Efficacy of Antioxidants in Treating Chemotherapy-Induced Toxicity

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Most chemotherapy drugs kill cancer cells by inhibiting the metabolic functions of those cells. In the process of killing cancer cells, chemotherapy drugs can also act upon healthy cells, which gives rise to adverse side effects. Current studies have examined the cytotoxic effect of a cancer drug, 5-fluorouracil, and the effect of a combination of vitamins A, E, and melatonin on abating oxidative stress on cellular membranes, thus minimizing toxicity. This study uses spot plating, microscopic analysis, and liquid turbidity test to examine the effects of antioxidants on 5-fluorouracil. Spot plating provides a visual representation of 5-fluorouracil's influence on cell growth in various conditions, over the period of one day. The microscopic analysis assesses the membrane integrity of cells at 2500x magnification. Liquid media tests provide a statistical representation of the cell growth trend (OD_{600}) in two hours. The addition of a combination of vitamins A, E, and melatonin prevented 94% of cell death caused by 5-fluorouracil, providing new insights into chemotherapy treatment. This study suggests that 5-fluorouracil toxicity is due to oxidative stress on cell membranes.

I. INTRODUCTION

During normal biochemical reactions, most of the oxygen taken up by the cells is converted to water by the action of cellular enzymes. However, under the exposure to most chemotherapy drugs, some of these enzymes leak electrons into oxygen molecules and lead to the formation of free radicals [2]. The various groups of these free radicals are collectively called reactive oxygen species (ROS). Although DNA is a stable and well-protected molecule, ROS can interact with it and cause several types of damage that result in cell death [2]. All forms of life maintain a steady concentration of ROS determined by the balance between their rates of production and removal by various antioxidants. However, this balance can be disturbed when the level of ROS exceeds its removal, or the level of antioxidants is diminished. This state is called "oxidative stress" and can result in serious damage to normal cells if the stress is substantial and persistent [5]. One approach in addressing this problem is the addition of a combination of certain antioxidants at specific dosages to lessen oxidative stress, thus alleviating the side effects of chemotherapy. Antioxidants neutralize free radicals by donating one of their own electrons and ending the electron "stealing" reaction [2]. This helps to prevent ROS-mediated cell and tissue damage as antioxidants neutralize the electrical charge and prevent the free radical from taking electrons from other molecules [2].

5-Fluorouracil is an antimetabolite chemotherapy drug that is commonly used for the treatment of leukemia, and cancers of the breast, ovary, and intestinal tract [3]. Despite its broad spectrum of anticancer activity against malignancies, 5-fluorouracil still presents numerous side effects due to it being cytotoxic. 5-Fluorouracil kills cancer cells by acting as a substitute for the normal building blocks of RNA and DNA. When this happens, DNA cannot replicate, and a cell cannot reproduce [1]. 5-Fluorouracil is converted into several active metabolites in the cell that can interfere with the synthesis and function of DNA and RNA [3]. 5-fluorouracil frequently results in myelosuppression, dermatitis, and cardiac toxicity, and it may cause toxicity in the gastrointestinal tract for 40–80% of patients [3]. Furthermore, 5-fluorouracil and its metabolites can also lead to the production of ROS, with subsequent release of pro-inflammatory cytokines, causing oxidative stress that leads to inflammation of the intestinal tissue [3]. 5-fluorouracil has various adverse effects such as cardiotoxicity, nephrotoxicity, and hepatotoxicity that restrict its extensive clinical usage. It causes marked organ toxicity combined with increased oxidative stress and apoptosis [4].

To address the potential negative effects of oxidative stress caused by 5-fluorouracil, we looked at three antioxidants: vitamin A, vitamin E, and melatonin. Previous studies have demonstrated that when used individually, vitamin A [6], vitamin E [7], and melatonin [8] decreased chemotherapy-mediated toxicity, enabled higher therapeutic response rate, and increased survival time in terminal cancer patients [6,7,8]. However, little research has been done to investigate the efficacy of a combination of vitamin A, vitamin E, and melatonin in inhibiting cytotoxicity induced by 5-fluorouracil. The antioxidant vitamin A prevents cell apoptosis caused by oxidative stress. It does this by activating signal-regulating pathways and interacting with ROS and acting as a scavenger of free oxygen radicals [2]. Vitamin E neutralizes free radicals by donating one of its own electrons to prevent cell and tissue damage caused by ROS [2]. And antioxidant melatonin corrects reduced glutathione concentration, thereby preserving membrane fluidity and relieving oxidative stress [2].

Given the potent effects of 5-fluorouracil toxicity on healthy cells, can a combination of antioxidants vitamin A, vitamin E, and melatonin in synergy with 5-fluorouracil abate oxidative stress on cellular membranes and subsequent cytotoxicity better than a single antioxidant? Since chemotherapy drugs present detriments to healthy cells due to stressed cell conditions and antioxidants counteract cytotoxicity, it is hypothesized that a combination of vitamin A, vitamin E, and melatonin alongside 5-fluorouracil will be more efficacious in decreasing cell toxicity, a consequence of 5-fluorouracil induced oxidative stress on cell membranes, and the prevention of cell death.

Thus, the objective of this study is to examine the cytotoxic effect of an antimetabolite, 5-fluorouracil, and how a combination of vitamins A, E, and melatonin could potentially interfere with 5-fluorouracil induced death of healthy cells.

II. METHODS

Three separate tests (spot plating, microscopic analysis, and liquid turbidity) were performed. For the spot plating test, ten Petri dishes were prepared with Yeast-Extract Peptone Dextrose (YEPD) media and the same

concentration of 5-fluorouracil, but a varying combination of vitamins A, E, and melatonin to study their effects on yeast cell growth. Control Dish 1 did not contain 5-fluorouracil or antioxidants. Dish 2 was treated with 5-fluorouracil only. Dishes 3 to 9 were treated with 5-fluorouracil and varying combinations of vitamins A, E, and melatonin as specified in Table I. Dish 10 was treated with antioxidants only. After the Petri dishes were set, 10 µL of yeast culture serial dilutions (1, 0.1, 0.01, 0.001) were pipetted in two rows onto each dish. Petri dishes were placed at 37 °C to grow overnight. Images of the growth were taken using a digital camera and growth data was recorded after one day.

TABLE I. TEST CONDITIONS FOR SPOT PLATING EXPERIMENT

Petri Dish No. & Test Condition	*Flu 5 mg/mL (µL)*	*Vita A 10 µg/mL (µL)*	*Vita E 100 mg/mL (µL)*	*Mel 25 mg/mL (µL)*
1. YEPD Control	-	-	-	-
2. Flu Control	50	-	-	-
3. Flu + A	50	50	-	-
4. Flu + E	50	-	50	-
5. Flu + Mel	50	-	-	50
6. Flu + A + E	50	50	50	-
7. Flu +A + Mel	50	50	-	50
8. Flu + E + Mel	50	-	50	50
9. Flu+A+E+ Mel	50	50	50	50
10. A+E+Mel Ctrl	-	50	50	50

Microscopic analysis was performed using methylene blue staining at 2500x magnification to assess the membrane integrity of yeast cells scraped from the spot plating test.

For the liquid turbidity testing, ten falcon tubes were prepared with YEPD media and the same concentration of 5-fluorouracil, but varying combinations of antioxidants. Control trial Tube 1 did not contain 5-fluorouracil or antioxidants. Tube 2 was treated with 5-fluorouracil only. Tubes 3 through 9 were treated with 5-fluorouracil and varying combinations of vitamins A, E, and melatonin as specified in Table II. Tube 10 was treated with antioxidants only. Then, 50 µL yeast culture of dilution 1 was added to each falcon tube and placed in an agitator. 3 mL of each culture was pipetted into its own cuvette to be measured. Every 30 minutes, the optical density at 600 nm of the culture was measured using a spectrophotometer and recorded for a period of two hours. The liquid media test was then repeated for the remainder of the yeast culture's serial dilutions (0.1, 0.01, 0.001).

TABLE II. TEST CONDITIONS FOR LIQUID MEDIA EXPERIMENT

Falcon Tube No. & Test Condition	*Flu 5 mg/mL (µL)*	*Vita A 10 µg/mL (µL)*	*Vita E 100 mg/mL (µL)*	*Mel 25 mg/mL (µL)*
1. YEPD Control	-	-	-	-
2. Flu Control	30	-	-	-
3. Flu + A	30	30	-	-
4. Flu +E	30	-	30	-
5. Flu + Mel	30	-	-	30
6. Flu + A + E	30	30	30	-
7. Flu +A + Mel	30	30	-	30
8. Flu + E + Mel	30	-	30	30
9. Flu+A+E+ Mel	30	30	30	30
10. A+E+Mel Ctrl	-	30	30	30

III. RESULTS AND DISCUSSION

The spot-plating image (Fig. 1) showed the growth of the yeast cells in different conditions over a period of one day.

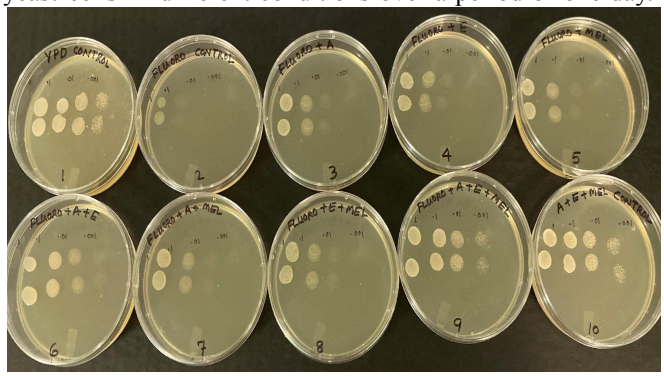

Figure 1. Spot-plating images of test samples. n=2.

The visible amount of cell growth in Dish 2 was less than that of the control Dish 1, indicating cell damage due to the presence of 5-fluorouracil. In Dish 2, serial dilutions 0.01 and 0.001 were not visible at all. Compared to those of the control plate, the yeast cells in Dish 2 were effectively killed by 5-fluorouracil. The addition of each antioxidant allowed more cells to survive. There was more visible cell growth in Dishes 3 through 9 than in Dish 2. All serial dilutions were clearly visible in Dish 9, indicating the prevention of cell death due to the combination of vitamins A, E, and melatonin. Dish 9, with a combination of vitamins A, E, and melatonin, was the most effective against 5-fluorouracil-induced yeast cell death. Cell growth in Dish 10 was comparable to the control Dish 1, reaffirming that antioxidants do not negatively affect cell growth.

The color, quantity, and structure of microscopic images of the yeast cells scraped from the spot plating test were compared to assess the membrane integrity. Figures 2b (serial dilution 1) and 2e (serial dilution 0.1) show the microscopic images of 5-fluorouracil-treated yeast cells scraped from Dish 2. When methylene blue dye is oxidized, it becomes a colorless compound in living cells, staining the dead cells. The blue dots in Figures 2b and 2e indicate dead cells. When the cell membrane is damaged, the functional properties of the cell will be lost. The number of cells in Figures 2b & 2e was fewer than that of the control Dish 1 (Fig. 2a & 2d) indicating membrane damage. Figures 2c (serial dilution 1) and 2f (serial dilution 0.1) are the microscopic images of yeast cells scraped from Dish 9 (treated with 5-fluorouracil, vitamins A, E, and melatonin). They show the recovery of cells with the combination of the three antioxidants, which are comparable to the control Dish

1 (Fig. 2a & 2d). Thus, microscopic images reaffirmed the results found with the spot plating test.

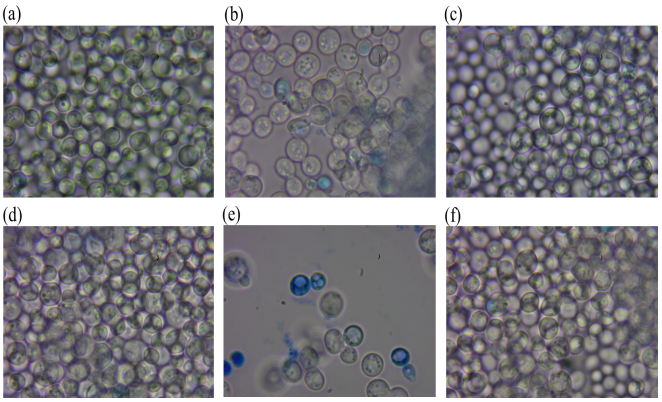

Figure 2. Yeast cell membrane integrity at 2500x magnification; (a) serial dilution 1 from control Dish 1; (b) serial dilution 1 from Dish 2 treated with fluorouracil only; (c) serial dilution 1 from Dish 9 treated with fluorouracil and vitamins A, E, and melatonin; (d) serial dilution 0.1 from control Dish 1; (e) serial dilution 0.1 from Dish 2 treated with fluorouracil only; (f) serial dilution 0.1 from Dish 9 treated with fluorouracil and vitamins A, E, and melatonin.

Table III shows the average growth (OD_{600}) of 5-fluorouracil and antioxidants-treated yeast culture samples in liquid media from all dilutions over a period of 2 hours.

TABLE III. GROWTH (OD_{600}) OF YEAST CULTURE IN LIQUID MEDIA

Test Condition	*0 Hour*	*0.5 Hour*	*1 Hour*	*1.5 Hours*	*2 Hours*
1. YEPD Control	0.138	0.234	0.339	0.358	0.369
2. Fluorouracil Control	0.124	0.128	0.138	0.146	0.159
3. Fluoro + Vitamin A	0.128	0.169	0.189	0.217	0.238
4. Fluoro + Vitamin E	0.129	0.175	0.203	0.233	0.259
5. Fluoro + Melatonin	0.127	0.158	0.171	0.195	0.223
6. Fluoro + A + E	0.134	0.199	0.275	0.291	0.314
7. Fluoro + A + Mel	0.130	0.179	0.212	0.244	0.276
8. Fluoro + E + Mel	0.132	0.186	0.234	0.258	0.294
9. Fluoro + A+E+ Mel	0.137	0.220	0.316	0.331	0.347
10. A+E+Mel Control	0.140	0.250	0.351	0.382	0.391

The optical density of the liquid media was measured over a period of two hours (Fig. 3). For each serial dilution, the rate of cell growth in Test Condition 2 (5-fluorouracil only) was consistently less than that in the control trial with no chemical added (Test Condition 1). On average, 57% of 5-fluorouracil-treated cells in Test Condition 2 (0.159) were damaged in comparison to the control after two hours.

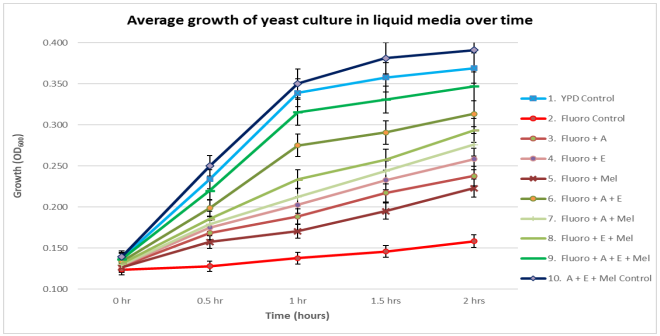

Figure 3. Compares the growth trend (OD_{600}) of 5-Fluorouracil-treated yeast culture samples in liquid media from all dilutions over a period of two hours.

After adding vitamin A to the sample, the cell damage was reduced to 36% in Test Condition 3 (0.238). With the addition of vitamin E to the sample, the cell death was further reduced to 30% in Test Condition 4 (0.259). When melatonin was added, there was a modest drop in cell damage to 40% in Test Condition 5 (0.223). All three antioxidants individually decreased cell death to an extent, with vitamin E demonstrating the strongest effect among the antioxidants. After adding a combination of vitamins A and E to the sample, only 15% of cells were damaged in Test Condition 6 (0.314). With the addition of a combination of vitamin A and melatonin, more than 25% of cells died in Test Condition 7 (0.276). When vitamin E and melatonin were added, cell death was reduced to 20% in Test Condition 8 (0.294). In comparison to a single antioxidant, the combination of two antioxidants was better in minimizing cell destruction, particularly the combination of vitamins A and E demonstrating the strongest effect. Out of all the test conditions, the combination of vitamins A, E, and melatonin was the most effective, reducing cell damage to only 6%, as seen in Test Condition 9 (0.347).

IV. CONCLUSION

Overall, this research examined the cytotoxic effect of 5-fluorouracil, and how a combination of antioxidants could possibly prevent the negative impact. The results indicated that a combination of vitamins A, E, and melatonin prevented 94% of cell death caused by 5-fluorouracil. Our results also suggest that a combination of vitamins A, E, and melatonin prevented cell death more effectively than a single antioxidant or the combination of any two antioxidants. As a result, a combination of vitamins A, E, and melatonin can be considered as a potential treatment for minimizing the cytotoxic side effects of 5-fluorouracil, thereby yielding faster recovery and minimal chemotherapy side effects for cancer patients that may lead to a more advantageous drug implementation method. Prior in vitro and in vivo studies of antioxidants and chemotherapy are consistent with this study's findings that antioxidants not only mitigate the toxicities induced by chemotherapeutic agents but also increase the therapeutic efficiency of chemotherapy [2].

While a combination of vitamins A, E, and melatonin can be effective in minimizing 5-fluorouracil-induced toxicity, high doses of antioxidants may protect both normal and cancer cells against ROS,

which could reverse the therapeutic effects of chemotherapy. This situation primarily depends on the antineoplastic mechanism of the drug as certain types of chemotherapy drugs use ROS as a means to kill malignant cells rather than ROS being an adverse effect. Therefore, future studies need to test the effect of vitamins A, E, and melatonin on other types of chemotherapy drugs such as alkylating agents, anthracyclines, and topoisomerase/mitotic inhibitors.

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