana et al. 2010).

“p53-Mediated DNA Repair Responses to UV Radiation: Studies of Mouse Cells Lacking p53, p21, and/or gadd45 Genes” provides information regarding p53, and the process of cells repairing themselves in response to UV radiation in mice without p53, p21, and/or gadd45 genes. This article involves the authors’ experiment with mice and UV radiation. They did an experiment in which they used in vitro cells to test the response in cells without the proteins related to p53 which had been treated with acute UV radiation. After their experiment, they used several methods of analysis to determine

the results, such as several types of assays and cell cycle analysis. The results of the source's experiment are that repair synthesis was detected in all non-S phase and non-G1/S transitional cells, cell cycle stage, cells that lacked the p53 proteins exhibited between 35% and 70% of the normal unscheduled DNA synthesis in response to UV radiation in comparison to wild-type cells (Smith et al. 2000).

UV light

Ultraviolet light is intimately linked to the health of the skin and the immune system that protects the skin from damage. UV radiation is capable of ionizing molecules in skin cells by chemically altering them. An example of possible chemical alteration is damage to a cell's DNA in which close pyrimidine bases (cytosine and thymine) bond to each other, known as pyrimidine dimers. UV radiation can have various effects on skin cells such as igniting pathogenic inflammatory pathways which may lead to autoimmune disorders and allergies while also having the possible effect of being used as a phototherapy to suppress pathogenic cutaneous immune response. This means that UV light provides a strong mutagenic source. The sun emits mostly visible light and infrared radiation while also emitting UVA, UVB, and UVC (Maverakis et al. 2010).

According to the article titled, "Characterization of the p53-mediated cellular responses evoked in primary mouse cells following exposure to ultraviolet radiation" depending on the wavelength of UV light, the damage to mammalian cells varies while all the UV light wavelengths are recognized as being a detriment to human health. Through this study on the effect of various wavelengths of UV light on primary mouse fibroblasts and showed that UVC (for example 254nm) elevated the p53 protein level and

the cells sequence specific DNA binding capacity while UVA did not have the same impact on the cells' p53 level or DNA binding (McFeat et al. 2013).

Zeaxanthin

Fruits and vegetables often contain colorful pigments that are natural lipid-soluble antioxidants named carotenoids. There are more than six hundred carotenoids that occur naturally, but there are about 20 that are detectable in human blood including lutein, lycopene, β -carotene, and zeaxanthin. Each has different properties that can assist the body in many ways such as helping in fetal development and adult homeostasis. Lutein and zeaxanthin are found plentifully in the human eye lens and while there these antioxidants provide eyes with protection against ophthalmic diseases and maintain eye health. The range of 400-200nm wavelength light is blocked in the eye by these ocular carotenoids which means that the retina and lens are protected from photochemical damage that may be induced from light exposure. The result of this is that these carotenoids are able to help the body in crucial ways, some being neutralizing free radicals produced in the body, offering protection against inflammation, apoptosis, oxidative stress, and mitochondrial dysfunction and in the eye (Johra 2020).

Zeaxanthin is a carotenoid in the xanthophyll family. It has the following characteristics: molecular weight of 568.8 Daltons, 11 conjugated double bonds and another name for this molecule is β , β -Carotene-3,3'-diol. Most of the time zeaxanthin is found with its isomer lutein. Sources of lutein include leafy green vegetables (contents as high as 40 mg per 100 g), yellow-orange fruits and vegetables (such as oranges, peaches,

and carrots, contents are often less than 1 mg per 100 g), egg yolks, and cheese (Murillo 2019).

In the article titled, “Dietary lutein/zeaxanthin partially reduces photoaging and photocarcinogenesis in chronically UVB-irradiated Skh-1 hairless mice” the experiment demonstrated that the mice fed a control diet had an increased amount of thickness of skin fold and infiltrating mast cell numbers after UVB irradiation compared to mice fed lutein/zeaxanthin-treated mice. The lutein/zeaxanthin-treated mice showed reduced total tumor volume and tumor multiplicity, and increased tumor-free survival time in comparison with control irradiated animals fed the control diet. UVB-induced photoaging and photocarcinogenesis of skin can be protected against using dietary lutein/zeaxanthin supplementation which is supported by this experiment (Astner 2007).

G2/M cell cycle arrest

In “Inactivation of p53 in normal human cells increases G2/M arrest and sensitivity to DNA-damaging agents” the experiment showed that without the presence of p53 mediated apoptosis there was an increase in cells in the G2/M phase while there was also a decrease in the number of cells in the G1 phase. This experiment was conducted using normal human fibroblasts (NHF) that had induction of their p53 proteins inhibited using p53 anti-sense oligonucleotides. The inhibition of p53 protein was suggested to enhance the sensitivity of the normal human cells to DNA-damaging agents such as UV light (C eraline et al. 1998).

Melatonin

Melatonin is produced by the pineal gland and functions include regulation of circadian rhythm, mood, and sleep. This article holds that there are oncostatic effects on human malignances from melatonin. Recent studies indicated that cancers such as prostate, breast, liver, oral, ovarian, and more had reduced invasiveness when melatonin treatments were added to medical treatment. (Targhazeh et al. 2022)

There has been evidence that melatonin had antiproliferative effects in human breast cancer cell lines. The concentration used in this experiment was near plasma physiological levels, at (1×10^{-11}) M to 1×10^{-9} M). In estrogen receptor-positive human breast cancer cell lines. There was a cytotoxic effect with doses higher than 1×10^{-4} M. This study showed that there was no inhibitory effect on human breast cancer cells at low(physiological) levels. (Papazisis et al. 1998)

There are several positive effects that this molecule has such as antioxidant, anticoagulopathic properties endothelial protective effects, and anti-inflammatory. Melatonin may be able to be used for a variety of diseases and disorders in a therapeutic method (Masters et al. 2014).

This article mentions that there were possible oncostatic effects properties of melatonin. These methods may include epigenetic alteration initiation, activation of apoptosis, angiogenesis and metastasis inhibition, free radical scavenger activity, and the modulation of melatonin receptors MT1 and MT2. Melatonin can act as an addition for cancer prevention and treatment through the previously mentioned pathways meaning that they could produce a growth inhibition effect in tumor cells. (Li et al. 2017)

IV. Methods and Materials

a. Background Information

Chinese hamster ovary cells (CHO) were obtained from L. Middleton, Eastern Kentucky University. CHO cells were thawed from minus eighty freezer. Preliminary cell culturing included the use of a solution using Dulbecco's Modified Eagle Medium (DMEM), penicillin and streptomycin to prevent bacteria from growing, and 10% FBS (fetal bovine serum) as a cell culturing supplement. T25 and T75 flasks were used for cell culturing. The CHO cells were cultured in vitro at 37°C in a humidified 6% CO₂ incubator. The cells were replated when the cells reached an 80% confluency. All treatments of cells were done in a sterile laminar flow to prevent contamination. Six-well plates were used for the experiments excluding the p53 testing where a ninety-six well plate was used.

b. Experimental Procedure

Flasks were removed from the incubator and placed in a sterile laminar flow. The used media in the T75's was removed. 2.5mL of trypsin was added to each flask. Flasks were incubated for 2 minutes in the CO₂ incubator. Next, the flasks were manipulated in such a way as to remove the cells' connection to the bottom of the flasks referred to as "smacked." The cells were viewed using a microscope to confirm that cells were no longer attached. The trypsin solutions containing the cells were divided into individual tubes, then media was added to each tube, so the total volume of the solution was 10mL. Tubes were centrifuged for five minutes at 300 X g. The supernatant was removed, and the cell pellets were resuspended into 10mL of media. Solutions were collected in a

pipette and redistributed to ensure that each well had an equal number of cells to begin the experiment. The centrifuging process was repeated. New media was added so that the total volume in each tube was 6mL. 1mL of solution was added to each well of two six-well plates.

In experiment 1, a six-well plate was selected to be a control and was placed into a sterile laminar flow without the UV light on and without a zeaxanthin treatment. A six-well plate was selected to be a UV light treated group.

In experiments 2 and 3, a six-well plate was selected to be a no UV treatment group which consisted of 2 wells of no zeaxanthin treatment, 2 wells of low (0.114mg zeaxanthin/mL) zeaxanthin treatment, and 2 wells of high (0.286mg zeaxanthin/mL) zeaxanthin treatment. The second six-well plate had UV light treatment and the same groups as the no UV group.

In experiment 4, a six-well plate was selected to be a no UV treatment group that consisted of 2 wells of an EtOH solvent group, 2 wells of low (0.01mg zeaxanthin/mL) zeaxanthin treatment, and 2 wells of high (0.05mg zeaxanthin/mL) zeaxanthin treatment. A second six-well plate had UV light treatment and the same groups as the no UV group.

In experiment 5, two six-well plates were selected to be a no UV treatment groups and two six-well plates were selected to be a UV treatment groups. Within these groups there were groups of no EtOH and no zeaxanthin treatment, an EtOH solvent group, low (0.01mg zeaxanthin/mL), and high (0.05mg zeaxanthin/mL) treatments. Each treatment group was done in triplicate so that there were three wells of each so statistics were able to be done.

In experiment with P53, there was a p53 level ELISA assay done with the same groups as experiment five, with each group done in triplicate. The treatment's concentrations were kept the same but decreased 10-fold because, in the six-well plate, 50 μ L of each treatment went into 1mL of media, while in the ninety-six well plate 5 μ L of each treatment went into 1mL of media. A solution containing cells was put into the flow cytometry to determine cells/ μ L; from this information, the amount of μ L per 10,000 cells was determined. Each of the ninety-six wells were given that amount of μ L.

In the melatonin experiment, a six-well plate was selected to be a no UV treatment group which consisted of 2 wells of no melatonin treatment, 2 wells of low treatment (1nM concentration), and 2 wells of high treatment (10nM concentration). The second six-well plate had UV light treatment and the same groups as the no UV group.

c. UV Light Treatment

Any six-well plate that underwent the UV light treatment was placed into a sterile laminar flow in the same position with the UV light on for two minutes with the lid off.

d. Zeaxanthin Treatment

Experiments 2 and 3 used two concentrations of zeaxanthin treatments (ZX): low (0.114mg/mL) and high (0.286mg/mL). Four zeaxanthin gel capsules (4mg each) were cut open using sterile scissors. The contents were added to a 50mL tube, and the gel capsule outer shells were discarded. 5mL of DMEM media and 2mL of 200 proof ethanol were added to the 50mL tube, which made a concentration of 2.286mg zeaxanthin/mL. The tube was mixed thoroughly. The solution was drawn up into a syringe and pushed

through a sterile individual use filter unit into another 50mL tube. 2mL of the 2.286mg/mL solution were added to one 50mL and 38mL of media was added to the tube for the low treatment concentration of 0.114mg zeaxanthin/mL. 5mL of the 2.286mg/mL solution were added to a 50mL and 35mL of media was added to the tube for the high treatment concentration of 0.286mg zeaxanthin/mL.

Experiments 4, 5, and 6 used two concentrations of ZX: low (0.01mg/mL) and high (0.01mg/mL). For the low concentration solution, one gel capsule (4mg) of zeaxanthin was cut open using sterile scissors. The contents were added to a 50mL tube, and the gel capsule outer shell was discarded. 20 mL of ethanol were added to the tube and the solution was mixed thoroughly using a pipette for a solution concentration of 0.2mg/mL. 50 μ L were added to the treated cells in the specified wells for a solution concentration of 0.01mg/mL. For the high concentration treatment, four gel capsules (4mg each) of zeaxanthin were cut open using sterile scissors. The contents were added to a 50mL tube, and the gel capsule outer shells were discarded. 16 mL of ethanol were added to the tube and the solution was mixed thoroughly using a pipette for a solution concentration of 1 mg/mL. 50 μ L were added to the treated cells in the specified wells for a solution concentration of 0.05mg/mL.

e. Melatonin Treatment

Melatonin tablets were obtained, 3mg each. One tablet was placed into 50mL of media then allowed to completely dissolve for a solution concentration of 260 μ M. This solution was filtered using a sterile filter to ensure sterility. Two stocks were made from

the previous solution; stock 1 was 260nM and stock 2 was 26nM. Stock 2 was used to make 1nM solution and 10nM solution which were used for the experiments.

f. Flow Cytometry used as Analysis Tool

The cells in each of the wells were allowed to grow in their new solution. Then 0.5mL of trypsin was added to each well and was rocked back and forth to ensure that the bottom was covered. The well plates were incubated for 2 minutes. Afterward, the well plates were smacked to remove the cells connection to the bottom of the flasks. The cells were viewed using a microscope to confirm that cells were no longer attached. The trypsin solutions containing the cells were put into individual tubes. New media was added to tube so that the total volume was 4mL. Tubes were centrifuged for 5 minutes at 300 X g. Supernatants were removed; the cell pellets were resuspended. The centrifuging process was repeated. Supernatants were removed; the cell pellets were resuspended in 1mL of Dulbecco's Phosphate Buffered Saline 1X. 100µL of each solution were added to individual tubes. 250µL of Dulbecco's Phosphate Buffered Saline 1X was added to each tube. 200µL of cold propidium iodide stain solution was added to each tube. Tubes were gently mixed by swirling and then they were put into a refrigerator to incubate in the cold darkness for 10 minutes. The samples were then analyzed on the flow cytometry for results right away.

g. P53 ELISA kit used as Analysis Tool

A Pierce™ p53 colorimetric in-cell ELISA kit from Thermo Fisher Scientific was used.

h. Cell Cycle test kit used as Analysis Tool

A Bioscience Pharmingen™ cell cycle test kit was used. This allowed for the percentages of cells in each stage of the cell cycle to be determined.

Table 1. Treatment groups from experiment 1

No UV/No ZX	No UV/No ZX	No UV/No ZX
No UV/No ZX	No UV/No ZX	No UV/No ZX

UV/No ZX	UV/No ZX	UV/No ZX
UV/No ZX	UV/No ZX	UV/No ZX

Group 1 was a control group(top). Group 2 was the UV group (bottom). Each was done in a six-well plate.

Table 2. Table of treatment groups from experiments 2 and 3

Group 1	Group 2	Group 3
No UV/No ZX	No UV/low ZX	No UV/high ZX
No UV/No ZX	No UV/low ZX	No UV/high ZX

Group 4	Group 5	Group 6
UV/No ZX	UV/low ZX	UV/high ZX
UV/No ZX	UV/low ZX	UV/high ZX

The six-well plates were divided into six categories as seen above.

Table 3. Table of treatment groups from experiment 4

Group 1	Group 2	Group 3
No UV/EtOH/no ZX	No UV/low ZX	No UV/high ZX
No UV/EtOH/no ZX	No UV/low ZX	No UV/high ZX

Group 4	Group 5	Group 6
UV/ EtOH/no ZX	UV/low ZX	UV/high ZX
UV/ EtOH/no ZX	UV/low ZX	UV/high ZX

The cell plates were divided into six categories as seen above.

Table 4. Table of treatment groups from experiments 5 and 6. The cell plates were divided into 24 categories as seen below.

Group Number	Treatment
1-3	No UV/ No ZX/ No EtOH
4-6	No UV/ No ZX/ EtOH
7-9	No UV/ low ZX
10-12	No UV/ high ZX
13-15	UV/ No ZX/ no EtOH
16-18	UV/ No ZX/ EtOH
19-21	UV/ low ZX
22-24	UV/ high ZX

V. Results

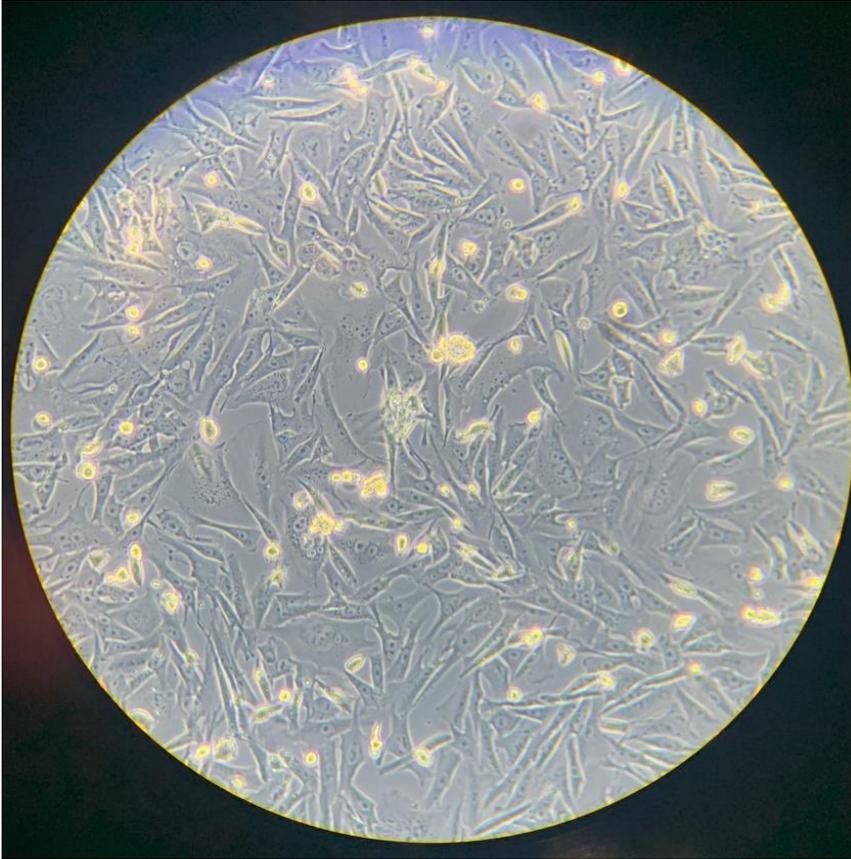


Illustration 1. Image of control group cells viewed at 40X which shows healthy cells covering most of the area being viewed.

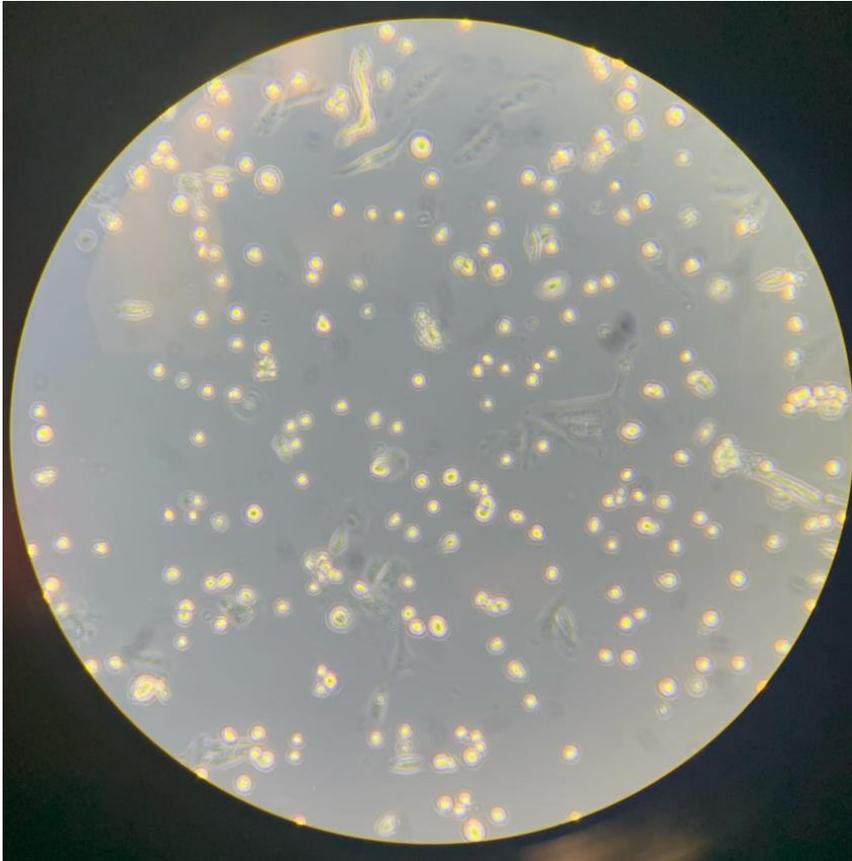


Illustration 2. UV light treated cells viewed at 40X shows many dead cells with some that are alive.

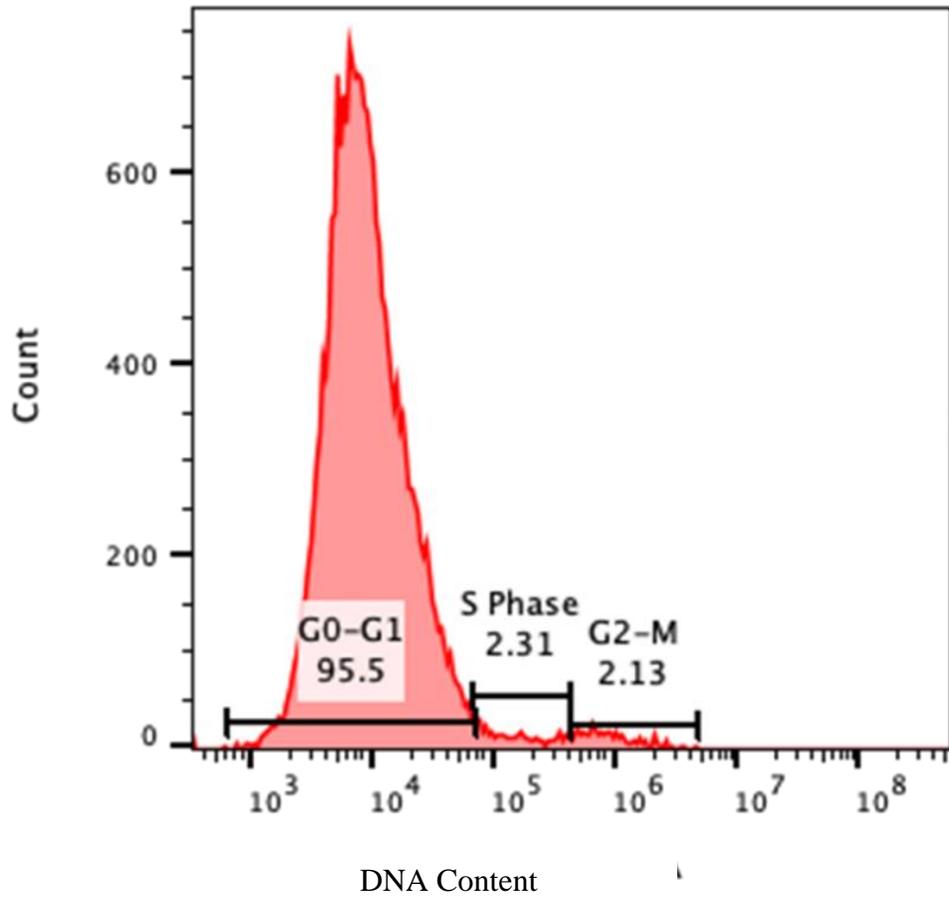


Figure 1. Flow cytometry data from the control set of cells from experiment 1.

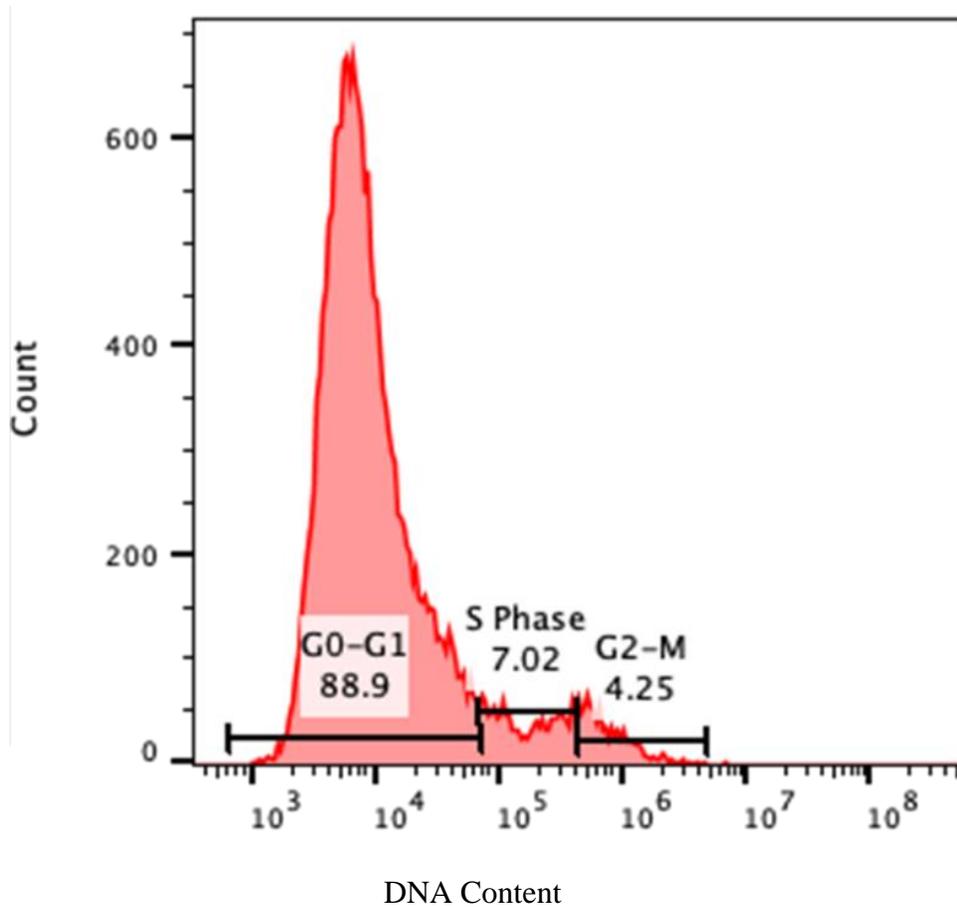


Figure 2. Flow cytometry data from the UV light treatment set of cells from experiment 1.

Table 5. Results from a no UV vs UV experiment that did not include zeaxanthin treatments. Cell number, G0/G1%, S%, and G2/M% data are presented.

	Cell number	G0/G1 %	S %	G2/M %
No UV	23361	95.5	2.31	2.13
UV	22445	88.9	7.02	4.25
Delta Δ	-916	-6.6	+4.71	+2.12

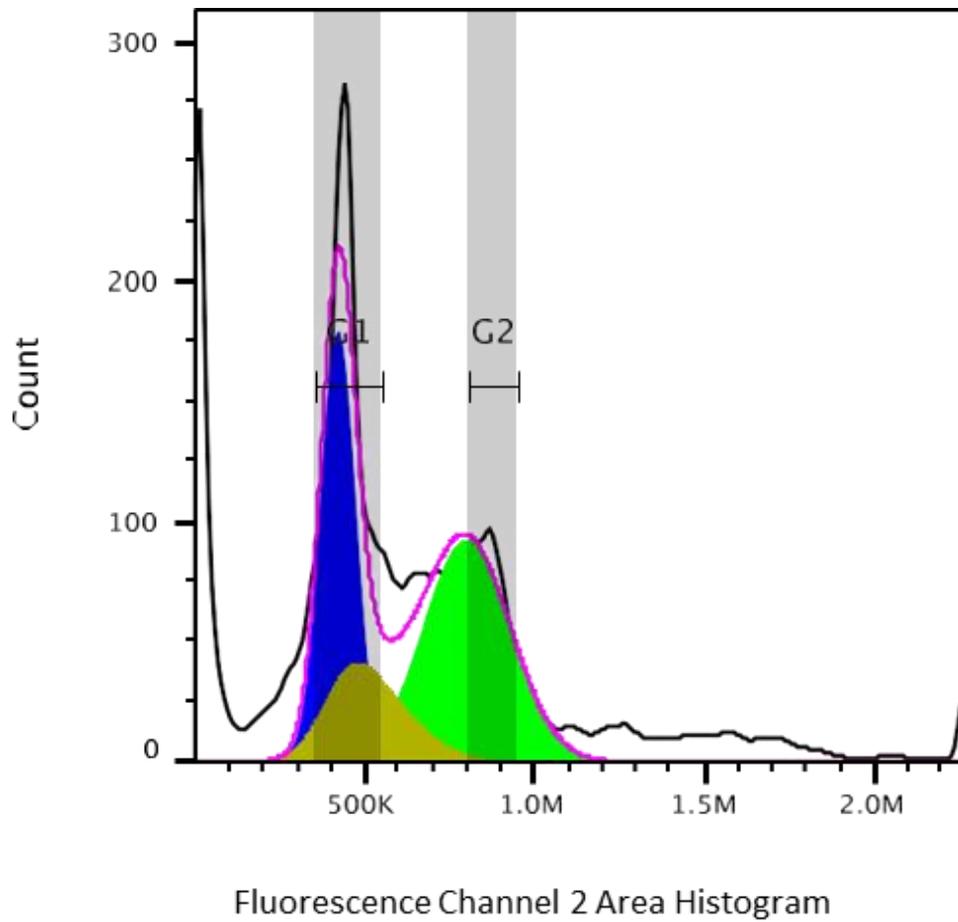
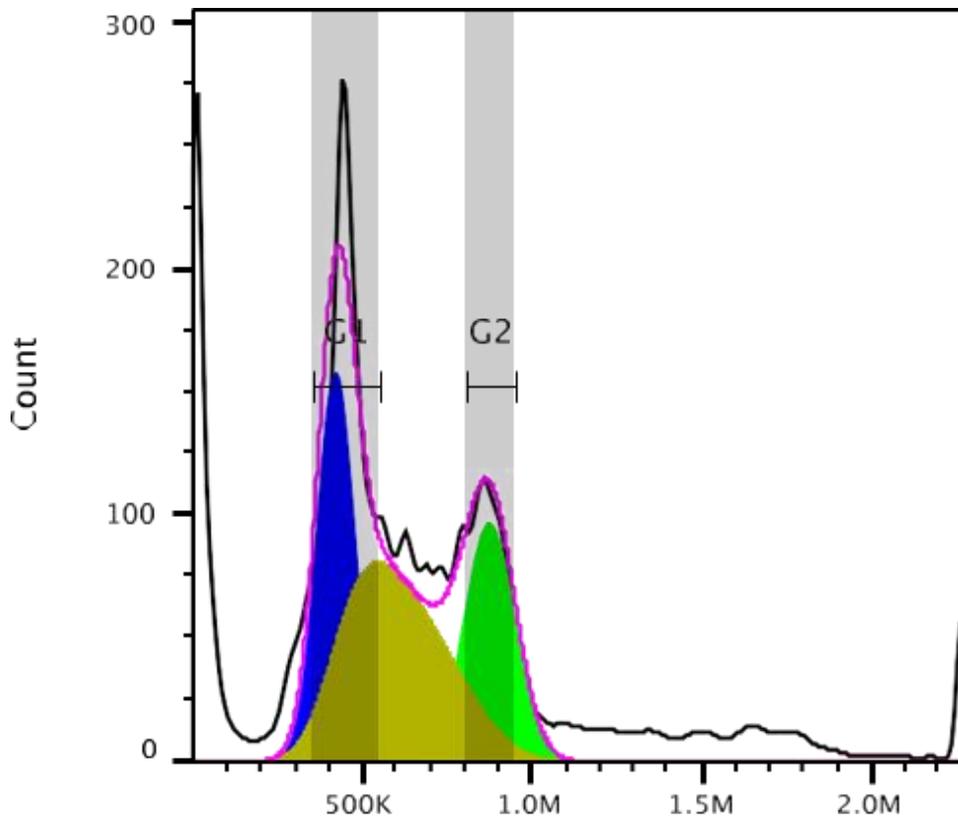


Figure 3. Histogram shows the flow cytometry data from the no UV EtOH treatment set of cells from experiment 4.



Fluorescence Channel 2 Area Histogram

Figure 4. Histogram shows the flow cytometry data from the UV EtOH treatment set of cells from experiment 4.

Table 6. Results from a no UV vs UV experiment that included EtOH. Cell number, G0/G1%, S%, G2/M%, %<G1, and %>G2 data are presented.

	Cell count	G1%	S%	G2%	%<G1	%>G2
No UV	11507	24.8	13.5	32.9	16.1	9.51
UV	12024	21.7	30.9	18.1	13.7	10.9
Delta Δ	+517	-3.1	+17.4	-14.8	-2.4	-1.39

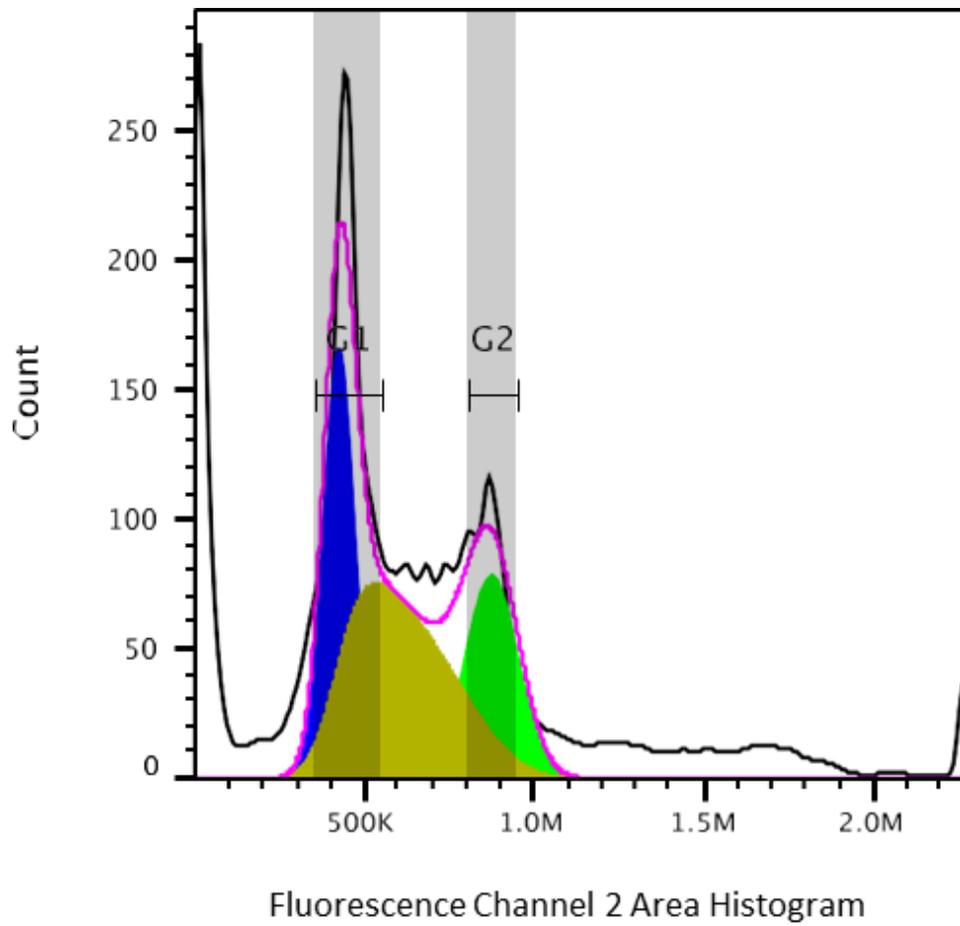
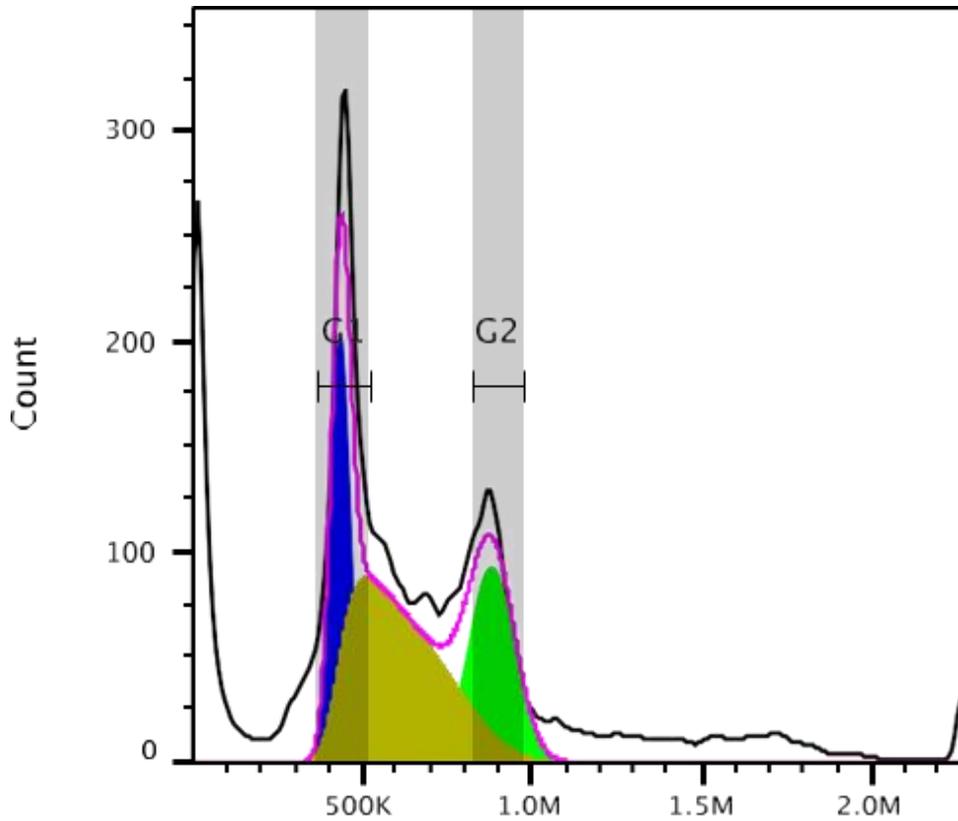


Figure 5. Histogram shows the flow cytometry data from the no UV low zeaxanthin treatment set of cells from experiment 4.

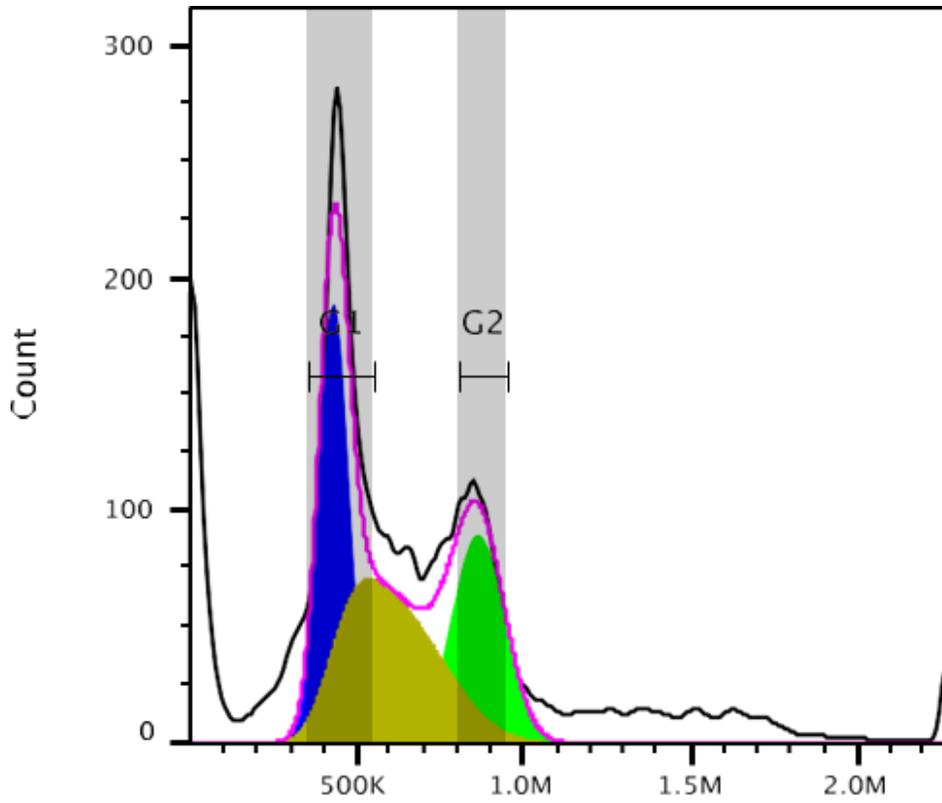


Fluorescence Channel 2 Area Histogram

Figure 6. Histogram shows the flow cytometry data from the UV low zeaxanthin treatment set of cells from experiment 4.

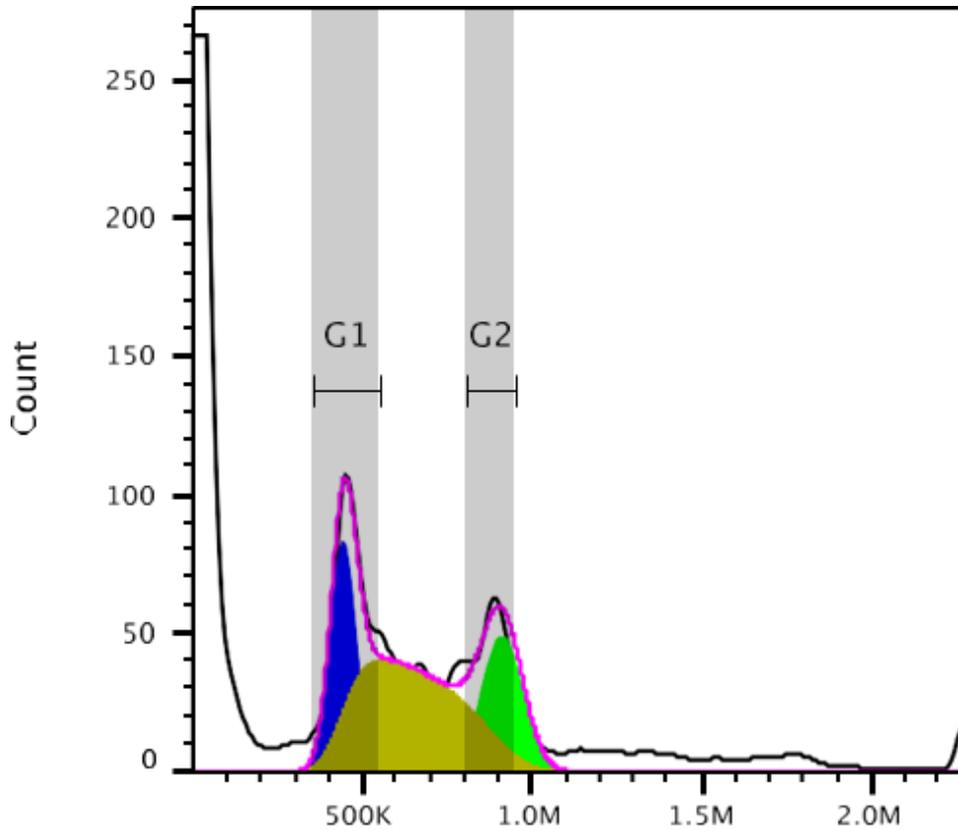
Table 7. Table of results from a no UV vs UV experiment that included low concentration of zeaxanthin treatments. Cell number, G0/G1%, S%, G2/M%, %<G1, and %>G2 data are presented.

	Cell count	G1%	S%	G2%	%<G1	%>G2
No UV	11705	21.0	31.2	16.9	16.0	10.5
UV	12226	16.0	32.1	17.4	19.0	11.0
Delta Δ	+521	-5.0	+0.9	+0.5	-2.4	+0.5



Fluorescence Channel 2 Area Histogram

Figure 7. Histogram shows the flow cytometry data from the no UV high zeaxanthin treatment set of cells from experiment 4.



Fluorescence Channel 2 Area Histogram

Figure 8. Histogram shows the flow cytometry data from the UV high zeaxanthin treatment set of cells from experiment 4.

Table 8. Shows the results from a no UV vs UV experiment that included high concentration of zeaxanthin treatments. Cell number, G0/G1%, S%, G2/M%, %<G1, and %>G2 data are presented.

	Cell number	G1%	S%	G2%	%<G1	%>G2
No UV	11676	23.2	28.8	19.4	13.8	11.0
UV	7978	11.2	13.6	10.5	43.7	8.46
Delta Δ	-3,698	-12.0	-5.2	-8.9	+29.9	-2.54

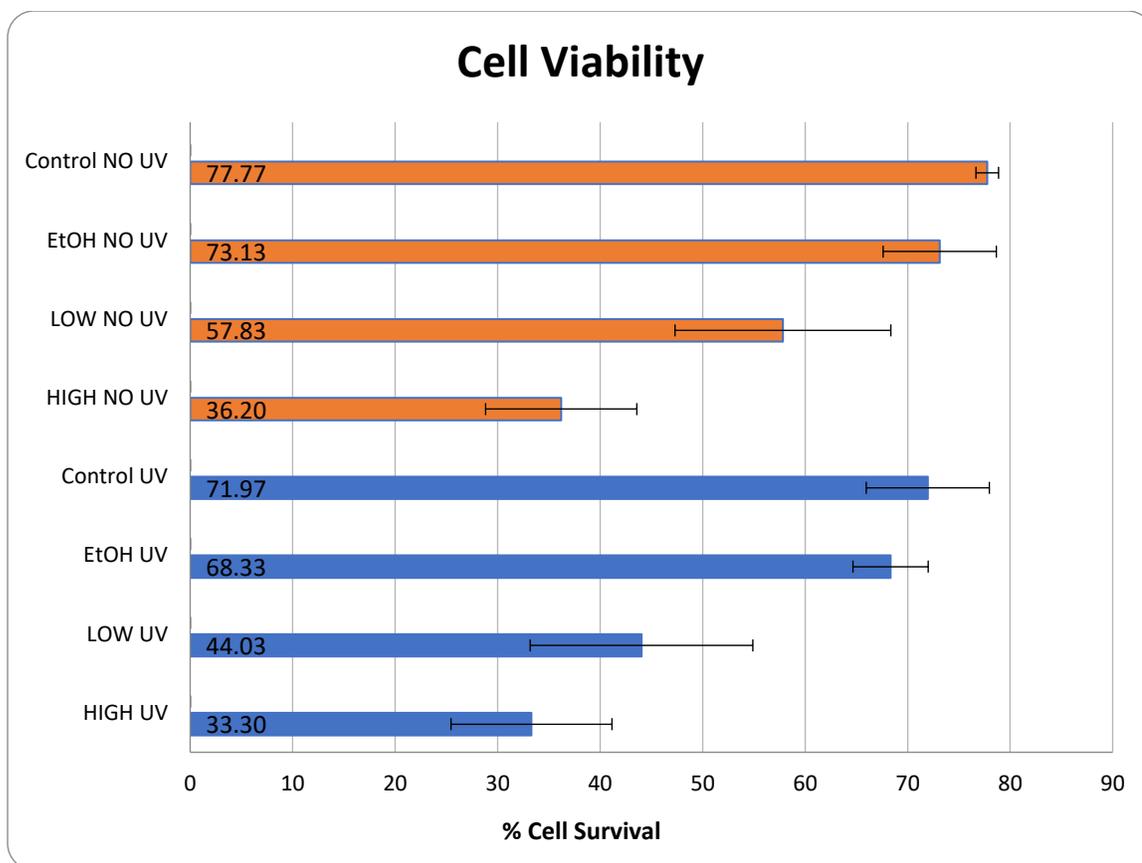


Figure 9. Bar graph of data from no UV vs. UV, each with the groups of control, EtOH, low zeaxanthin, and high treatment groups

Table 9. Shows the cell cycle results from the no UV experiment that included control, low concentration treatment of melatonin, and high concentration treatment of melatonin. Cell number percent in the cell cycle analysis, G0/G1%, S%, G2/M%, data are presented.

	Cell Cycle	G0_G1	S	G2_M
Control	75.40%	46.50%	16.30%	36.20%
Control Low	77.80%	46.90%	15.90%	36.10%
Control High	75.70%	47.00%	15.60%	36.20%

Mean	76.30%	46.80%	15.93%	36.17%
SD	1.31%	0.26%	0.35%	0.06%

Table 10. Shows the cell cycle results from the UV experiment that included control, low concentration treatment of melatonin, and high concentration treatment of melatonin.

Cell number percent in the cell cycle analysis, G0/G1%, S%, G2/M%, data are presented.

	Cell Cycle	G0_G1	S	G2_M
UV	62.90%	35.50%	16.20%	47.40%
UV Low	64.20%	35.30%	15.90%	47.80%
UV High	60.70%	34.30%	14.60%	50.30%
Mean	62.60%	35.03%	15.57%	48.50%
SD	1.77%	0.64%	0.85%	1.57%

VI. Discussion

Some strengths of the experiment are that it determined a concentration of zeaxanthin treatment which is not cytotoxic to the cells treated, gave statistics on the cell viability of the treatment groups, and found cell cycle arrest results for each of the treatment groups. Some shortcomings or limitations of the experiment were contamination, polarity of the lipid gel capsule, and the concentrations of the treatments being too toxic to the cells in the p53 experiment.

From the results of the no UV vs UV experiment that included low concentration the data indicates that there was a large decrease in alive cells from the no UV group to

the UV group showing that the low concentration solution of zeaxanthin did not have a positive effect on cell viability and instead a negative effect. This perceived negative effect may be that the free radical scavenger induced apoptosis in more damaged cells providing some protection but P53 data would be needed to confirm.

UV light for the amount of time used in this experiment caused more replication and more cell cycle blocks (specifically G2/M cell cycle arrest) than no UV light treatment.

Experiment with p53 was conducted to determine the p53 level of the treatment groups. The treatments concentrations were kept the same as experiment 5 just decreased 10-fold because, in the six-well plate, 50 μ L of each treatment went into 1mL of media, while in the ninety-six well plate, 5 μ L of each treatment went into 1mL of media. The control groups' cells survived, but the EtOH, low, and high zeaxanthin treatments' cells died. No data was collected from this experiment.

The results of the melatonin experiments showed that at the concentrations used no effect was evident. It did further provide evidence that there was G2/M cell cycle arrest occurring and that there was a decreased number of cells that were viable for cell cycle analysis after the UV light treatment.

These pieces of data support some of my hypotheses including an increase in cell cycle arrest in UV light treated cells and the p53 data would be required for confirmation of other conclusions.

VII. Conclusion

UV light treatments showed increased numbers of cells in cell cycle arrest and an increase in cell replication. Both treatments with zeaxanthin resulted in increased cell replication and increased cell cycle arrest. We used the same zeaxanthin concentrations to determine p53 levels in the cells. However, the control group were the only cells that survived the treatment and all zeaxanthin treated cells died. These preliminary results indicate that zeaxanthin did not provide protection from the UV light damage since there was an increase in cell death and cell cycle arrest. The effects of zeaxanthin on preventing free radical damage need further investigation, as current literature indicates that treatments of these free radical scavenging products reduce potential lethal damage from UV light.

In conclusion, these experiments were important for determining the effects of zeaxanthin at different concentrations and UV light on cell viability and cell cycle arrest. This is an emerging area of research for supplements that may lead to the elucidation of many vital facts when it comes to cancer prevention that involves UV light and this may give motivation to investigate the use of other supplements as cancer preventatives.

VIII. Limitations

These experiments had several technical difficulties/limitations while planning and executing the experiments. Experiments 2 and 3, which were the first to try and incorporate the zeaxanthin treatment (both low and high concentrations), were both contaminated with fungus or bacteria. Both solutions were put through a sterile filter to ensure sterility before treatment of the cells, and the source of the contamination is still

unclear. No results were able to be gathered from these contaminated experiments; however, we determined that the concentration of zeaxanthin was too high, and we reduced the concentrations in subsequent experiments.

The acquired sample of zeaxanthin was a rapid release gel capsule which consisted of 4mg of zeaxanthin each. It is a substance contained in nonpolar carriers which consist of corn oil, vegetable glycerin, and yellow beeswax. This raised some issues when it came to solubilizing the zeaxanthin in media. After adding portions of the 2.286mg zeaxanthin/mL solution to two tubes which became the low (0.114mg/mL) and high (0.286mg/mL) concentrations and adding in over 30 mL of media to each, the non-polar components of the capsule came out of solution and made the emulsion not suitable for using *in vitro*.

After consulting several sources, we determined that ethanol (EtOH) would be a suitable solvent for the capsules since EtOH has a polar -OH group and a non-polar ethyl group. The concentrations of each solution were decreased so that it was 0.01 mg/mL or 0.05mg/mL of EtOH solutions with zeaxanthin.

The media being used at a certain point was much older than had been thought and negatively affected the cell growth in the culturing flasks. Once discovered, new media was made and used instead. As a result, the cells that were being incubated did not have the same cellular growth supplements and some whole plates of cells died due to poor media, causing the experiments to be delayed.

The CO₂ incubator used is a sealed and climate-controlled area that has the ability to help the cells' media to remain at a relatively stable neutral pH by combining the H₂O in the cells with the CO₂ to make a buffer named carbonic acid (H₂CO₃) and bicarbonate

(HCO₃⁻). Through the use of CO₂ at the right level the pH, of the cells can be maintained at a point around neutral, which is best for their growth. Acidic and alkaline conditions for the cells inhibit the cells' ability to grow (gaslab.com, 2021). The CO₂ tank was allowed to run empty during one set of experiments which caused the cells to die and delay our subsequent experiments.

Old/non-optimal fetal bovine serum had a similar negative effect on the cells as the media and our cells were not growing at optimal rates. Media that contained old/ non-optimal fetal bovine serum was used, but a newly made bottle of media was used when that had been used completely. Due to this issue the experiments may have been delayed.

Originally, the flasks that were used for cell culturing were integrated vented caps, when we switched to manually vented caps, human error led to our cultures dying. This was due to the cells in the T75 flasks not receiving the airflow exchange needed for growth. After this error, vented-cap-T75 flasks were acquired for cell culturing use. Which eliminated the need for manual venting of the caps and eliminated the human error which caused cell flasks to be negatively affected when no ventilation occurred. The number of T75 flasks being kept at this time in the experiment continued to increase to an excessive degree which then caused the number of vented-cap flasks to be depleted soon afterward. This led to the return to the use of non-vented cap T75 and the return of manually vented caps, which again negatively affected the cell health when venting was not done or not done correctly. In the process of culturing cells in T75 flasks, there were some flasks that had minor cracks in the plastic, but those that were completely cracked were discarded. This did not lead to any contamination in the cells that were cultured in the remaining T75 flasks used.

One limitation came with long term implications regarding cell health was that of cell senescence in the flasks that had high passage number, the number of times the culture has been subcultured. This was not confirmed in any way but there appeared to be a decrease in number of cells, the rate of cell division, and the appearance of the cells that had a high passage number such as those which were seven and above. Cellular senescence is a state of being for cells which is characterized by the lack of cell division. Senescence can be a positive for organisms because it may mean that the cells have undergone tumor suppression and other processes, but senescence may also mean that the cells have undergone cellular damage from which they are not able to recover, and cells stop cell division/die prematurely.

The experiment to determine p53 levels in cells did not result in p53 data. It showed that 10,000 cells (in 100 μ L) were killed by 5 μ L of EtOH, 5 μ L of low, and 5 μ L of high zeaxanthin treatments, while the control cells remained alive. The ELISA was not able to be completed due to the treated groups not containing enough healthy and attached cells. If it had been done, only the control groups would give p53 data.

IX. References

- Astner, S., Wu, A., Chen, J., Philips, N., Rius-Diaz, F., Parrado, C., Mihm, M. C., Goukassian, D. A., Pathak, M. A., & González, S. (2007). Dietary lutein/zeaxanthin partially reduces photoaging and photocarcinogenesis in chronically UVB-irradiated Skh-1 hairless mice. *Skin pharmacology and physiology*, 20(6), 283–291. <https://doi.org/10.1159/000107576>
- Athar, M., Elmets, C. A., & Kopelovich, L. (2011). *Pharmacological activation of p53 in cancer cells. Current pharmaceutical design*, 17(6), 631–639. <https://doi.org/10.2174/138161211795222595>
- Aubrey, B. J., Kelly, G. L., Janic, A., Herold, M. J., & Strasser, A. (2017, November 17). How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? Retrieved February 14, 2022, from <https://www.nature.com/articles/cdd2017169#Sec10>
- Céraline, J., Deplanque, G., Duclos, B., Limacher, J. M., Hajri, A., Noel, F., Orvain, C., Frébourg, T., Klein-Soyer, C., & Bergerat, J. P. (1998). Inactivation of p53 in normal human cells increases G2/M arrest and sensitivity to DNA-damaging agents. *International journal of cancer*, 75(3), 432–438. [https://doi.org/10.1002/\(sici\)1097-0215\(19980130\)75:3<432::aid-ijc17>3.0.co;2-a](https://doi.org/10.1002/(sici)1097-0215(19980130)75:3<432::aid-ijc17>3.0.co;2-a)
- Johra, F. T., Bepari, A. K., Bristy, A. T., & Reza, H. M. (2020). A Mechanistic Review of β -Carotene, Lutein, and Zeaxanthin in Eye Health and Disease. *Antioxidants* (Basel, Switzerland), 9(11), 1046. <https://doi.org/10.3390/antiox9111046>

- Li, Y., Li, S., Zhou, Y., Meng, X., Zhang, J. J., Xu, D. P., & Li, H. B. (2017). Melatonin for the prevention and treatment of cancer. *Oncotarget*, 8(24), 39896–39921. <https://doi.org/10.18632/oncotarget.16379>
- Masters, A., Pandi-Perumal, S. R., Seixas, A., Girardin, J.-L., & McFarlane, S. I. (2014). Melatonin, the hormone of darkness: From sleep promotion to ebola treatment. *Brain disorders & therapy*. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4334454/>
- Maverakis, E., Miyamura, Y., Bowen, M. P., Correa, G., Ono, Y., & Goodarzi, H. (2010). Light, including ultraviolet. *Journal of autoimmunity*, 34(3), J247–J257. <https://doi.org/10.1016/j.jaut.2009.11.011>
- McFeat, G. D., Allinson, S. L., & McMillan, T. J. (2013). Characterisation of the p53-mediated cellular responses evoked in primary mouse cells following exposure to ultraviolet radiation. *PloS one*, 8(9), e75800. <https://doi.org/10.1371/journal.pone.0075800>
- Murillo, A. G., Hu, S., & Fernandez, M. L. (2019). Zeaxanthin: Metabolism, Properties, and Antioxidant Protection of Eyes, Heart, Liver, and Skin. *Antioxidants (Basel, Switzerland)*, 8(9), 390. <https://doi.org/10.3390/antiox8090390>
- Oren, M., & Rotter, V. (2010). *Mutant p53 gain-of-function in cancer. Cold Spring Harbor perspectives in biology*, 2(2), a001107. <https://doi.org/10.1101/cshperspect.a001107>
- Otsuka, K., & Ochiya, T. (2014, September 11). Genetic networks lead and follow tumor development: MicroRNA regulation of cell cycle and apoptosis in the p53

pathways. *BioMed Research International*. Retrieved February 22, 2022, from <https://www.hindawi.com/journals/bmri/2014/749724/>

Papazisis, K. T., Kouretas, D., Geromichalos, G. D., Sivridis, E., Tsekreli, O. K.,

Dimitriadis, K. A., & Kortsaris, A. H. (1998). Effects of melatonin on proliferation of cancer cell lines. *Journal of pineal research*, 25(4), 211–218.

<https://doi.org/10.1111/j.1600-079x.1998.tb00390.x>

Silva, A. da, Piccinato, C. de A., Sardinha, L. R., Aloia, T. P. A., & Goldberg, A. C.

(2020, October 16). Comparison of senescence progression in mesenchymal cells from human umbilical cord walls measured by immunofluorescence and flow cytometry of P16 and P21. *Einstein (São Paulo)*. Retrieved February 22, 2022,

from <https://www.scielo.br/j/eins/a/HpMk8whnFVhhPfQ8SLqJTsR/?lang=en#>

Smith, M. L., Ford, J. M., Hollander, M. C., Bortnick, R. A., Amundson, S. A., Seo, Y.

R., Deng, C. X., Hanawalt, P. C., & Fornace, A. J., Jr (2000). p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. *Molecular and cellular biology*, 20(10), 3705–3714.

<https://doi.org/10.1128/MCB.20.10.3705-3714.2000>

Targhazeh, N., Reiter, R. J., Rahimi, M., Qujeq, D., Yousefi, T., Shahavi, M. H., & Mir,

S. M. (2022). Oncostatic activities of melatonin: Roles in cell cycle, apoptosis, and autophagy. *Biochimie*, 202, 34–48.

<https://doi.org/10.1016/j.biochi.2022.06.008>

Tavana, O., Benjamin, C. L., Puebla-Osorio, N., Sang, M., Ullrich, S. E., Ananthaswamy,

H. N., & Zhu, C. (2010). Absence of p53-dependent apoptosis leads to UV radiation hypersensitivity, enhanced immunosuppression, and cellular senescence.

Cell cycle (Georgetown, Tex.), 9(16), 3328–3336.

<https://doi.org/10.4161/cc.9.16.12688>

What is a CO₂ incubator? Gaslab.com. (2021, July 23). Retrieved November 7, 2022,

from <https://gaslab.com/blogs/articles/what-is-a-co2-incubator>