Apple Extract Impedes the Proliferation and / or Metastasis of Oral Carcinoma Cells

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Abstract

The research into nutraceuticals has the potential to radically improve cancer treatments. Whereas current methods, such as chemotherapy and radiotherapy, have detrimental effects on healthy cells and debilitating holistic effects on the body, nutraceuticals can assist in preventing the growth and metastasis of cancerous cells without causing harm to healthy cells. *In vitro* experiments showed that the apple extract nutraceutical successfully prevented the proliferation and / or migration of the oral carcinoma cells without causing significant harm to healthy cells. Research in the laboratory of *in vitro* toxicology at Stern College for Women demonstrated that apple extract functions via an alternative mechanism to the antioxidant or prooxidant routes. Apple extract has the potential to prevent or cure oral cancers.

Introduction

I- Prevalence and General Causes of Cancer

i- The Prevalence of Cancer

There are numerous kinds of cancers that affect different parts of the body. In the United States, the second leading cause of death is cancer. Cancer is so common that one out of two men and one out of three women develop cancer (Siegel *et al.,* 2016). The cost of treating cancer patients is growing astronomically. It was predicted that the United States would spend $156 billion on cancer treatments during the year 2020 (Sahdeo *et al.,* 2017).

ii- General Causes of Cancer

Cancer is the term for a genetic disease in which cells continuously proliferate. The body can develop cancer as a result of internal or external factors. Internal factors are inherited genetic mutations that lead to the development of cancerous cells. There are numerous external factors that can cause cancer, such as dyes, tobacco, and exposure to various harmful chemicals. These agents can cause a mutation in a gene that controls cell proliferation, leading to uncontrolled cell proliferation. Both internal and external factors can impact the regulation of specific genes, such as genes designated to repair DNA damage. (Sahdeo *et al.,* 2017).

iii- Reactive Oxygen Species

Cellular signaling pathways consist of molecules that are called cellular intermediates. Reactive Oxygen Species (ROS) are one category of cellular intermediates.

ROS include oxygen radicals, such as $O2^{\bullet-}$; $OH^{\bullet}$; $RO_2^{\bullet}$; and $RO^{\bullet}$ (Carocho and Ferreira, 2013). A radical is a molecule that contains an atom with an unpaired electron in its outermost orbital. Free radicals are charged molecules or ions due to their gain or loss of an electron. These ROS attempt to take an additional electron from another molecule in the cell or donate the extra electron to another cellular molecule (Fumar *et al.,* 2017). ROS can also be non-radical molecules that can easily be reduced into radicals, such as HOCl; O_3; and H_2O_2. ROS can be produced via endogenous and exogenous sources. Endogenous production of ROS includes byproducts in reactions carried out by the mitochondria and peroxisomes within the cells. Additionally, ROS is a major product produced by the NADPH oxidase enzyme system. Exogenous sources that lead to ROS production include pollutants, tobaccos, drugs, and radiation. Cells balance this buildup of ROS with cellular antioxidants (Sahdeo *et al.,* 2017).

An excessive accumulation of ROS, however, can lead to oxidative stress within the cell (Sahdeo *et al.,* 2017). ROS can cause damage to proteins, DNA, RNA, sugars, and lipids by removing or donating an electron from or to these molecules. Proteins can be oxidatively modified via different processes: an amino acid in a peptide chain can be impacted and free radicals can lead to peptide cleavage or formation of a protein cross linkage. DNA and RNA damage can occur when a free radical modifies a purine or pyrimidine base, breaks the DNA or RNA strand, or leads to the formation of a cross-link between the DNA or RNA and a protein. Lipid peroxidation occurs when a free radical removes a hydrogen atom from a fatty acid chain, which turns the fatty acid chain into a free radical. This altered fatty acid becomes an unstable free radical, and it will react with oxygen to form a peroxyl radical. These

processes result in a continuous production of free radicals within the cell (Carocho and Ferreira, 2013).

Oxidative stress is linked to a myriad of diseases, including: cancer, cardiovascular diseases, neurological disorders, asthma, and diabetes mellitus (Carocho and Ferreira, 2013). Oxidative stress can damage DNA, and an accumulation of damaged DNA can lead to transformation, which is the cellular changes that occur when a healthy cell becomes malignant (Sahdeo *et al.,* 2017).

iv- ROS and Tumor Survival

Reactive Oxygen Species (ROS) are implicated not only in the transformation of normal cells to malignant ones, but also in the survival of cancerous cells (Sahdeo *et al.,* 2017). Lee, et al. conducted an *in vivo* study in 2007 on pancreatic cancer cell lines. This study showed that the NADPH oxidase enzyme system produced ROS which mediated the antiapoptotic process, which prevented cell death. The accumulated ROS inhibited the protein tyrosine phosphatases, which maintained the activation of kinases. This prevented the process of apoptosis in the pancreatic cancer cells (Lee *et al.,* 2007). ROS are also involved in metastasis. High levels of ROS are present in metastatic cancer cells (Sahdeo *et al.,* 2017). In a 2010 study by Xu *et al*., the authors assert that exposure to ethanol has been correlated with an increase in ROS in breast cancer cells. ROS promotes the migration and invasion of breast cancer cells. Furthermore, the researchers demonstrated that the antioxidant Cyanidin-3-glucoside prevented the metastasis of the breast cancer cells (Xu *et al.,* 2010). These research studies demonstrate that the cancer cells have higher levels of ROS than

non-cancerous cells. ROS are a byproduct of cellular metabolism, and cancer cells have higher proliferation rates than non-cancerous cells. Therefore, cancer cells have a higher level of ROS than non-cancerous cells. Antioxidants can combat the heightened levels of ROS and kill the cancer cells. Alternative methods are to further increase the levels of ROS beyond the viable point for the cancerous cells or to introduce prooxidants that counteract the cells' antioxidants (Kim *et al.,* 2019). Some commonly used cancer treatments increase the levels of ROS in order to kill the cancer cells.

II- Methods of Cancer Treatment

i- Radiation Therapy

Radiation therapy is used in the treatment of more than 50% of cancer patients. Radiation therapy targets high-energy particles, such as protons, or waves, such as x-rays or gamma rays, to destroy the cancerous cells within a specific region of the body. This method of treatment is advantageous as relatively few healthy cells in the body are destroyed. The radiation causes breaks within the cellular DNA. Cancerous cells have a higher rate of cellular division than non-cancerous cells. The breaks within the DNA stop the cancerous cells from dividing and ultimately leads to the death of those cells. An unfortunate side effect of radiation therapy is that the radiation itself can cause damage to a healthy cell which itself could then become cancerous. The radiation is used to treat the current cancer, but there is a risk that the patient will develop another cancer as a result of exposing the patient to these high-energy particles or waves (American Cancer Society Radiation, n.d.).

ii- Chemotherapy

Chemotherapy uses drugs to kill cells by interfering with a specific stage of the cell cycle. The drugs are not specific to cancerous cells, so healthy cells are also harmed during treatment Chemotherapy drugs can be classified according to how they interfere with the cell cycle. Alkylating agents are a group of chemotherapy drugs that damage the DNA and thereby prevent the cells from replicating. A concern with the use of alkylating agents is that the bone marrow, which produces new blood cells, can become damaged and years later the patient may develop leukemia. A sub-category of alkylating agents is nitrosoureas. These drugs follow the same mechanism as the other alkylating agents, but they are uniquely able to cross the blood-brain barrier and thereby kill cancerous cells located in the brain. Another category of chemotherapy drugs is antimetabolites. These drugs prevent DNA and RNA replication. While chemotherapy drugs have proven to be beneficial in killing cancerous cells, healthy cells are also killed with this form of treatment (American Cancer Society Chemotherapy, n.d.).

iii- ROS and Antioxidant Interactions and Cancer Therapy Efficacy

Many cancer therapeutics induce ROS production as a means of killing the cancerous cells (Sahdeo *et al.,* 2017). According to some studies, ROS induce apoptosis in cancerous cells (Harvie, 2014). ROS can induce cell death in some cell types depending on the source of the ROS, the site of ROS production, the specific species, concentration, and time (Sahdeo *et al.,* 2017). Consuming antioxidants during radio or chemotherapy may lower the efficacy

of these treatment methods. Phase two and three trials do not support using antioxidants in cancer patients (Harvie, 2014).

ROS play a role in killing cells that have already become cancerous. 2-methoxyestradiol, buthionine sulfoximine, cisplatin, doxorubicin, imexon, and motexafin gadolinium are examples of anti-cancer drugs that increase the level of ROS in cancer cells (Kim *et al.,* 2019). According to this view that ROS kill cancerous cells there is a role for prooxidants in cancer treatment.

Some research studies support the use of antioxidants to kill cancerous cells, while others cancer treatments increase the levels of ROS to kill cancerous cells. The study of cancer is complex and not yet fully understood. There are numerous types of cancer, so it is plausible that different cancers may necessitate different kinds of treatment.

III- Oral Cancer

Cancer can affect any part of the body. Each year there are over 1.5 million new cases of cancer in the United States (Sahdeo *et al.,* 2017). Globally, there are approximately 405,000 new cases of oral cancer each year. The American Cancer Society predicted that in the United States alone there would be 42,220 new cases of oral cancer resulting in 8,220 deaths in the year 2014. This means that in the United States oral cancer is fatal in approximately 20% of the cases. Smoking tobacco, consuming alcohol, and poor oral hygiene are commonly associated with the development of oral cancer. Men over the age of 50 are at the highest risk of developing oral cancer (Montero and Patel, 2015).

One method of treating oral cancer is by surgically removing the cancerous cells. In some cases the surgeon also needs to remove a portion of the patient's tongue or jawbone. The patient will subsequently have difficulty with eating and speaking. A patient may receive radiation or chemotherapy in addition to or in place of surgery (Mayo Clinic, n.d.). As discussed above there are drawbacks to these forms of treatments, as well. Therefore, it is imperative to explore an alternative natural method to treat oral cancer.

IV- Nutraceuticals

i- Nutraceuticals

The term nutraceutical is a combination of the words nutrition and pharmaceutical. Foods with medicinal properties are categorized as nutraceuticals. Some nutraceuticals, as discussed previously, function as antioxidants or proxidants, while others provide benefit via other mechanisms.

ii- Antioxidants

Antioxidants are molecules that scavenge Reactive Oxygen Species (ROS) and result in a new radical that can be stabilized by intramolecular hydrogen bonding (Carocho and Ferreira, 2013). Therefore, antioxidants counter oxidative stress, thereby protecting the DNA, RNA, sugars, lipids, and protein molecules from oxidative damage. Examples of antioxidants include: glutathione (GSH), thioredoxin, ascorbic acid, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Ali *et al.,* 2020). A particular antioxidant may target a specific ROS (Fumar *et al.,* 2017). For instance, SOD is an enzyme that breaks

down superoxide into hydrogen peroxide, CAT enzyme degrades the hydrogen peroxide into water and oxygen, and GSH inactivates organic pollutants by altering their molecular structure. Natural antioxidants present in fruits and vegetables include: tocopherols, ascorbic acid, carotenoids, and flavonoids (Ali *et al.,* 2020).

Antioxidants can be classified as hydrophilic or hydrophobic according to their molecular properties. Hydrophilic antioxidants react with free radicals located in the cell cytosol and blood plasma. Examples of hydrophilic antioxidants include: ascorbic acid, glutathione, lipoic acid, and uric acid. Hydrophobic antioxidants are used to prevent lipid peroxidation and cell membrane damage. Examples of hydrophobic antioxidants include: vitamin A, carotenes, ubiquinol, and vitamin E (Fumar *et al.,* 2017).

iii- Prooxidants

Prooxidants induce oxidative stress by forming reactive species, such as ROS, or by combating antioxidants. Some antioxidants also possess prooxidant properties. For example, vitamin C is typically classified as an antioxidant. However, vitamin C can reduce Fe^{3+} or Cu^{3+} to Fe^{2+} or Cu^{2+}, which can then react with oxygen and then produce H_2O_2. The H_2O_2 can be reduced into the prooxidant hydroxyl radicals. Sulfur and nitrogen can also form reactive species and are referred to as reactive sulfur species (RSS) and reactive nitrogen species (RNS) (Carocho and Ferreira, 2013). [See above section I-iii-Reactive Oxygen Species.]

iv- Antioxidant Nutraceuticals and Cancer Research

Numerous research studies show that antioxidants can reduce oxidative damage and prevent cancer. Antioxidants reduce cell proliferation and increase apoptosis in cancerous cells. Numerous antioxidants have been implicated in preventing cancer initiation, progression, and metastasis (Harvie, 2014).

Research studies demonstrate that antioxidants can prevent metastasis of cancer cells. For example, pancreatic cancers are not sufficiently vascularized, which results in hypoxic conditions. Tumor hypoxia maintains cancer cells, increases metastasis, and promotes resistance to chemotherapy and radiotherapy. Unfortunately, this results in a four month survival rate for pancreatic cancer patients. Hypoxia induces the production of mitochondrial Reactive Oxygen Species (ROS), which are involved in pathways that promote cancer cell metastasis. Shimojo *et al.* conducted an *in vitro* study in 2013 and demonstrated that antioxidant *N*-acetylcysteine (NAC) suppressed the excessive levels of mitochondrial ROS in xenograft nude mice with implanted human pancreatic cancer cells. NAC suppressed metastasis from the cancerous pancreas cells to liver cells. The same experiment was repeated with antioxidant ebselen to further suggest that antioxidant activity prevented metastasis (Shimojo, *et al.,* 2013).

v- Prooxidant Nutraceuticals and Cancer Research

As noted above, many experiments suggest that antioxidants have anticarcinogenic properties. Other experiments demonstrate that prooxidants can also possess anticarcinogenic properties. In 2008 Babich, *et al.* conducted an *in vitro* study analyzing the impact of black

tea extract on oral carcinoma cells. They noted that the polyphenols in the black tea displayed prooxidant properties, as they raised the levels of reactive oxygen species in the cancerous cells. Notably, the black tea increased the ROS levels to a greater extent in the cancerous cells than in the non-cancerous cells. Their research suggests that the prooxidant properties found within the black tea led to the spontaneous death of the cancerous cells (Babich *et al.,* 2008). In 2009 Babich, *et al.* conducted an *in vitro* study on the effects of the prooxidant *Ginkgo biloba* leaf extract. They found that the extract was cytotoxic to carcinoma HSC-2 cells by increasing the ROS levels. The oxidative stress led the cancerous cells to undergo apoptosis (Babich *et al.,* 2009). This study demonstrates that the prooxidant activity of nutraceuticals can cause cell death in some types of cancers.

vi- Apple Extract Nutraceutical

Apples contain a variety of antioxidant phytochemicals, biologically active molecules found in plants. The polyphenol group of antioxidants can be divided into subclasses; the two largest subclasses are flavonoids and phenolic acids. The subclass of flavonoids is further divided into subgroups based upon their molecular structures. Flavonoid subgroups include flavanols, flavonols, anthocyanidins, dihydrochalcones, and hydroxycinnamic acids. An assortment of flavonoids are present in apples, such as flavanols, flavonols, and anthocyanidins. Apple polyphenols prevent a myriad of illnesses by countering the cellular oxidative stress, such as diabetes mellitus (Tu *et al.,* 2017).

vii- Apple Extract and Cancer Research

Numerous research studies have been conducted on the phytochemical antioxidants contained in apples (Tu *et al.,* 2017). An *in vivo* human study was conducted by Yuan, *et al.* in 2011. The 25 participants each drank 300 ml of apple juice and 300 ml of grape juice daily for two weeks. After two weeks the participants had an overall increase in their plasma total antioxidant capacities (Yuan *et al.,* 2011). This demonstrated the presence of antioxidants in apple and grape juices. A study was conducted by Lin, *et al* in 2016. Phloretin is a phytochemical found in apples that displays antioxidant activity. In an *in vitro* study the researchers noted that phloretin inhibited the proliferation of human colorectal and liver cancer cells by inhibiting the type 2 glucose transporter (GLUT2). Inhibiting the transporter resulted in apoptosis of the cancerous cells. Furthermore, *in vitro* and *in vivo* mouse studies demonstrated that phloretin inhibited GLUT2, which prevented the cell proliferation, migration, and invasion of colon cancer cells (COLO 205) (Lin *et al.,* 2016). Similarly, Kobori, *et al.* conducted an *in vitro* study in 1997 and noted that phloretin was cytotoxic to melanoma cells by inhibiting a glucose transporter. This induced the cancerous cells to undergo apoptosis (Kobori, *et al.,* 1997). In 2014 Miura, *et al.* conducted studies on apple polyphenol extract and liver cancer. An *in vitro* experiment demonstrated that the apple extract decreased the proliferation and invasion of hepatoma cancer cells in a dose-dependent manner up to the concentration of 200 mg/mL. They conducted an *in vivo* study that further demonstrated that orally administering apple extract resulted in a reduction in the proliferation and metastasis of hepatomas in rats transplanted with a tumor from the hepatoma cell line AH109A. Additionally, the rats with the administered apple polyphenols

had lower levels of hydrogen peroxide, which is classified as an ROS (Miura, *et al.,* 2007). These research studies suggest that apple extract contains antioxidant properties that can prevent the growth and metastasis of cancer cells. Although there are numerous research articles that suggest that the phenolic compounds in apple extract exert antioxidant activity, Babich, *et al.* assert that some phenolic compounds possess prooxidant properties. Polyphenols can induce an *increase* in the level of ROS, causing oxidative stress, and ultimately causing the cancerous cells to undergo apoptosis (Babich *et al.,* 2011). In 2017 D'Angelo conducted an *in vitro* study on apple polyphenol extract and human breast cancer cells. They determined that the polyphenols acted as prooxidants and induced apoptosis in the cancer cells. Depending upon the particular environment, such as pH or concentration, the polyphenols can exert antioxidant or prooxidant properties (D'Angelo *et al.*, 2017).

Preliminary Data

Cancerous cells are known to have higher levels of ROS than healthy cells. If the ROS level is increased beyond the capacity that the cancerous cells can handle then the cells die (D'Angelo *et al.*, 2017). Additionally, Babich, *et al.* had previously conducted research on nutraceuticals, such as pomegranate extract, black tea, and *Ginkgo biloba* tea extract. Their research suggested that these nutraceuticals were cytotoxic to cancerous cells via their prooxidant properties.

Apples are a commonly consumed fruit. Similar studies were therefore conducted on an apple extract (*Malus pumila mill*) to ascertain its anticarcinogenic properties. A Neutral Red (NR) assay was used to assess apple extract for prooxidant properties. An NR assay

measures cell viability, as only viable cells can uptake the neutral red dye. Apple extract was added in various concentrations to cancerous HSC-2 cells. Then catalase, pyruvate, and buthionine sulfoximine (BSO) were individually added to some of the wells containing apple extract and HSC-2 cells. The percentage of live cells in wells containing only apple extract or apple extract plus catalase, pyruvate, or BSO was not significantly different. Catalase, pyruvate, and BSO protect the cells from prooxidants. This suggested that apple extract does not induce the death of cancerous cells by interfering with the ROS mechanism, and apple extract does not function as a prooxidant. The apple extract causes cell death via an alternative method. Additionally, this research study demonstrated that apple extract is cytotoxic to cancerous cells when added at a concentration greater than 200 ug/mL (Figure 1) (Friedman *et al.,* 2015).

This preliminary data served as the backdrop for the subsequent experiments which assessed for the antiproliferative and anti-migration properties in apple extract.

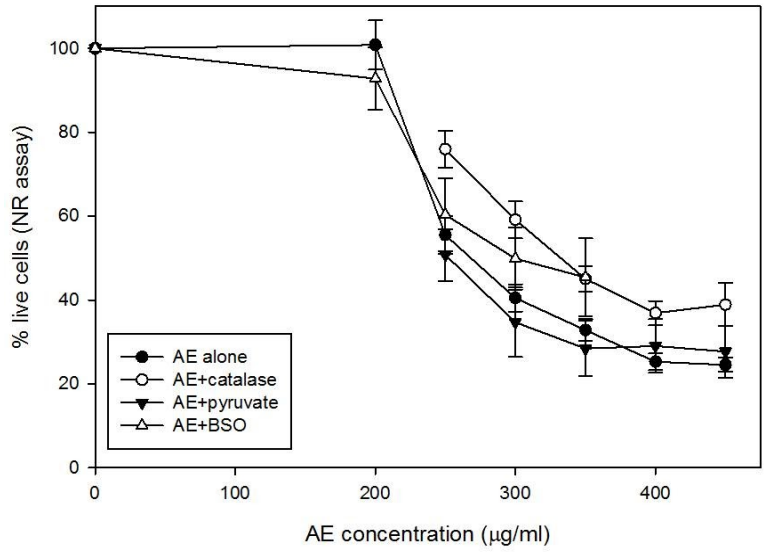

Figure 1. The percentage of live cells with the addition of varying concentrations of apple extracts. Some wells had apple extract alone. Catalase, pyruvate, and BSO were individually added to the remaining wells (Friedman *et al.,* 2015).

Research conducted Fall 2019 in Dr. Schuck's laboratory of *in vitro* toxicology at SCW

In the Fall of 2019 experiments on apple extract were conducted in Dr. Schuck's lab. Experiments were conducted to demonstrate that apple extract (AE) can prevent the growth and migration of the cancerous cells. Previous research established that the apple extract does not prevent the growth of healthy, non-cancerous cells at the same concentrations at which it does so to cancer cells. This is in contrast to other cancer treatments, such as radiotherapy and chemotherapy, which can harm the non-cancerous cells. Prior experiments demonstrated that the apple extract does not exhibit prooxidant qualities. Rather, the apple extract prevents the growth of the cancerous cells via an alternative method, which has not yet been identified.

Metastasis is a property of cancer cells, and we wanted to see if the AE can possibly prevent it. One *in vitro* way to evaluate metastasis is using a wound-healing assing / scratch assay. A scratch assay is implemented to remove a section of a monolayer of cancerous cells grown in petri dishes. It is expected that without the addition of an anticancer agent the remaining cancerous cells will proliferate and thereby fill in the gap. A scratch assay is used in cancer research to mimic migration, as the remaining cells can migrate to the cell-free zone. A scratch assay was performed by Shimojo, *et al.* in 2013 to assess the migration of PANC-1 cells (human, pancreatic cancer cells). The antioxidant *N*-acetylcysteine (NAC) was added to some of the PANC-1 cells. The wells containing NAC maintained a larger gap than those without this nutraceutical. This demonstrated that NAC lessened the migration of PANC-1 cells (Shimojo, *et al.,* 2013).

If the apple extract prevents the growth and / or migration of cancerous cells then it is expected that the addition of apple extract will maintain the gap. Additionally, it is expected that as the concentration of apple extract increases the percentage of closure of the gap (growth and / or migration of cancerous cells) should decrease.

HF-1 normal oral fibroblasts and HSC-2 oral carcinoma cells were seeded in 12-well plates in complete Dulbecco's Modified Eagle Medium (DMEM) and grown to 80-90% confluency in a monolayer overnight. After 24 hours, a scratch was created along the diameter of each well using a 200 µl pipette tip. Cell debris was removed, followed by the addition of fresh media alone or containing 25, 50, or 75 µg/mL apple extract (Figures 2 and 3). As the concentration of apple extract increased the width of the gap decreased to a lesser extent in a dose-dependent manner.

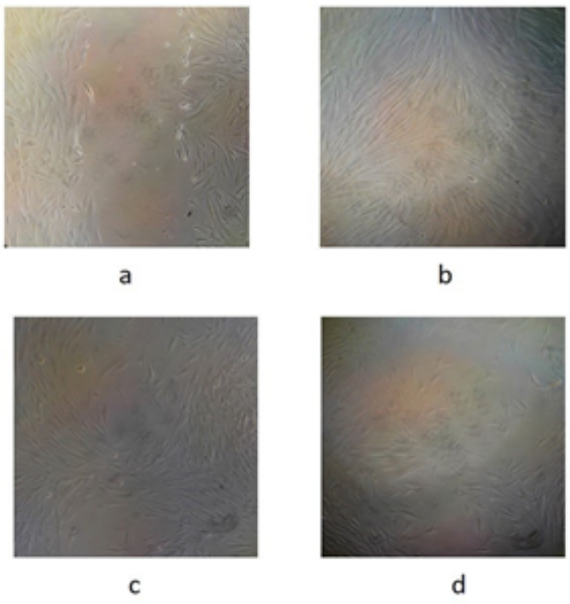

Figure 2. Closure of wound in HF-1 monolayer. HF-1 cells were seeded in 12-well plates in complete DMEM media and grown to 80-90% confluency in a monolayer overnight. After 24 hours, a wound / scratch was created along the diameter of each well using a 200 µl pipette tip (a). Cell debris was removed, followed by addition of fresh media alone (b) or containing (c) 25, (d) 50, or (not shown) 75 µg/mL AE.

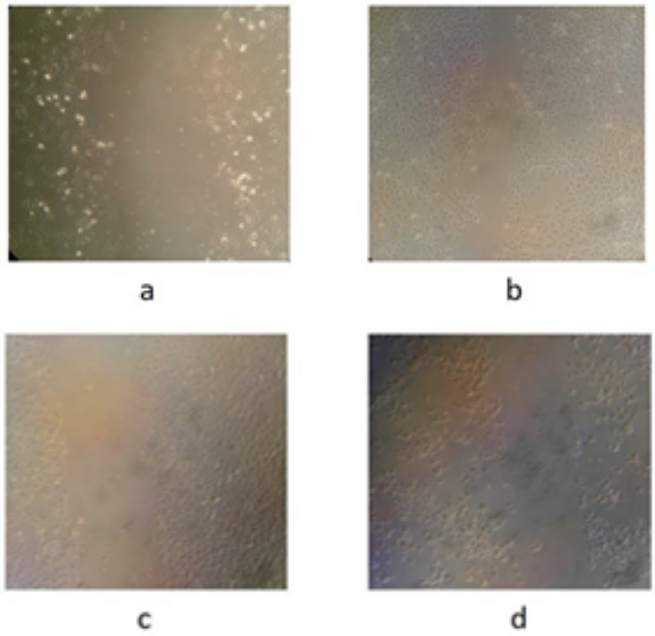

Figure 3. Closure of wound in HSC-2 monolayer. HSC-2 cells were seeded in 12-well plates in complete DMEM and grown to 80-90% confluency in a monolayer overnight. After 24 hr, a wound / scratch was created along the diameter of each well using a 200 µl pipette tip (a). Cell debris was removed, followed by addition of fresh media alone (b) or containing (c) 25, (d) 50, or (not shown) 75 µg/mL AE.

24 hours after the HF-1 and HSC-2 wells were scratched and treated wound healing was measured by phase contrast microscopy using an ocular micrometer. The distance across the scratch was measured at three different areas along the wound. The mean values are shown (Figure 4).

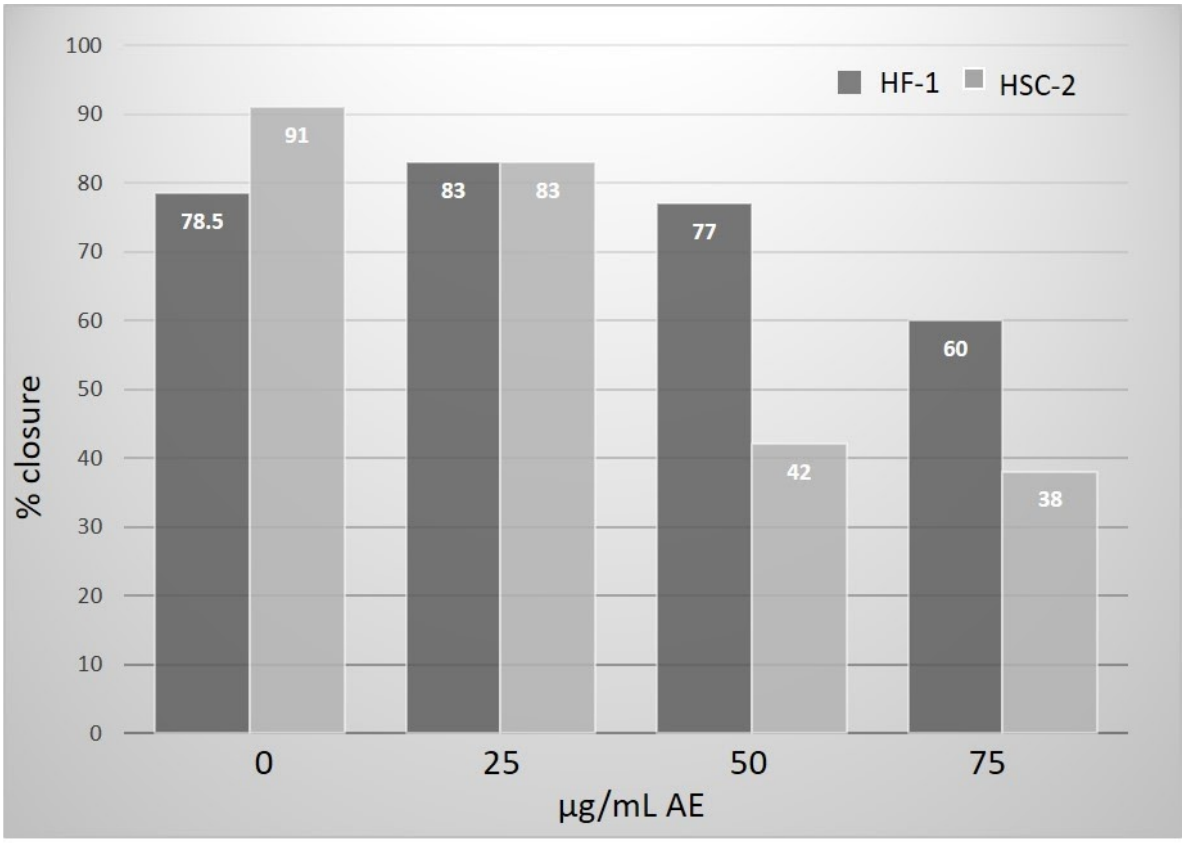

Figure 4. Wound healing was measured 24 hours after scratch and treatment by phase contrast microscopy using an ocular micrometer. The distance across the scratch was measured at three different areas along the wound. Mean values are shown.

As the concentration of apple extract increased the width of the scratch decreased to a lesser extent in a dose-dependent manner. A greater percentage of closure means that there was greater proliferation and / or migration of the cancerous cells. The control wells did not contain any apple extract, and, as expected, there was a high percentage of closure, 78.5% for HF-1 and 93% for HSC-2. Cancerous cells have faster proliferation rates and migrate more than non-cancerous cells. Therefore, it is understandable that in the controls the cancerous HSC-2 cells had a higher percentage of closure than the non-cancerous HF-1 cells.

Overall, as the concentration of apple extract increased the percentage of closure decreased. The addition of 75 ug/mL apple extract resulted in a closure of 60% for HF-1 and 38% for HSC-2. These results indicate that the addition of apple extract effectively mitigated the growth of the cancerous cells. At a concentration of 75 ug/mL there was a lower percentage of closure in HSC-2 than HF-1. This difference suggests that the cell line HSC-2 was more affected by the addition of apple extract than HF-1.

These findings indicate that the use of apple extract may be beneficial in the treatment of oral cancer. Further research is necessary to determine if specific oral cancers will require greater concentrations of apple extract. Different oral cancers may require various doses of apple extract to mitigate the growth of those cancerous cells.

Discussion

I- Implication of Preventing Metastasis

The experiment conducted in Dr. Schuck's lab suggests that apple extract is beneficial in preventing the growth and / or migration of cancer cells, but it did not kill any of the cells. The cells grew to 80-90% confluence in a monolayer on each well. Then a scratch was made along the diameter of each well to remove a section of the cells. After 24 hours of treatment with varying concentrations of apple extract, the cells were observed and the percent of closure of the gap was calculated. Maintaining a larger percentage of the gap represented a greater prevention of cell proliferation. However, the fact that there were not any new empty spots in the wells suggests that the cells were not killed by the addition of the apple extract. Higher concentrations of apple extract are necessary to kill the cancerous cells. This research

finding that non-cytotoxic levels of apple extract can still impede the growth / migration is an important finding. A critical component to caring for cancer patients is preventing the spread of the cancer to other areas of the body. Many cancer patients may start out with a cancer in one area of the body, but the cancer can metastasize to another location. The apple extract prevented the proliferation of the cancerous cells, so it may be able to help prevent an oral cancer from spreading to the neck or other locations in the patient's body. According to the Oral Cancer Foundation, the primary cause for cancer patient death is due to metastasis. Cells from the primary tumor can splinter off and develop an entirely new tumor elsewhere in the body. What may have started out as a small tumor in the oral cavity can turn into a lethal situation if the cancer spreads to the patient's lungs or brain. An important element in treating all cancer patients is attempting to remove or treat the tumor before it has the ability to travel through the patient's bloodstream or lymphatic system and grow a secondary tumor. Metastasis is particularly a concern for oral cancers. In a healthy cellular environment the epithelial cells adhere to one another and to the extracellular matrix. Cancer cells have fewer cadherin proteins, which are necessary to hold adjacent cells together. The lack of sufficient cadherin proteins enables a cancerous cell to break away from the primary tumor and invade another area of the body. Additionally, patients with oral cancer are at a heightened risk of developing secondary tumors. The reason is because saliva contains the molecule hyaluronic acid which can bind to a cell and enable it to escape the hold of the other adjacent cells. The saliva helps a cancerous cell break away from the primary tumor. Once the cancerous cell is free it can travel and develop into another tumor. The patient who was initially only dealing with one cancer in the oral cavity now has the challenge of additional tumors (The Oral

Cancer Foundation, n.d.). A difficulty in treating cancer patients is that it is not readily apparent if the cancer has spread to other areas. The patient may then receive treatment for the known cancer and only sometime later discover that the cancer had in fact spread to additional locations. The patient will then need to once again go through the pain and challenges of treating those newly discovered cancers. The research on nutraceuticals suggests that consuming the right produce may lower the risk of cancer metastasis. Specifically, apple extract may provide an effective means to prevent the spread of the oral cancerous cells. The survival rate of cancer patients will drastically increase when the medical community develops a means to prevent the metastasis of cancerous cells.

II- Further Research

There are numerous research studies on nutraceuticals and cancer. As noted above, research studies are conflicting regarding the role of reactive oxygen species. Some studies demonstrate that ROS maintain the growth of cancerous cells. They have also noted that antioxidants are beneficial in counteracting the effects of the ROS and, thereby, preventing the growth and metastasis of the cancer cells. Other researchers have noted that antioxidants are beneficial in preventing the initiation of cancer, but prooxidants are necessary to inhibit the growth of cancerous cells. The differences in the findings of whether antioxidants or prooxidants are necessary to prevent the growth of cancerous cells suggests that perhaps different cancers need to be treated individually. Research should focus on the source of the cancer and then determine if the cancerous growth can be curtailed by the addition of which specific nutraceutical.

The scratch assay analysis did not distinguish between proliferation and migration. The experiments demonstrated that as the concentration of apple extract increased a wider gap was maintained. The apple extract prevented the proliferation and / or migration of the cancerous cells. Further research should be conducted to determine if the apple extract prevented proliferation, migration, or both processes.

Previous research demonstrated that apple extract does not function as a prooxidant. Future research is necessary to determine how this nutraceutical successfully prevents the growth of oral cancer cells without harming healthy cells.

III- Determining the Appropriate Apple Extract Concentration

The research conducted in Dr. Schuck's lab suggests that healthy, non-cancerous cells and cancerous cells are impacted differently by the presence of the apple extract nutraceutical. When 75 ug/ mL of apple extract was individually added to the strains HF-1 and HSC-2 there was a quantitative difference in the percentage of closure of the gap. The same concentration of apple extract prevented the growth of cells to various extents. The HSC-2 maintained a wider gap than the HF-1 strain when the same concentration of apple extract was added to each cell type. This finding can have significant implications when applying the use of apple extract to treat or prevent the growth of various oral cancers. Further research will be necessary to determine the precise concentration of apple extract that will be necessary to curtail the growth of any given oral cancer. Whenever medications are administered to a patient there is a precise dose according to the specific disease and age or weight of the patient. Medication is only effective at combating the disease if the correct dose

is administered. The research on apple extract demonstrates that it will also be necessary to determine what concentration of the nutraceutical will need to be consumed. It will also be important to assess if there are differences in the necessary dosing of apple extract according to the site of the oral cancer. For example, a cancer located on the cheek may react differently to the nutraceutical than a cancer located in the gums.

IV- Administering the Nutraceutical *In Vivo*

The *in vitro* apple extract research conducted in Dr. Schuk's lab demonstrates potential for this nutraceutical to prevent the growth of cancerous cells. The apple extract was applied to cover the entire surface of the cancerous cells for 24 hours. Additionally, the cancerous cells in the Petri dish were in a monolayer. The apple extract was therefore able to be absorbed into the cancerous cells. When a higher concentration of apple extract was added there was a lower percentage of closure of the gap. This showed that the cancerous cells were impacted by the addition of this nutraceutical. This finding suggests promising results for the use of apple extract in oral cancer patients. Future research will need to be conducted to determine the effects of apple extract in an *in vivo* study. One point to consider is that apple extract will not be able to simply sit in a patient's mouth for 24 hours. The saliva in the patient's mouth will pretty quickly wash away any amount of the applied nutraceutical. It is therefore important to assess the level of benefit that the apple extract has on the oral cancer once the extract is swallowed and no longer concentrated in the oral cavity. Additionally, the actual growth of the cancer may not be a single monolayer as it is in the Petri dish. Therefore, it will be necessary to consider how the addition of the apple extract or any given

nutraceutical will be able to have an effect on the cancerous cells that are not on the surface of the mouth. When conducting an *in vivo* study it will be important to assess various means of administering the nutraceutical. In medicine there are various routes of drug administration, such as oral, topical creams, and injections. Each medication or vitamin is given in the manner that enables the compound to successfully accomplish the necessary cure or prevention. So too, it will be important to determine how to best administer apple extract to maximize the medicinal benefit of this nutraceutical.

V- Consuming Produce

The current research on apple extract suggests that there are medicinal benefits to consuming nutraceuticals. As seen in the research in Dr. Schuck's lab, the concentration of apple extract impacted the percentage of closure of the gap. In order to extend these research findings to a general suggestion that people consume produce, and specifically apples, it would be beneficial to assess how the consumption of nutraceuticals can successfully inhibit the growth of an oral cancer. It would also be important to assess how consuming an apple will be able to actually provide the necessary protection against the spread of oral cancer.

VI- Determining the Quantity of Apples

The research conducted in Dr. Schuck's lab demonstrated that higher doses of apple extract were more effective at preventing the proliferation / migration of the cancerous cells. The wells that contained the highest concentration of apple extract had the lowest percentage of closure of the scratch. This finding shows that the correct dose will need to be given to

cancer patients in order to gain the maximum benefit from this nutraceutical. Furthermore, in order to recommend that cancer patients consume apples or other fruits it is necessary to research how many apples will need to be consumed in order to gain the optimal medicinal benefit. If cancer patients would need to consume a large quantity of apples then this may not be a realistic recommendation. An alternative would be to explore a product similar to the apple extract that would be safe for cancer patients to drink. Additionally, it will be important to assess if peeling, cooking, or blending the apples reduces its medicinal qualities. In order to maximize the benefits of the apples it is important to know what forms of processing will negatively affect the anti-cancerous properties.

VII- Different Nutraceuticals Impact the Same Cancer in Different Ways

Different nutraceuticals can have different effects on a particular cancer. Some nutraceuticals can be water soluble while others are fat soluble. In order for the nutraceutical to have the desired beneficial impact it must be able to mix with the surroundings at the site of the cancer. It is therefore important to assess specifically which nutraceuticals are beneficial in preventing the spread of oral cancer. On a larger scale it is necessary to explore which nutraceuticals are helpful in curtailing the growth of any given cancer. The medicinal benefits vary amongst the different nutraceuticals. This may help to explain why there are some research studies that did not find a significant difference in the size of a tumor when administering a nutraceutical. It is possible that the nutraceutical they were using was not the right match for the cancer that they were working on. Further research should assess how

different nutraceuticals compare to one another in order to determine which nutraceuticals are most effective against which cancers.

VIII- Recommendations for Cancer Patients

In January 2020 I shadowed a group of genetic counselors at Hackensack University Medical Center. During one of the genetic counseling consultations with a cancer patient the genetic counselor discussed with her patient the importance of consuming an assortment of produce. The genetic counselor handed the cancer patient a brochure which outlined the benefits of cancer patients increasing the number of fruits and vegetables consumed each day. There is some debate amongst researchers if cancer patients should be consuming an increased quantity of produce while receiving treatments. If the cancer patient is receiving a form of treatment that increases the level of prooxidants in order to kill the cancerous cells, then it may be counterproductive for the patient to consume high levels of produce that contain antioxidants. The American Cancer Society notes that patients receiving chemotherapy or radiation therapy should discuss with their doctor before taking vitamins and minerals, as it can impact the efficacy of the cancer treatments (American Cancer Society Nutrition, n.d.). This recommendation is in line with the researchers that assert that antioxidants are beneficial in preventing the initiation of cancer, but prooxidants are the compounds that can help to prevent the continuous growth of cancerous cells. These cancer therapeutics increase the level of reactive oxygen species in the cancer cells in order to kill those cells. It would be interesting to further assess if there is a benefit for cancer patients to specifically consume the produce or vitamins that possess prooxidant properties.

IX- Natural Produce Versus Supplements

The American Cancer Society notes that if it is beneficial for a patient to consume nutraceuticals before, during, or after treatment then it is preferable to consume the natural fruits, vegetables, and herbs instead of supplements (American Cancer Society Nutrition, n.d.). This can be due to the fact that the body is more readily able to absorb the beneficial compounds found in the food products than in the form of a pill.

X- Organic Products

In addition to determining which particular fruits and vegetables are beneficial in preventing the initiation or metastasis of a given cancer it is also important to assess the difference between organic and conventionally grown produce. In 2006 Olson, et al. conducted an *in vitro* study of the antioxidants present in strawberries. The researchers assessed for the level of various antioxidants present in organic and conventionally cultivated strawberries. The organic strawberries contained higher levels of each measured antioxidant than the conventionally cultivated strawberries. The researchers found that both categories of strawberries prevented the growth of colon and breast cancer cells, but the organic strawberries had a statistically significant higher anti-proliferative activity than the non-organic strawberries (Olsson *et al.*, 2006). This research shows that organically grown produce may possess a significant advantage over conventionally grown produce in terms of their ability to prevent the growth of cancerous cells. The researchers found that a critical distinction between the group of strawberries was the level of antioxidants. This furthers the side of the argument that these antioxidants, as opposed to prooxidants, have an ability to

fight against cancer proliferation. It would seem to follow that if cancer patients are recommended to consume fruits and vegetables then it would be more beneficial for them to eat organically grown produce in place of the conventionally grown fruits and vegetables. Further research will be necessary to determine if the disparity in the level of antioxidants is present in other organic products in comparison to their conventionally grown counterparts.

XI- Nutraceuticals Decrease Medical Interventions

Any form of medical intervention poses a potential risk to the body. Oral cancer is often treated by surgically removing the cancerous cells and an additional protective layer of the adjacent, healthy cells. The surgery can result in complications for the patient because it is sometimes necessary to remove a portion of the patient's tongue or jaw bone. Radiation therapy presents the potential problem for inducing DNA damage, such that a healthy cell can then become cancerous. Chemotherapy also presents the challenge that healthy cells can be killed during treatment. These methods of treatment are commonly used to remove cancer from the body. It would be beneficial to the medical community if there would be methods that are less invasive and pose a lower risk of harm to the patient. Additionally, there are painful and uncomfortable side effects associated with surgery, radiation, and chemotherapy. In contrast, consuming fruits and vegetables is a natural means of allowing the body to heal itself and lower the proliferative activity of the cancerous cells. It is not likely that consuming specific produce or supplements will ever fully replace the current medical care for cancer patients. However, if a patient knew which particular food products to consume in order to prevent the continuous growth of the cancer then the patient may not require as large of a

surgery or as much radiation or chemotherapy. There is a significant medical difference in the health of a patient who has a relatively small versus a larger cancerous growth in the body. Nutraceuticals have the potential to lower the levels and intensity of medical interventions.

XII- Preventative Medical Approach

Consuming fruits and vegetables can prevent the initiation of cancer. Simply eating the right foods can save numerous lives each year. There are many research studies that show that nutraceuticals can prevent the initiation of cancer. Therefore, the research on nutraceuticals is not only applicable to those who are currently suffering from cancer. Nutraceutical research is an important field for all members of society, as the discoveries can prevent the onset of cancer. An apple a day may truly keep the doctor away.

XIII- Lowers Financial Costs

The research conducted in Dr. Shuck's lab on apple extract may serve as a means of preventing the proliferation and / or migration of oral cancer. This finding can significantly lower the financial costs of medical care for treating cancer patients. In the United States alone billions of dollars are exhausted each year caring for cancer patients. There are many other countries where even if the medical care is available the cancer patients do not have a means to pay for the extraordinarily expensive treatments. These impoverished individuals do not have any means of treating a cancerous growth. These patients are more likely not to survive even a small cancerous growth because they cannot afford the medical treatments. The research on nutraceuticals may significantly increase the survival rate of poor cancer

patients who cannot pay for radiation or chemotherapy. The research on apple extract may enable even the poorest members of society to live longer with oral cancer. Consuming apples is a significantly cheaper means of medical care. In the United States the poorest members of society are eligible for medicaid and the elderly receive medicare. However, there are still many individuals whose salaries are above the threshold to qualify for government paid insurance, but they still cannot afford their own medical insurance. This group of individuals do not possess any insurance and certainly cannot afford to pay for cancer treatments. Therefore, it is imperative to research effective cancer treatments that are more affordable. Consuming a few apples may be one way to increase treatment for all cancer patients.

XIV- Nutraceuticals Provide Cancer Care to Third-World Countries

Research on apple extract and other nutraceuticals will enable those who live in third-world countries to have a potential means to lower the rate of cancer deaths. There are many countries who still do not have any of the expensive radiation devices or doctors with the expertise to treat cancer patients. This means that even if citizens of those countries suspect that they have cancerous growths they do not have the doctors with the expertise or medical equipment to diagnose or treat these patients. There may not be any radiologists or surgeons in the nearby area, but they are far more likely to have access to fresh produce. If the scientific community can spread a global message about the importance of consuming apples then even those without access to medical facilities may see a decrease in the number of people dying from cancer.

XV- Impact on the Consumer Market

The research in nutraceuticals continues to develop and provide new insight into the tremendous benefits encapsulated in an assortment of fruits and vegetables. It is therefore important to consider how the consumer market will be impacted if specific produce are endorsed by doctors, and specifically by oncologists. The food industry will often advertise a product according to its potential health benefits. For example, Cheerios are advertised as a low cholesterol food which may be beneficial to those dealing with heart problems. This technique is beneficial to the public as the cereal prominently displays this information. Another example is V8 vegetable juice. The center of the label states in capital letters "essential antioxidants." V8 is not even mentioning the benefits of antioxidants, but by including the term "essential" they are marketing their product as one that contains compounds beneficial to one's health. If oncologists indeed recommend eating apples as part of a daily diet then the consumer market for apples may be positively affected. The marketing teams may be a means for the scientific community to spread awareness about the benefits of consuming apples. If they are successful in convincing customers to buy more apples then there will be a positive impact on those involved in growing and selling apples.

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