Antioxidants rescue carcinogen induced mitotic defects in both chromosomally stable and unstable cells

By

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ABSTRACT

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Tumor cells are characterized by an increase in genomic instability, brought about by both chromosomal rearrangement and chromosomal instability. Both of these broad changes can be induced by exposure to carcinogens. During mitosis, cells can exhibit early and late lagging chromosomes, multipolar spindles or anaphase bridges, all of which contribute to genomic rearrangement. We have studied the link between exposure to carcinogen and prevalence of mitotic defect in both chromosomally stable and unstable cell lines as well as examined the restorative effects of antioxidants in preventing mitotic defects. We have exposed MES-SA uterine cancer cells to vinyl chloride followed by exposure to an antioxidant: ascorbic acid, β-carotene, or lycopene. Treated cells were then scored for the prevalence of mitotic defects within the population and compared to controls. We have also investigated whether pre-treatment with the antioxidants will weaken the effects of carcinogen exposure in these cell lines.

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Introduction

Cancer is the second most common cause of death in the United States, exceeded only by heart disease, accounting for nearly 1 in every 4 deaths. According to the American Cancer Society, about 1,638,910 new cancer cases are projected to be diagnosed in 2012. Because of these startling statistics modern medicine has turned its focus towards improving treatments and preventative measures.

In the human body, most cells undergo a simple cycle of growth. They grow, divide, and then they die. This cycle allows for a continuous supply of fresh cells that replace the dying cells. Cancerous cells result when cells deviate from this normal cell cycle. In cancer, cells grow then divide without end. Due to the fact that highly regulated constraints that prevent normal cell growth do not limit cancerous cells, they continue to divide and outlive their counterparts leading to more abnormal cells.

Cancerous cells deviate from the normal rules of human cells. It is common for cellular defects to arise from imbalances in cell functioning resulting in genetic mutations, unrestrained proliferation, and abnormal cellular shape. These can result due to abnormalities found in common cellular events like the cell cycle, apoptosis and cell signaling (Evan &Vousden, 2001; Hanahan& Weinberg, 2000). Cancer cells display several common properties; in addition to the changes to their growth cycle they also tend to invade regions of normal, healthy cells (Hanahan& Weinberg, 2000; Ruoslahti, 1996). As cancer cells grow into areas of normal cells, they will destroy those cells and form an abnormal mass of cells called a tumor.

Often, an external element can act as catalysts for certain types of cancer. This external element is frequently a chemical agent that an individual is exposed to and is

designated as a carcinogen. A carcinogen is defined as any compound or agent that is involved in damaging or disrupting the normal processes of the cell and increasing its ability to proliferate (Loeb & Harris, 2008). Disease generally does not result from just one exposure but from continuous exposure. With this in mind, careful analysis of the effects of these external chemicals on cellular processes must be examined in order to understand their effects on the development of cancer.

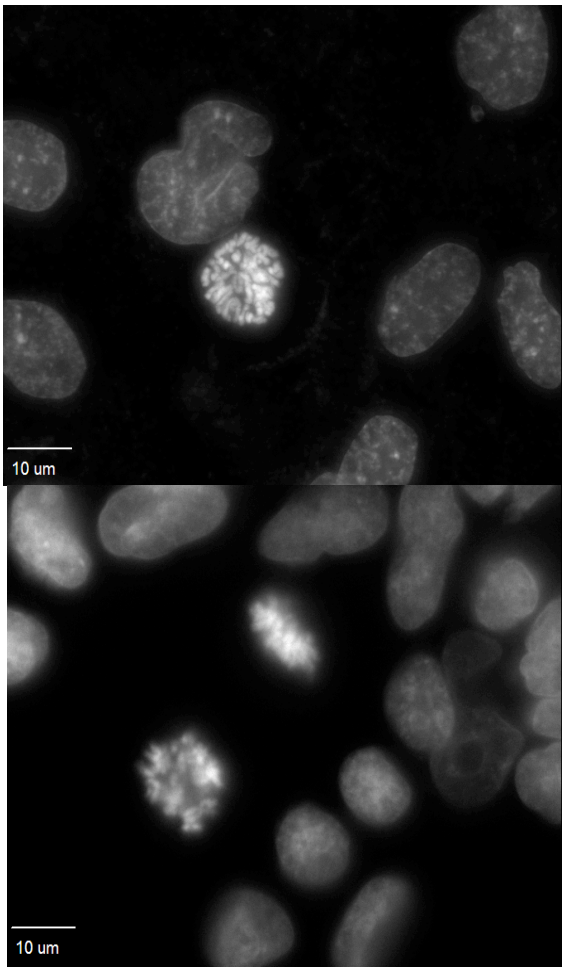
Carcinogen exposure can increase the likelihood of cancer via a number of mechanisms. One way is to increase the occurrence of mitotic defects within the cell, thus altering the genome. The three main types of defects exhibited are lagging chromosomes, multipolar spindles, and anaphase bridges (Hanahan& Weinberg, 2000; Saunders et al., 2000; Gollin & Reshmi, 2005). Some of these instabilities are associated with the formation of reactive oxygen species, which are known to damage DNA in an attempt to reduce themselves. Reactive oxygen species can be defined as a chemical species that contains oxygen and have reactive chemical properties. These commonly include free radicals like the hydroxyl radical and superoxide. These are produced when electrons escape from a given reaction pathway and react with molecular oxygen found in the cell. These reactive oxygen species are continually generated as long as the cell is functioning but are generally eradicated by other components of the cell's physiology (Toyokuni et al., 1995; Pelicano et al., 2004).

There is an increase in the build-up of the products associated with ROS reactions in cancer cells. This is evident in the detection of these products in the urine and plasma of cancer patients (Pelicano et al., 2004). Primary leukemia cells have been shown to posses a significant increase in reactive oxygen species when compared with normal

lymphocytes (Pelicano et al., 2004). By increasing the production of reactive oxygen species, the cancer cells are provided with a continual source of compounds that damage DNA (Toyokuni et al., 1995; Pelicano etal., 2004). This new source constantly promotes a cycle of genetic anomalies (Solomonet al., 1991; Ruoslahti, 1996; Hanahan & Weinberg, 2000).

Reactive oxygen species can therefore cause more damage in the cell which can lead to the development and progression of cancer. Reactive oxygen species can also act to enhance cellular proliferation by acting as messengers in a variety of signaling pathways throughout the cell (Pelicano et al., 2004). This would mean that any aberrant increase in ROS concentration could encourage cellular growth and proliferation in addition to the aforementioned role in promoting DNA damage and genetic instability (Toyokuni et al., 1995; Pelicano et al., 2004).

Mitosis is the process by which a eukaryotic cell separates the chromosomes in its nucleus into two identical sets, in two separate nuclei. It is generally followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal shares of these cellular components. Mitosis consists of five stages: prophase, prometaphase, metaphase, anaphase, and telophase. Examples of these cellular events are provided in Figure 1.



A

B

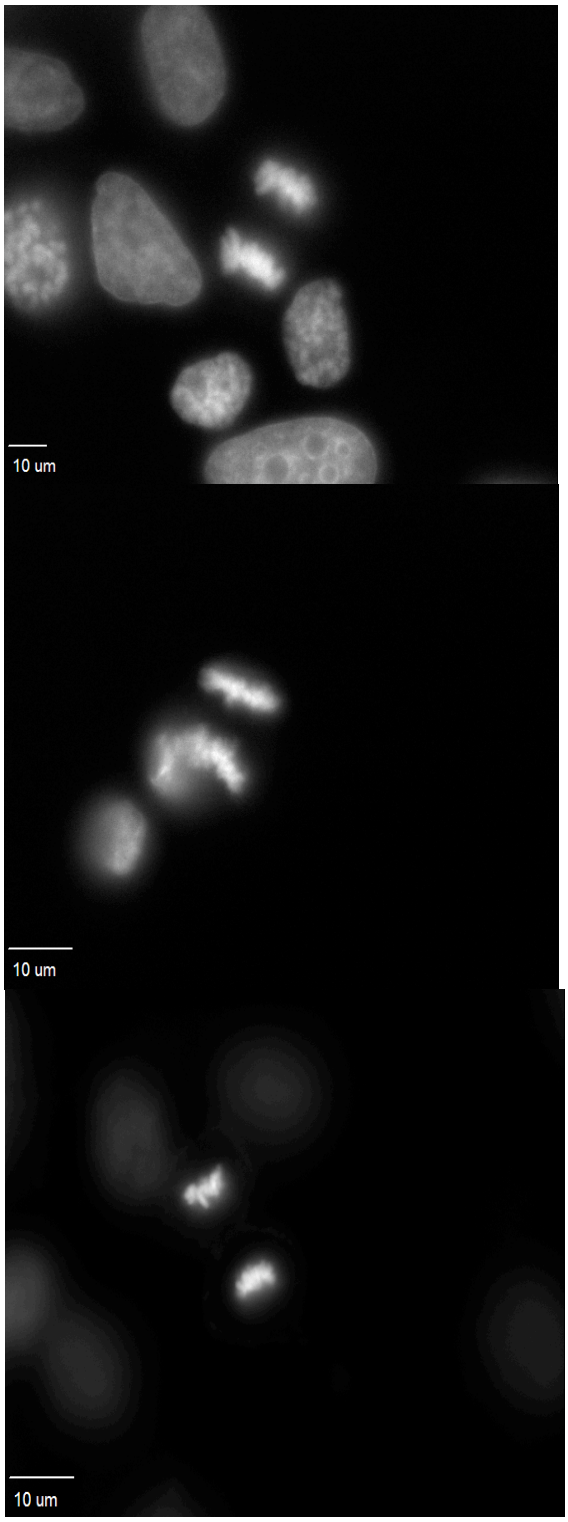


Figure 1: Representative images of MES-SA cells in mitosis stained with HOECHST.
A: Prophase, B: Prometaphase,
C: Metaphase, D: Anaphase, E: Telophase

During prophase the chromosomes condense and the nuclear envelope breaks down. During prometaphase microtubules attach to the kinetochore in a process termed search and capture. At metaphase, sister chromatids are aligned at the middle of the cell. Then, in anaphase chromosomes are pulled apart. Finally, telophase is defined by the rearrangement of microtubules, decondensation of chromatin and the reformation of the nuclear envelope, undoing what is seen in prophase. Cytokinesis, the process of splitting the cell into two, begins in anaphase and concludes in telophase. This series of heavily-controlled events occurs in a normal, healthy cell under strict regulatory control. However, when cells are cancerous or have been exposed to carcinogens there is an increase in the number of mitotic defects present in the cell. Mitotic defects result when different mechanical issues occur during mitosis.

The most common mitotic defects are anaphase bridges, lagging chromosomes and multipolar spindles. Anaphase bridges occur when chromosomes are pulled in the direction of both spindle poles by the microtubules during chromosome segregation, during anaphase in mitosis. These result from the fusion of broken chromosome ends, leading to the formation of dicentric chromosomes (Luo et al., 2004). A Lagging chromosome is either a whole chromosome or portion thereof that has been lost due to the failure of the microtubules to attach to the chromosomes (Gollin&Reshmi, 2005). These lagging chromosomes can lead to the formation of micronuclei. Recent studies show that there are two different types of lagging chromosomes occurring during the first three stages of mitosis including prophase, prometaphase, and metaphase and the late lagging chromosomes occurring during the anaphase and telophase stages of mitosis (Compton, 2010). Multipolar spindle formation occurs when there is a deregulation in the

number of centrosomes and the failure of the regulatory machinery that maintains spindle pole number, eventually leading to the formation of aneuploid cells (Saunders et al., 2000; Quintyne et al., 2005; Kwon et al., 2008). In all of these cases, the outcome can be that the new cells will have extra or fewer copies of particular chromosomal regions than in normal cells.

Vinyl Chloride is a known carcinogen. The chemical naturally occurs as a sweet smelling, colorless gas that is soluble in water. It is used primarily in the industrial production of plastics and is used to make polyvinyl chloride (PVC), which is used in a variety of consumer products. Vinyl chloride has been linked to a variety of cancer types such as liver, brain, lung, and the lymphatic system (Viola et al., 1971; Velez-Cruz et al., 2005). Further evidence linking vinyl chloride to cancer has been provided by numerous experimental studies involving the exposure of laboratory animals (Maltoni et al., 1981). Exposure to vinyl chloride of the general public occurs in a variety of ways. Individuals can be exposed by inhaling contaminated air, consuming drinking water that is contaminated, or by exposure to the skin (Viola et al., 1971; U.S. Department of Health & Human Services, 2001). It has been estimated by the United States government that approximately 1 million pounds of vinyl chloride is released into the environment each year (U.S. Department of Health & Human Services, 2001).

A helpful opponent against reactive oxygen species are antioxidants. An antioxidant is any molecule capable of inhibiting the oxidation of other molecules. These compounds act as reactive oxygen species scavengers and aid the cell in reducing the harmful oxygen species to a less destructive state. They are defined as any type of molecule that is able to terminate a series of reactions that involve the transfer of

elections from one substance to a reactive agent. Examples of antioxidants are Ascorbic Acid, β-Carotene, and Lycopene.

Ascorbic acid is commonly known as Vitamin C. Vitamins are one of the primary compounds responsible for scavenging reactive oxygen species and also for stimulating the activity of reductive enzymes commonly found in human body cells (Fang et al., 2002). Vitamin C is commonly found in citrus fruit such as oranges, lemons, and grapefruits. This vitamin is an essential nutrient for humans and helps to prevent diseases like scurvy. The primary component of its chemical makeup is the ascorbate ion, which is an antioxidant and a co-factor in the upregulation of several vital enzymes (Maramag et al., 1997; Fang et al., 2002).

β-Carotene is a type of carotenoid found in many plants. β-Carotene belongs to a category called tentraterpeniod and are commonly found in the form of a polyene chain. β-carotene has been shown to react with peroxyl radical to form a stabilized oxygen species. By terminating the destructive chain of chemical reactions required to reduce reactive oxygen species to an nonharmful state, this carotenoid exhibits strong anti-oxidant properties (Burton &Ingold, 1984; Fang et al, 2002).

Lycopene is a red carotenoid pigment present in tomatoes and many berries and fruits. Previous studies indicate that lycopene can afford protection against DNA damage induced by xanthine, a carcinogen, at low concentrations of 1-3 μM (Lowe et al., 1999). Lycopene may also protect against the onset of prostate cancer, as demonstrated by both *in vivo* and *in vitro* studies (Hall, 1996).

The goal of this project is to understand the correlation between exposure to specific carcinogenic chemicals and antioxidants cocktails on mitotic defects in order to gain a better idea of cancer progression.

Methods

Cell Culture

MES-SA cells obtained from the American Type Cell Culture Collection (ATCC; Manassas, VA) were grown in McCoy's 5A Medium (Sigma-Aldrich Chemical Company; St. Louis, Missouri) supplemented with 60 ml FBS (Sigma) and 5.5 ml Penicillin-Streptomycin (MP Biomedical; Solon, Ohio). Cells were grown at 37°C in 5% CO_2. The cells were passaged with 0.25% Trypsin-EDTA (Sigma).

Immunofluorescent staining and slide preparation

Cells were seeded on coverslips and allowed to incubate overnight. Medium was removed and the appropriate carcinogen/antioxidant containing media was added and the cells were allowed to incubate for 24 hours. If a second treatment was required, cells were washed and the second media-with additives was applied for 24 hours. The same protocol was used for a third treatment as necessary. Upon completion of carcinogen or antioxidant treatment, coverslips were fixed with cold methanol.

Cells were stained with Hoechst 33342 (AnaSpec, San Jose, California) in order to stain chromatin. An Olympus IX-81 Inverted Fluorescence Microscope (Olympus America Incorporated, Center Valley, Pennsylvania, 100x objective, N.A.=1.65) was used to analyze the immunofluorescent cells, with a Hammamatsu C4742-95 CCD Camera (Hammamatsu Corp Bridgewater, New Jersey) and Slidebook 5.0 software (Intelligent Imaging Innovations Incorporated, Denver, Colorado).

Antioxidant Stock Solution Preparation:

- *Vitamin C (Ascorbic Acid):*
    - The stock solution was prepared by creating a 100 mM solution. This was accomplished by adding 0.88g of powdered Vitamin C (Sigma) to 50 mL of growth medium. From this stock solution, dilutions were prepared and used in experiments.

- *β-Carotene:*
    - The stock solution was prepared by creating a 10 mM solution. This was accomplished by adding 0.2685g of powdered β-Carotene (Sigma) to 50 mL of growth medium.
- *Lycopene:*
    - The stock solution was prepared by creating a 500x solution. This was accomplished by adding 1 mg of powdered lycopene(Sigma) to 3.724 ml of nanopure water.
- *Ascorbic Acid/Lycopene:*
    - Cocktails were made by mixing 1 ml of the Ascorbic Acid stock solution and 5 µl of the lycopene stock solution.
- *β-Carotene /Ascorbic Acid:*
    - Cocktails were made by mixing 1 ml of the β-Carotene stock solution and 1 ml of the Vitamin C stock solution.

Carcinogen Stock Solution Preparation:

- *Vinyl Chloride:*
    - The stock solution was prepared by creating a 10 mM solution. This was accomplished by adding 7.8 µl of liquid Vinyl Chloride (Restek, Bellefonte, Pennsylvania) to 50 mL of growth medium.

Antioxidant trials

A medium was prepared that was specific to a particular antioxidant; Ascorbic Acid, Lycopene, or β-Carotene. From stock cell cultures, cells were pipetted into a six-well plate, on top of glass coverslips, and allowed to grow for 24 hours. After 24 hours of untreated, regular cell growth, the medium was replaced with the antioxidant solution. Slides were then prepared as described. The resulting effects of the antioxidant solution on the cells were analyzed by counting 50 mitotic cells per slide and scoring the number of those cells that showed defects.

Carcinogen trials

From stock cell cultures, cells were pipetted into a six-well plate, on top of glass coverslips, and allowed to grow for 24 hours. After 24 hours of untreated, regular cell growth, the media was replaced with vinyl chloride containing solution for 24 to 48 hours. Slides were then prepared as described in the fluorescent microscopy section. The resulting effects of the carcinogenic solution on the cells were analyzed by counting 50 mitotic cells per slide and scoring the number of those cells that displayed defects.

Antioxidant rescue of carcinogen-induced defects

From stock cell cultures, cells were pipetted into a six-well plate, on top of glass coverslips, and allowed to grow for 24 hours. After 24 hours of untreated, regular cell growth, the medium was replaced with the carcinogenic solution for 24 hours. The carcinogenic stock solution was then replaced by either an antioxidant solution or antioxidant cocktail. Slides were then prepared and scored as previously described.

Antioxidant pre-treatment of carcinogen-induced defects

From stock cell cultures, cells were pipetted into a six-well plate, on top of glass coverslips, and allowed to grow for 24 hours. After 24 hours of untreated, regular cell growth, the medium was replaced with an antioxidant solution for 24 hours. The antioxidant solution was then replaced by a carcinogenic solution for 24 hours and then prepared and scored as before.

Results

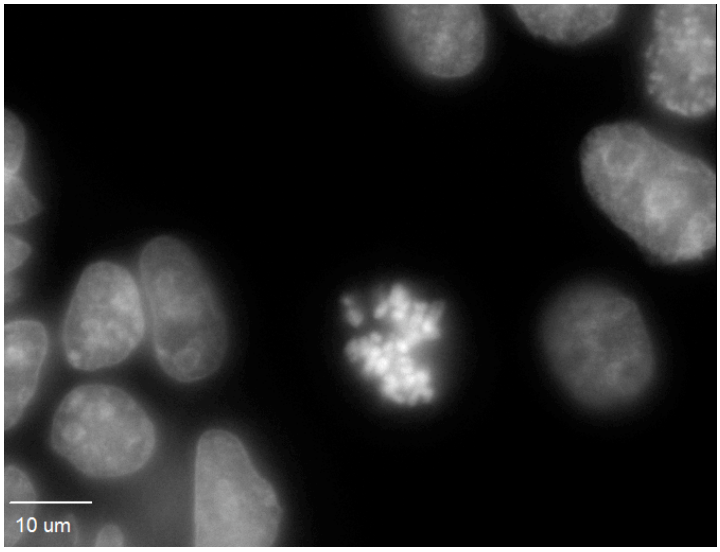
In order to examine the effects of antioxidants and carcinogens on mitotic defects, we defined four distinct types of defect based upon HOECHST staining of chromosomes. The first was early lagging chromosomes (Figure 2A). Cells exhibiting this defect showed a number of chromosomes that had drifted from the early three phases of mitosis. Because these chromosomes drifted too far from where the spindle poles and microtubules can recapture this genetic material it will likely fail to be split evenly amongst the daughter cells, with either both copies of the chromosome going to a single cell, or being lost.

The second type of defect was the late lagging chromosome (Figure 2B). Cells exhibiting this defect showed a number of chromosomes that had not been captured by the spindle poles and microtubules during the last two stages of mitosis. Because these chromosomes missed the opportunity to be captured by the spindle poles, this genetic material will be lost upon completion of cytokinesis.

The third type of defect was the multipolar spindle pole formation (Figure 2C). Extra spindle poles have formed within the cell and this results in the chromosomes being pulled in multiple directions. Subsequently, there is an uneven distribution of genetic material between the daughter cells. This means that a loss of function or amplification of certain genes can occur (Lengauer et al., 1998; Saunders et al., 2000).

The fourth defect was anaphase bridges (Figure 2D). These occur when microtubules from opposite spindle poles attach to a single chromosome or fused pair of sister chromatids, and it is subsequently pulled towards both poles. This stress of pulling results in these chromosomes breaking, leading to a loss of function or amplification of

certain genetic traits in the new daughter cells. We used these four types of mitotic defects as the primary criteria, to examine and analyze the results of other experiments.



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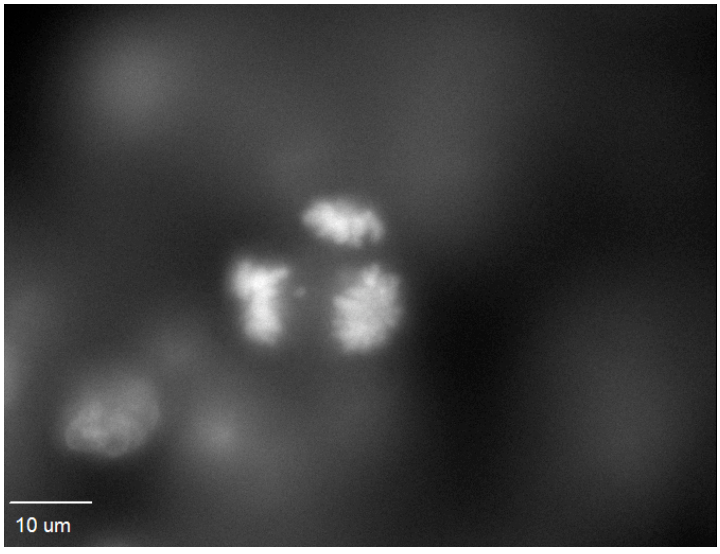

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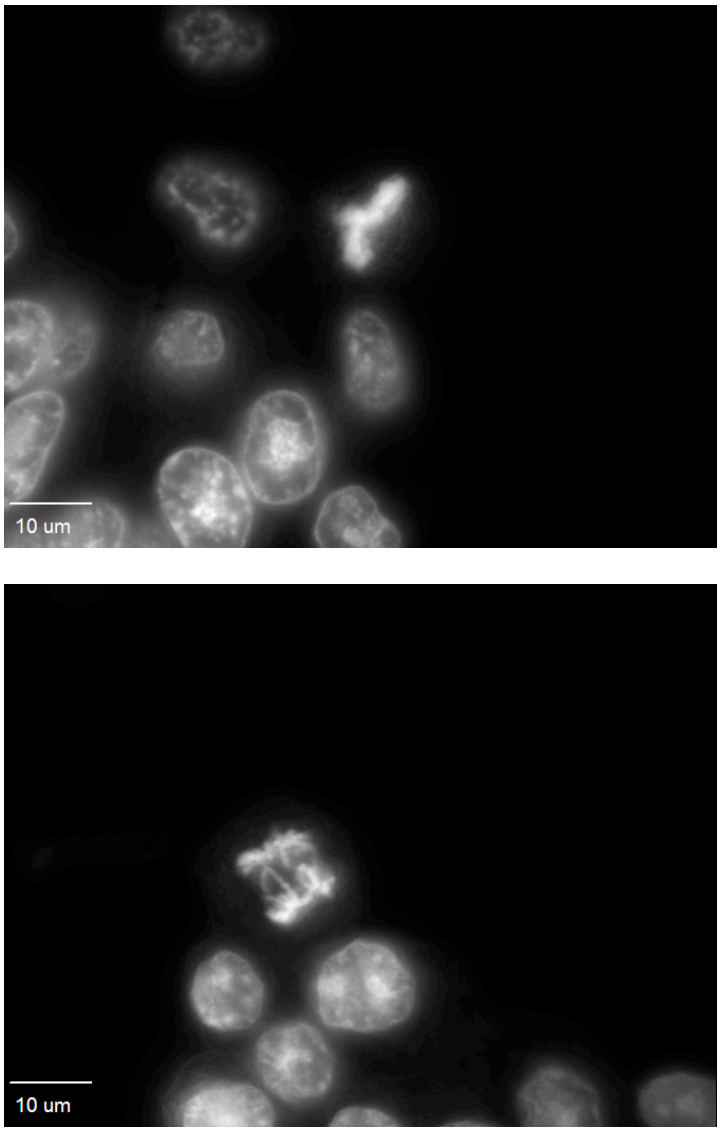

Figure 2: Representative images of mitotic defects stained with HOECHST. A: Early Lagging Chromosome, B: Late Lagging Chromosome, C: Multipolar, D: Anaphase Bridge

With these scoring criteria established, we wanted to determine the number of mitotic defects that are commonly found in the cancerous cell line MES-SA. Additionally, we wanted to establish during which phase of mitosis these defects occurred. Untreated MES-SA cells have a mitotic index of 7%. This mitotic index indicates that in any total given population that 7 percent of all the cells will be in mitosis. This high mitotic index

is indicative of a cancerous cell line. When untreated MES-SA cells were scored, we saw early lagging chromosomes in 2.4% of mitotic cells, late lagging chromosomes in 2.3% of mitotic cells, multipolar defects in 2% of mitotic cells and anaphase bridges in 8% of mitotic cells (Table 1).

Table 1: Percentage of mitotic cells exhibiting defects in untreated MES-SA cells

	Normal	Lagging	Multipolar	Bridges
Prophase	46.0	1.7	1.0	N/A
Prometaphase	10.0	0.0	0.3	N/A
Metaphase	9.3	0.7	0.0	N/A
Anaphase	3.0	2.0	0.0	7.0
Telophase	17.7	0.3	0.3	7.0

Next, we wanted to examine the effects of vinyl chloride treatment on the frequency of mitotic defects. Cells were incubated in a vinyl chloride containing medium for 48 hours and then processed for analysis. Treatment with vinyl chloride increased the number of early lagging chromosomes from 2.4% to 11% and increased the number of anaphase bridges from 8% to 14%. This carcinogen did not alter the frequency of late lagging chromosomes (3%) and multipolar (1%) defects (Table 2).

Table 2: Mitotic defects observed in MES-SA treated with Vinyl Chloride for 24 and 48 hours

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	8.5	1.0	1.0	N/A
Prometaphase	20.0	2.5	1.0	N/A
Metaphase	11.0	1.0	0.0	N/A
Anaphase	6.0	1.0	0.0	3.5
Telophase	3.5	0.0	0.0	1.5

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	17.0	2.0	0.0	N/A
Prometaphase	11.0	2.0	0.0	N/A
Metaphase	15.0	5.0	1.0	N/A
Anaphase	5.0	2.0	0.0	11.0
Telophase	8.0	0.0	0.0	0.0

For the next experiment, we wanted to establish if the antioxidants had any restorative properties. Cells were grown in the antioxidant solutions for 48 hours and were processed for analysis, which were then compared to controls. Compared to the control, β-Carotene treatment showed a reduction in early lagging chromosomes from 1.7% to 0.0%, while other defects were consistent with control data (Table 3). Ascorbic acid treatment showed a reduction in anaphase bridges from 8% to 6% (Table 4). Finally, lycopene treatment showed a reduction in anaphase bridges from 8% to 5% although an increase in early lagging chromosomes from 2.4% to 9% was observed (Table 5).

Table 3: Percentage of MES-SA cells exhibiting mitotic defects after being treated with β-Carotene for 24 and 48 hours.

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	16.0	3.5	1.0	N/A
Prometaphase	4.0	0.5	3.5	N/A
Metaphase	8.5	0.5	1.0	N/A
Anaphase	6.5	1.0	0.5	4.0
Telophase	1.5	0.5	0.0	1.0

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	7.0	0.0	1.0	N/A
Prometaphase	3.0	0.0	2.0	N/A
Metaphase	3.0	0.0	0.0	N/A
Anaphase	6.0	2.0	0.0	7.5
Telophase	2.0	1.0	0.0	0.0

Table 4: Mitotic defects observed in MES-SA after treatment with Ascorbic Acid for 24 and 48 hours

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	12.0	1.0	0.0	N/A
Prometaphase	7.0	0.0	5.0	N/A
Metaphase	13.0	1.0	0.0	N/A
Anaphase	7.0	4.0	0.0	5.0
Telophase	0.0	0.0	0.0	1.0

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	17.0	1.0	0.0	N/A
Prometaphase	3.0	0.0	2.0	N/A
Metaphase	7.0	0.0	1.0	N/A
Anaphase	14.0	1.0	0.0	3.0
Telophase	1.0	0.0	0.0	0.0

Table 5: Mitotic defects in MES-SA cells treated with Lycopene for 24 hours and 48 hours.

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	21.5	1.5	2.0	N/A
Prometaphase	9.5	0.0	0.5	N/A
Metaphase	9.0	1.0	0.0	N/A
Anaphase	1.5	1.5	0.0	2.5
Telophase	4.5	0.0	0.0	0.0

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	14.5	2.5	0.5	N/A
Prometaphase	5.5	0.5	0.0	N/A
Metaphase	21.5	2.5	0.5	N/A
Anaphase	3.5	1.0	0.5	3.0
Telophase	3.0	0.0	0.0	0.0

For the next set of experiments, we wanted to establish if there was any type of cumulative or combinatorial restorative properties for antioxidant cocktails. Cells were grown in the antioxidant cocktail solutions for 48 hours and were processed for analysis and comparison as before. Several concentrations and combinations of antioxidants proved to be lethal to the cells. Compared to the untreated control, treatment with a cocktail of β-Carotene and Ascorbic Acid showed a reduction in anaphase bridges from 8% to 3.7%. This treatment also showed a reduction in early lagging chromosomes from 2.4% to 1.8% and a reduction in late lagging chromosomes from 2.3% to 1.8%. There were no changes observed in multipolar defects (Table 6). MES-SA cells were then exposed to a cocktail of Lycopene and Ascorbic Acid for 48 hours which showed a reduction in anaphase bridges from 8% to less than 1% as well as a reduction in late lagging chromosomes from 2.3% to 0% (Table 7).

Table 6: Percentage of mitotic MES-SA cells with mitotic defects after simultaneous treatment with β-Carotene and Ascorbic Acid for 24 hours and 48 hours.

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	7.0	0.5	0.5	N/A
Prometaphase	3.0	0.5	2.5	N/A
Metaphase	12.0	1.0	1.5	N/A
Anaphase	7.0	1.0	0.5	5.0
Telophase	2.5	0.5	0.0	1.5

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	12.0	1.0	0.0	N/A
Prometaphase	3.0	0.0	1.0	N/A
Metaphase	13.0	0.0	2.0	N/A
Anaphase	10.0	1.0	0.0	2.0
Telophase	9.0	0.0	0.0	0.0

Table 7: Mitotic defects in MES-SA cells treated with Lycopene and Ascorbic Acid for 24 hours and 48 hours.

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	21.5	3.5	0.5	N/A
Prometaphase	12.0	1.0	1.5	N/A
Metaphase	1.5	0.0	0.0	N/A
Anaphase	2.0	0.0	0.0	5.5
Telophase	7.0	0.0	0.0	0.0

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	16.5	5.0	0.5	N/A
Prometaphase	9.5	1.0	1.0	N/A
Metaphase	10.5	0.5	0.5	N/A
Anaphase	5.0	0.0	0.5	0.5
Telophase	5.0	0.0	0.0	0.0

Next, we wanted to see if pre-treating cells with a cocktail of antioxidants before exposure to carcinogen would have a preventative effect. We exposed MES-SA cells to a cocktail of β-Carotene and Ascorbic Acid for 24 hours followed by treatment with vinyl chloride for 24 hours. The cells were processed and mitotic defect frequencies were compared to those found in cells treated with only vinyl chloride. Here, we saw a

reduction in early lagging chromosomes from 11% to 1.4%, but no effects on any of the other mitotic defects (Table 8).

Table 8: MES-SA cells exhibiting mitotic defects after treatment with β-Carotene and Ascorbic Acid for 24 hours followed by treatment with vinyl chloride for 24 hours.

	Normal	Lagging	Multipolar	Bridges
Prophase	28.0	1.0	1.0	N/A
Prometaphase	6.0	0.0	4.0	N/A
Metaphase	8.0	0.0	1.0	N/A
Anaphase	6.0	3.0	0.0	9.0
Telophase	1.0	0.0	0.0	0.0

In addition to observing any preventative effect, we wanted to see if antioxidants had a restorative effect on cells that had been exposed to carcinogen. Therefore, we treated cells with a cocktail of antioxidants after being treated with a carcinogen. We exposed MES-SA cells to vinyl chloride solution for 24 hours and then replaced this solution with a cocktail of β-Carotene and Ascorbic Acid for 48 hours. Compared to vinyl chloride control, this treatment reduced the number of early lagging chromosome from 11% to 1.0% but had no effects on any of the other mitotic defects (Table 9).

Table 9: MES-SA cells exhibiting mitotic defects after treatment with vinyl chloride for 24 hours followed by treatment with β-Carotene and Ascorbic Acid for 24 hours.

24 hours	Normal	Lagging	Multipolar	Bridges
Prophase	17.0	2.0	0.0	N/A
Prometaphase	9.0	1.0	1.0	N/A
Metaphase	10.5	1.0	2.5	N/A
Anaphase	7.0	1.0	0.0	7.0
Telophase	2.5	0.0	0.0	2.5

48 hours	Normal	Lagging	Multipolar	Bridges
Prophase	28.0	1.0	1.0	N/A
Prometaphase	6.0	0.0	4.0	N/A
Metaphase	8.0	0.0	1.0	N/A
Anaphase	6.0	3.0	0.0	9.0
Telophase	1.0	0.0	0.0	0.0

For the final set of experiments, we wanted to observe any changes in frequency of mitotic defect under conditions of both preventative and restorative treatment with antioxidants. We treated MES-SA cells with β-Carotene and Ascorbic Acid for 24 hours followed by treatment with vinyl chloride for 24 hours and then β-Carotene and Ascorbic Acid again for 24 hours. Compared to the control, this treatment shows a reduction in anaphase bridges from 13% to 9.5%, a reduction in late lagging chromosomes from 4.4% to 0%, and a reduction in multipolarity from 9% to 4.7% (Table 10).

Table 10: Mitotic defects in MES-SA cells treated with β-Carotene and Ascorbic Acid for 24 hours followed by treatment with vinyl chloride for 24 hours and then with β-Carotene and Ascorbic Acid for 24 hours.

	Normal	Lagging	Multipolar	Bridges
Prophase	15.0	1.0	0.0	N/A
Prometaphase	3.5	0.0	1.0	N/A
Metaphase	8.0	0.5	1.0	N/A
Anaphase	6.5	0.0	0.0	2.5
Telophase	1.5	0.0	0.0	1.5

Discussion

We chose to use the uterine cancer cell line MES-SA was because we could see all four distinctive types of mitotic defects within the mitotic population (Figure 2). All of these defects can lead to a number of problems for the cells and can cause the cell to deviate from the normal restraints of metabolism.

When discussing a correlation between the occurrence of specific mitotic defects when treated with a carcinogen and a reduction in specific mitotic defects when treated with an antioxidant, we were able to establish baseline levels of mitotic defects in the untreated MES-SA cells. Furthermore, we could increase specific mitotic defects in response to vinyl chloride, meaning our model system of cell line and carcinogen was a valid choice and we could be confident that it would be straightforward to observe changes in defects under our proposed antioxidant experiments. Specifically, treated cells showed a marked increase in the number of early lagging chromosomes and an increase in the number of anaphase bridges when treated with the carcinogen. Interestingly, we saw no change in the rate of multipolarity, which may be due to the lack of extra centrosomes, which are a requirement for high levels of multipolarity (Quintyne et al., 2005).

When we treated our cells with antioxidants, we saw that all the antioxidants reduced at least one defect. However, we did not see the same effect with each antioxidant: when the cells were treated with Ascorbic Acid or lycopene, we saw a reduction in the frequency of anaphase bridges, but when cells were treated with β-Carotene for 48 hours the number of early lagging chromosomes were reduced. This

suggests that each antioxidant is likely more effective in scavenging particular ROS species from different reactions.

When discussing a correlation in the reduction of specific mitotic defect when treated with a cocktail of antioxidants, we determined that several cocktails reduced at least one defect, including defects that were not affected by either of the antioxidants when used alone. When the MES-SA cells were treated with β-Carotene and Ascorbic Acid, there was a reduction in anaphase bridges and early lagging chromosomes as was seen with the individual treatments. However, we also saw a decrease in the frequency of late lagging chromosomes. Similarly, when the cells were treated with lycopene and Ascorbic Acid there was a reduction in the number of anaphase bridges, as was seen for both antioxidants when used on their own, but also a reduction in the number of late lagging chromosomes. This additive effect is of particular note, since it suggests that if the antioxidants target specific ROS species, lagging chromosomes are produced by a wider variety of those harmful species, making them less susceptible to prevention. This result could also be due to the prolonged exposure or increased concentration of the antioxidants to the cell. Alternatively, this could be a result of the total concentration of antioxidants used on the cells.

When we examined the antioxidant pretreatment of cells prior to exposure to vinyl chloride, we again saw a reduction in the number of mitotic defects. Both β-Carotene and Ascorbic Acid were able to reduce the rate of early lagging chromosomes. This likely means that the antioxidants are blocking the effect of the vinyl chloride treatment. Curiously, we did not see a decrease in the frequency of anaphase bridges in the Ascorbic Acid experiment as we had observed when normal cells were treated with

that antioxidant. This outcome may be due to an increase in the number of DNA breaks caused by the exposure to vinyl chloride. Under such conditions, Ascorbic Acid may be incapable of preventing all of those breaks.

Next, we examined antioxidant rescue of pretreated carcinogenic cells. Cells that were exposed to vinyl chloride for 24 hours and then treated with β-Carotene and Ascorbic Acid for 48 hours showed a drastic decrease in the numbers of mitotic defect. Based on these results, we see that a cocktail of antioxidants does have the ability to rescue the cells after treatment with a carcinogen and that multiple antioxidants are more efficient at scavenging ROS species. Compared to the previous results for the pretreatment of this cocktail β-Carotene and Ascorbic Acid, we see that regardless of whether we add the antioxidant cocktail before or after the carcinogen the antioxidants have a profound effect on blocking the harmful activity of the carcinogen.

Finally, we wanted to see if treatment with antioxidants both before and after carcinogen exposure would increase the cell's resistance to damage. Here, we saw very strong effects, with three out of four mitotic defects (late lagging chromosomes, anaphase bridges and multipolar spindles) showed significant decreases in their frequencies. This shows that pretreatment and post-treatment of an antioxidant cocktail is highly effective in reducing the number of mitotic defects in cancer cells. We could extend these results to suggest that antioxidants are a viable source as both a powerful treatment for cancer as well as a preventative measure to be taken. A diet which contains a variety of different antioxidant-rich foods may well help prevent cancer from developing as well as decrease the rate at which a tumor progresses if one has begun to form. Translation from *in vitro* to *in vivo*, however, is challenging as it is important to fully understand if the antioxidant

effects observed are due to the prolonged exposure of the antioxidants to the cell or the concentration of antioxidants used. It will be important to further these studies by examining more combinations of antioxidants and different carcinogenic molecules, as well as by further varying the concentrations and times of exposure for both harmful and helpful molecules on the cells.

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