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14. ABSTRACT Adjuvant chemotherapy often causes cognitive decline in breast cancer survivors. Although the cognitive deficits are often temporary, it appears that for at least a subset of survivors, the deficits last for years and can have a deleterious impact on survivor quality of life. Recent evidence shows that chemotherapy agents can have long-lasting neurotoxic effects: increase in cell death and decrease in cell division/proliferation in the SVG, the DG, and the CC, as well as delayed myelin degeneration. Which chemotherapy agents or combinations of agents cause CNS damage remains unclear. Our study was designed to determine 1) if doxorubicin or cyclophosphamide cause a decrease in neurogenesis and/or myelin damage and 2) if neurogenesis and/or myelin damage caused by 5-Fluorouracil can be prevented by pre and co-treatment with antidepressants or antioxidants. The results from our auditory brainstem response experiments suggest that 5-Fluorouracil causes lasting myelin damage and that doxorubicin and cyclophosphamide do not. In addition, our results suggest that co-treatment with antioxidants does					
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Introduction

Adjuvant chemotherapy often causes cognitive decline in breast cancer survivors. Although the cognitive deficits are often temporary, it appears that for at least a subset of survivors, the deficits last for years and can have a deleterious impact on survivor quality of life. Recent evidence shows that chemotherapy agents can have long-lasting neurotoxic effects: increase in cell death and decrease in cell division/proliferation in the SVG, the DG, and the CC, as well as delayed myelin degeneration. Thus, at least one underlying mechanism of chemotherapy-induced cognitive dysfunction may be a decline in neuro and glialgenesis, and/or delayed myelin degeneration. With this in mind, a critical question becomes *what can prevent/reduce that negative mechanism **before** it occurs rather than waiting to try to alleviate the problem after the damage has occurred?* Given that most antidepressants cause an increase in neuro/glialgenesis it seems plausible that antidepressants might reduce some of the neurotoxic effects listed above, and thereby reduce cognitive decline. In addition, given that chemotherapy agents have, at least to some extent, a free radical mechanism, it seems plausible that antioxidants might reduce the neurotoxic effects of chemotherapy. Melatonin appears to protect against demyelination in general and to stimulate neurogenesis and is thus a good candidate antioxidant. Our research project takes advantage of the recent development of the mouse models of chemotherapy induced neurotoxicity to explore the possibility that antioxidants and antidepressants might prevent or decrease the severity of chemotherapy-induced long-lasting neurotoxicity.

Body

Aim 1: Can antioxidant or antidepressant treatment prevent or decrease the neurotoxicity caused by Fluorouracil (5-FU) treatment in mice.

1a. Measurement of **cell death** in the lateral subventricular zone (SVZ), the corpus callosum (CC), and the dentate gyrus (DG) at 1 day, 14 days, 56 days, and 6 months after 5-fluorouracil (5-FU) treatment using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) as a function of pre & co-treatment with 1) N-acetyl cysteine (NAC) 2) Melatonin & 3) Fluoxetine.

Saline Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU + NAC Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU + Melatonin Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

5-FU + Fluoxetine Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

1b. Measurement of **cell proliferation** in the SVZ, the CC, and the DG at day 1, 14 days, 56 days, and 6 months after 5-FU treatment using Ki-67 as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

1c. Measurement of **white matter density** in the CC and white matter tracts of the striatum at 1 day, 14 days, 56 days, and 6 months after 5-FU treatment using optical density of anti-myelin basic protein (Anti-MBP) as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

1d. Measurement of **transcriptional regulation** in oligodendrocytes at 1 day, 14 days, 56 days, and 6 months after 5-FU treatment assessed with labeling for both Olig2 and CC-1 as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

1e. Measurement of **myelin function** prior to treatment, and at 1 day, 14 days, 56 days, and 6 months after 5-FU treatment as assessed by auditory brainstem response (ABR) as a function of pre & co-treatment with 1) NAC 2) Melatonin & 3) Fluoxetine.

Saline Group: (n=8 C57BL/6J mice)

5-FU Group: (n=8 C57BL/6J mice)

5-FU + NAC Group: (n=8 C57BL/6J mice)

5-FU + Melatonin Group: (n=8 C57BL/6J mice)

5-FU + Fluoxetine Group: (n=8 C57BL/6J mice)

We have completed the majority of data collection and data analysis for the auditory brainstem response (ABR) experiment in Aim 1 (i.e., Aim 1-1e.). The ABR is a robust response making it

Fig. 1

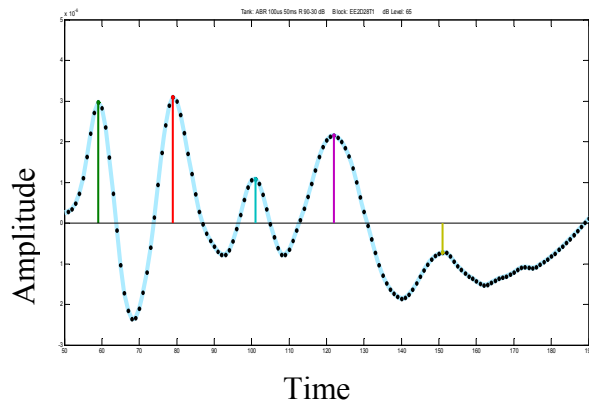


Fig. 2

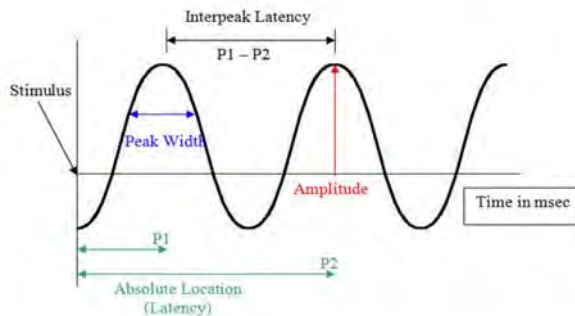
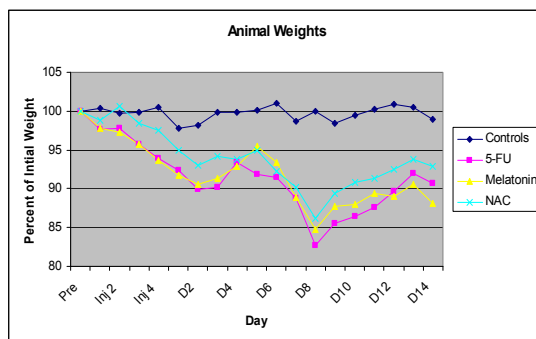


Fig. 3



ABR and suggests myelin damage. A negative change indicates a speeding up of the ABR and suggests enhancement of transmission speed. In general, the more peaks included in the interpeak analysis the more sensitive it should be to myelin damage, however, peak 5 is the least stable of the 5 ABR peaks. Thus we have included our results for peak 4 – peak 1 rather than peak 5 – peak 1. As seen in Figure 4 the 5-FU treated animals were significantly slower than the control animals at all time-points so far collected. Unfortunately, with the possible exception of the Melatonin group on day 14, the antioxidant treatments were not preventative.

a useful technique in both clinical and research settings for studying disorders of the CNS, including myelin damage. Previously it was shown that 5-FU caused both myelin damage and a corresponding increase in ABR inter-peak latencies in mice. Figure 1 shows a representative ABR from one of our mice. There are 5 main peaks of interest in the ABR indicated by the green, red, blue, purple, and yellow lines. The main indication of myelin damage is an increase in the distance between the peaks, although we have also analyzed other measures such as peak width and amplitude. Figure 2 summarizes the various measures we use, although it should be noted that it shows the analysis for just the first 2 peaks. We treated mice ($n = 8$ per group) with either 5-Fluorouracil (70 mg/kg) + Saline (0.9%), 5-Fluorouracil (70 mg/kg) + Melatonin (25mg/kg), 5-Fluorouracil (70 mg/kg) + N-acetyl-cysteine (NAC) (400mg/kg), or Saline (0.9%) + Saline (0.9%) and recorded ABRs. We have completed recording data for all time points except our last point (6 months). As mentioned in our request for a no-cost extension, although pilot data suggested that our dosing caused weight-loss but no significant animal loss, during the experiment proper our dosing caused systematic animal death which resulted in the chemotherapy treated groups having just 5 or 6 animals rather than 8. As shown in Figure 3, the chemotherapy treated groups lost significantly more weight than did the control animals indicating therapeutic (i.e., toxic) effect (as did the loss of some animals). Figure 4 shows change in interpeak latency (peak 4 – peak 1) on day 1, 14, and 56. The change is relative to baseline data collected 2 days prior to treatment. A positive change indicates a slowing down of the

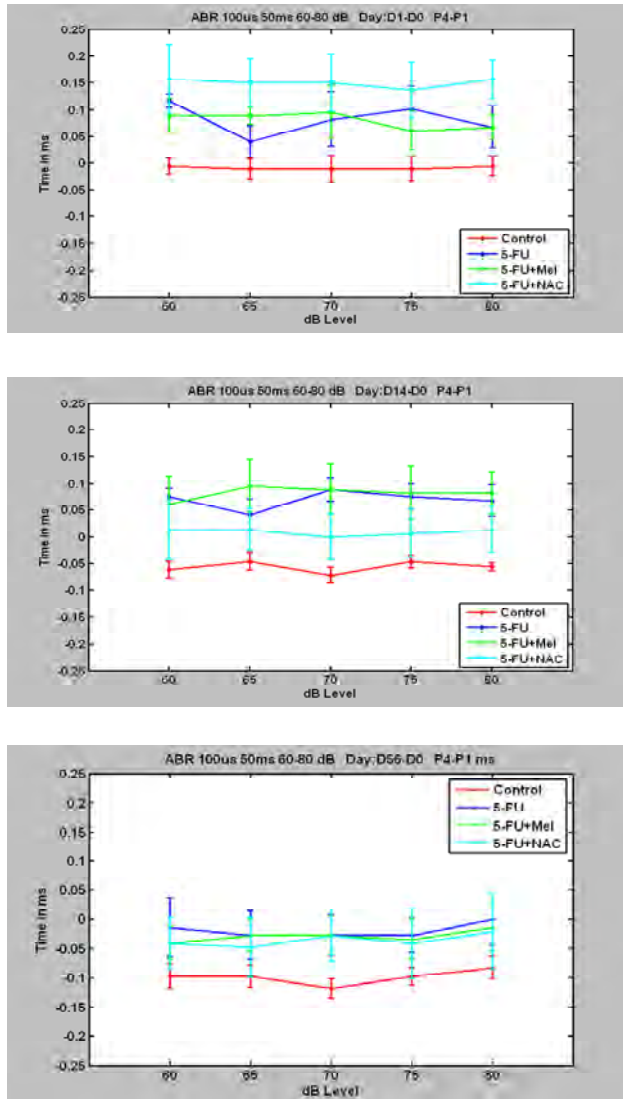


Figure 4: Change in P4 – P1 interpeak latency compared to day 0 for days 1, 14, and 56, top to bottom figures. Positive scores indicate an increase in interpeak latency and negative scores indicate a decrease in latency. Error bars represent ± 1 standard error of the mean.

Day 1 5-FU, 5-FU+Mel, & 5-FU+NAC > Control all $ps < .05$;

Day 14 5-FU, 5-FU+Mel > Control all $ps < .003$

Day 56 5-FU, 5-FU+Mel, & 5-FU+NAC as a group > Control $p < .05$

Because of dosing issues and unexpected lack of colony space at the start of the project period (as described above and in our previous request for a non-cost extension) we are not as far along on the immunocytochemistry experiments (Aim 1 a-d) as we had expected. We changed to a lower dose of 5-FU (60 mg/kg) for those experiments and have treated, perfused, frozen, and begun slicing brains for half the animals in the 1 day, 14 day and 56 day time points. Half the animals for the 6 month time point have been treated and we are waiting for them to reach the 6 month time-point.

Aim 2: Do doxorubicin & cyclophosphamide cause neurotoxicities similar to that of 5-FU in mice?

2a. Measurement of **cell death** in the SVZ, the CC, and the DG at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment using TUNEL.

Saline Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

Doxorubicin Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

Cyclophosphamide Group: (n=5 x 4 time points for a total of 20 C57BL/6J mice)

2b. Measurement of **cell proliferation** in the SVZ, the CC, and the DG at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment using Ki-67.

2c. Measurement of **white matter density** in the CC and white matter tracts of the striatum at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment using optical density of Anti-MBP.

2d. Measurement of **transcriptional regulation** in oligodendrocytes at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment assessed with labeling for both Olig2 and CC-1.

2e. Measurement of **myelin function** prior to treatment, and at 1 day, 14 days, 56 days, and 6 months after doxorubicin or cyclophosphamide treatment as assessed by ABR.

Saline Group: (n=8 C57BL/6J mice)

Doxorubicin Group: (n=8 C57BL/6J mice)

Cyclophosphamide Group: (n=8 C57BL/6J mice)

We have completed data collection and the majority of data analysis for the auditory brainstem response (ABR) experiment in Aim 2 (i.e., Aim 2-2e.). The ABR is a robust response making it a useful technique in both clinical and research settings for studying disorders of the CNS, including myelin damage. Previously it was shown that 5-Fu caused both myelin damage and a corresponding increase in ABR inter-peak latencies in mice. Figure 5 shows a representative ABR from one of our mice. There are 5 main peaks of interest in the ABR indicated by the

Fig. 5

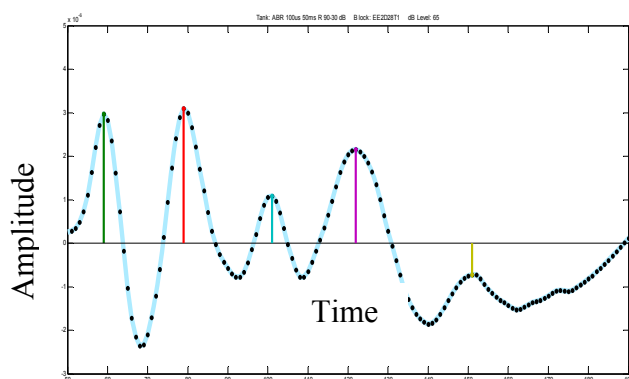


Fig. 6

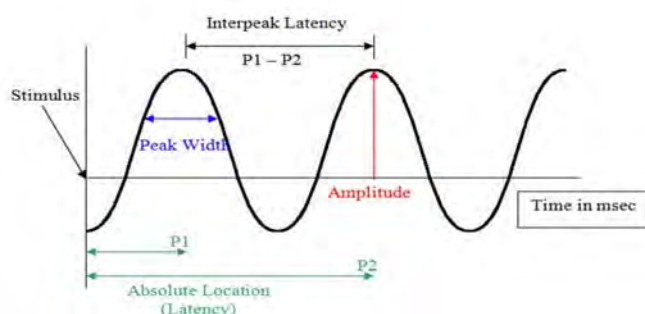
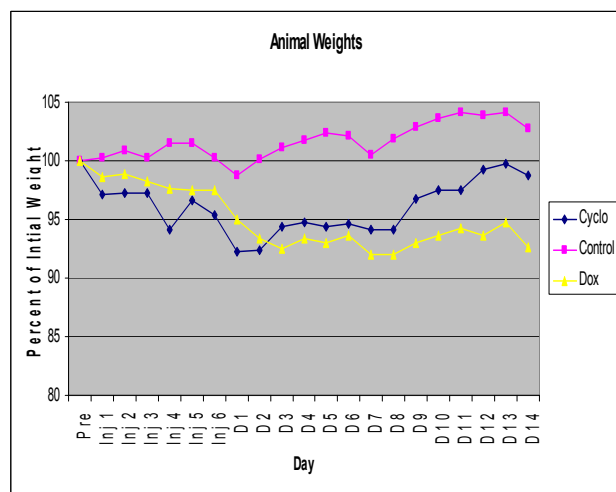


Fig. 7



green, red, blue, purple, and yellow lines. The main indication of myelin damage is an increase in the distance between the peaks, although we have also analyzed other measures such as peak width and amplitude. Figure 6 summarizes the various measures we use, although it should be noted that it shows the analysis for just the first 2 peaks. We treated mice with either cyclophosphamide (120 mg/kg per i.p. injection; n=8), doxorubicin (5 mg/kg per i.p. injection; n=8) or saline (0.9%; i.p.; n=8) and recorded ABRs. We have just completed recording data for our last time-point (6 months), however, we have not completed the analysis of the 6 month time point yet. As shown in Figure 7 the chemotherapy treated group lost significantly more weight than did the control animals indicating therapeutic (i.e., toxic) effect. In addition, 5 of the 8 cyclophosphamide treated animals had gray hair by 4 months whereas none of the control animals did. Finally, 3 doxorubicin treated animals showed significant weight loss during months 4 and 5 and we had to place them on a liquid diet. Despite the significant toxic effects of the chemotherapy, we found no evidence of differences in the ABRs at the 1 day, 14 day, or 56 day time-points between the control animals and the doxorubicin or cyclophosphamide treated animals. Figure 8 shows change in interpeak latency (peak 4 – peak 1) on day 1, 14, and 56. The change is relative to baseline data collected 2 days prior to treatment. A positive change indicates a slowing down of the ABR and suggests myelin damage. A negative change indicates a speeding up of the ABR and suggests enhancement of transmission speed. In general, the more peaks included in the interpeak analysis the more sensitive it should be to myelin damage, however, peak 5 is the least stable of the 5 ABR

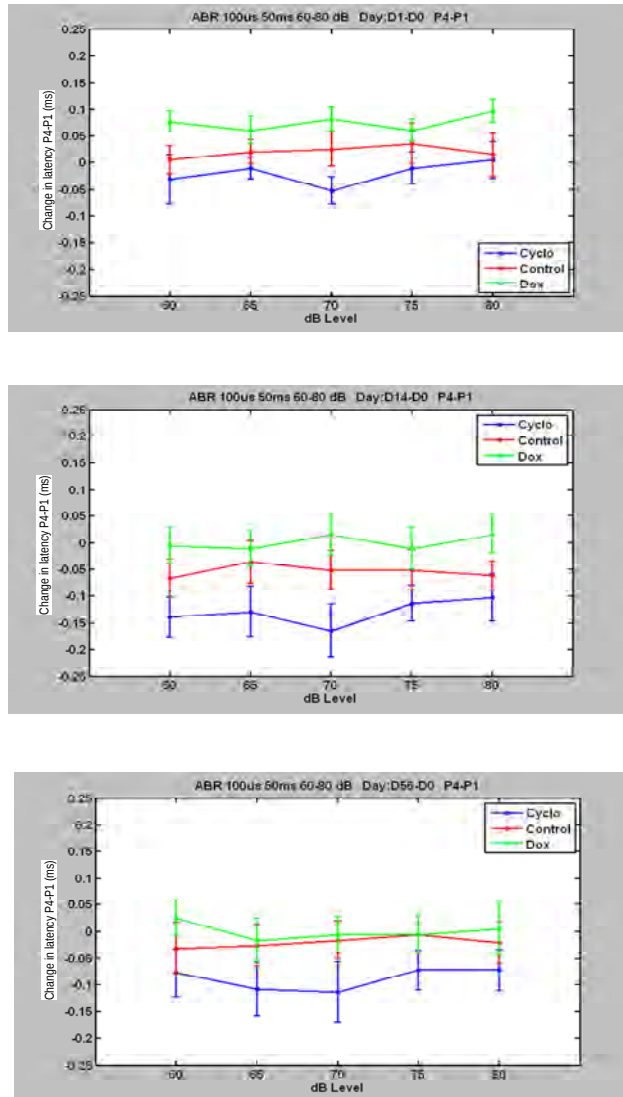


Figure 8. Change in P4 – P1 interpeak latency compared to day 0 for days 1, 14, and 56, top to bottom. Positive scores indicate an increase in interpeak latency and negative scores indicate a decrease in latency. Error bars represent ± 1 standard error of the mean. Doxorubicin (Dox) & Cyclophosphamide (Cyclo)

Day 1 Dox > Cyclo $p < .02$;

Day 14 Dox > Cyclo $p < .02$;

Day 56 No significant differences

peaks. Thus we have included our results for peak 4 – peak 1 rather than peak 5 – peak 1. As sign in Figure 8 there were no significant differences between the control group and either chemotherapy group. There was, however, a significant difference between the doxorubicin group and the cyclophosphamide group at day 1.

For our immunohistochemistry experiments (Aim 2 a-d) we have treated, perfused, frozen, and begun slicing brains for half the animals at all time points.

Key Research Accomplishments

- 1) Completed data collection plus majority of data analysis for the ABR experiments in Aim 2
- 2) Completed majority of data collection and data analysis for the ABR experiments in Aim 1
- 3) Treated and have begun processing about half the mice for the immunohistochemistry experiments for Aims 1 and 2

Reportable Outcomes

Conferences

Upcoming: Maxwell Hennings, Hawk Cambron, Robert Ferguson, and Thane Fremouw.
The effects of single agent doxorubicin, cyclophosphamide, or 5-Fluorouracil treatment on the auditory brainstem response in mice. Chemo Brain: Mechanisms & Assessments (University of Kentucky, KY) October 2011.

Maxwell Hennings, Hawk Cambron, Robert Ferguson, and Thane Fremouw.
Chemotherapy, Neurotoxicity, and Cognitive Decline: Developing a Mouse Model and Potential Interventions. Era of Hope Conference (Orlando, FL) August 2011.

Maxwell Hennings, Hawk Cambron, Robert Ferguson, and Thane Fremouw. *Of mice, chemotherapy, and the auditory brainstem response.* Mainely Data (and some theory) (Bates College, ME) May 2011

Conclusion

Adjuvant chemotherapy often causes cognitive decline in breast cancer survivors. Although the cognitive deficits are often temporary, it appears that for at least a subset of survivors, the deficits last for years and can have a deleterious impact on survivor quality of life. Recent evidence shows that chemotherapy agents can have long-lasting neurotoxic effects: increase in cell death and decrease in cell division/proliferation in the SVG, the DG, and the CC, as well as delayed myelin degeneration. Which chemotherapy agents or combinations of agents cause CNS damage remains unclear. The results from our auditory brainstem response experiments suggest that 5-Fluorouracil causes lasting myelin damage and that doxorubicin and cyclophosphamide do not. In addition, our results suggest that co-treatment with antioxidants does not prevent 5-Fluorouracil related myelin damage. Our immunocytochemistry experiments are ongoing and will help us determine if doxorubicin or cyclophosphamide cause a decline in neurogenesis, as does 5-Fluorouracil, and if antidepressants or antioxidants can prevent such damage.