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TITLE: Randomized Trial of Neuroprotective Effects of Erythropoietin in Patients Receiving Adjuvant Chemotherapy for Breast Cancer: Positron Emission Tomography and Neuropsychological Study

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Introduction

In the United States approximately 60-80% of patients diagnosed with breast cancer will receive adjuvant chemotherapy. Of these patients more than 30% will experience short-term and long-term cognitive impairment (e.g., problems with memory and concentration) for at least 1-2 years after completion of chemotherapy. Despite the effects cognitive impairment can have on a patient's quality of life very few studies have been conducted to learn more about this side effect.

This study aimed to evaluate the pathophysiology of cognitive dysfunction in patients receiving adjuvant chemotherapy with Adriamycin and cyclophosphamide for breast cancer using [^{15}O] water PET scans and neuropsychological tests.

The intent of the study was to enroll a total of 24 eligible patients with early stage breast cancer who were candidates for adjuvant chemotherapy. It was planned for each patient to undergo [^{15}O] water PET scans at baseline and after completing 4 cycles of adjuvant chemotherapy to measure the differences in regional blood flow of the brain during working memory. Neuropsychological tests were to be done to determine attention, speeded processing, memory, and executive functions outside of the [^{15}O] water PET scans.

In April 2004 this study was amended due to published data regarding the safety of Epoetin alfa (EPO). This data showed that patients with normal hemoglobin levels who receive treatment with EPO have increased morbidity and mortality. Due to this information the Investigators were prompted to change the protocol to look at pre-post chemotherapy changes in the brain with PET scan, but without treating patients with EPO. Therefore the protocol aims to understand the pathophysiology of cognitive dysfunction without studying the role of EPO. The final report for this study was submitted in September 2006.

An amendment to the study was initiated in April 2005 to include animal experiments. As per published literature, proinflammatory cytokines play a role in the pathogenesis of cognitive dysfunction. The experiments are designed to assess the cytokines before and after chemotherapy in a rat model, as explained below. We have established an experimental animal model to study chemotherapy-induced cognitive dysfunction observed in the clinical setting. In this model administration of four weekly doses of clinical chemotherapeutics, i.e., the combination of adriamycin and cytoxan, results in impaired memory function in rats. This study was completed in February 2007 and the results of the study was sent to DOD with the report on September 2007. The study results were published in the Journal Met Brain Dis (23:325-333).

After completion of Task 4, we initiated Task 5 as described below.

We proposed to further characterize the mechanisms of this cognitive dysfunction by molecular genomics approach. We analyzed global gene expression in the hippocampi from treated vs. control rats using the microarray methodology. This analysis allowed us to identify genes whose expression is altered by the chemotherapeutic treatment.

Subsequently, expression of these genes was analyzed by real time RT-PCR to confirm the microarray results, to precisely quantify the differential expression of these genes, and to further streamline the selection of putative genes that underscore the memory impairment. This study will provide important information for future, more clinically oriented endeavors to determine the correlation between the polymorphism of these genes and the susceptibility of patients to chemotherapy-induced cognitive dysfunction.

Body

Task 1. Study the baseline cognitive function **Completed**

- Five patients with early stage breast cancer receiving adjuvant chemotherapy were enrolled in this study. One patient was withdrawn due to the inability to complete the baseline PET scan. The patient became claustrophobic during the PET scan, therefore, the scan was not completed.
- Baseline cognitive function assessments with neuropsychological measures were completed by each patient.
- The baseline study of regional blood flow of the brain using [^{15}O] water PET scans during working memory were completed by each patient.

Task 2. To study the cognitive function after 4 cycles of chemotherapy with [^{15}O] water Positron Emission Tomography (6-12 months) **Completed**

- Each patient completed PET scans and neuropsychological measures 2-4 weeks after the completion of 4 cycles of AC.

Task 3. Analysis of the data and writing of the final report (12-18 months) **Completed**

- All data is maintained in a secure database. The human study data will not be analyzed due to the small sample size.

Task 4. Animal Study **Completed Prior to Amendment (02/2007)**

- Baseline cytokine assay in the peripheral blood was completed.
- Animals were treated with Adriamycin and cytoxan every two weeks for 4 cycles.
- Cytokines in the blood were measured weekly while the animals were being treated with chemotherapy.
- Analyzed the brains of chemo-treated rats by histology.

Task 5. Animal Study - Identifying target genes involved in chemotherapy-induced cognitive dysfunction **Completed**

- Rats were treated with weekly intraperitoneal injections of adriamycin/cytosine for four weeks, and memory impairment was evaluated by behavioral testing.
- Brain tissue was collected and hippocampal mRNA was extracted and purified.
- Gene expression was profiled using microarrays to identify genes that are differentially expressed in control vs. treated hippocampi.

Key Research Accomplishments (Task 4)

Proinflammatory cytokine expression as a possible mechanism of chemotherapy induced cognitive dysfunction

In this preliminary study we tested the effect of commonly used chemotherapeutics on rats to elucidate the mechanisms of chemotherapy-induced brain damage leading to cognitive dysfunction, and ultimately, to understand how these mechanisms can be controlled for therapeutic purposes. We used ten month old Sprague-Dawley female rats (retired breeders) injected intraperitoneally with a combination of adriamycin and cytoxan (1:10 w/w) as an experimental system. The initial experiments were carried out to establish non-lethal and non-morbid dosage of the chemotherapeutics. Three groups of rats were treated weekly with the following doses of adriamycin/cytosine (mg/kg): (A) 2.5/25, (B) 5/50 and (C) 10/100. Control group was injected with saline only. All rats (n=4) in group B and C died after three and two injections, respectively, but no mortality was observed in group A. Over four weeks of the experiment the body weight increased by approximately 2 and 4% in the A vs. control group, respectively indicating no significant morbidity. Therefore, the dosage of 2.5 mg/kg of adriamycin and 25mg/kg of cytosine was used in the subsequent experiments. This treatment is further referred to as “chemo-treatment”.

To address Specific Aim 1 we measured the level of proinflammatory cytokines in the peripheral blood of the treated vs. control animals. Blood samples were collected 1, 2 and 7 days after each injection, and the level of TNF α was determined by ELISA. The baseline level of the cytokine was 6.8 ± 1.8 pg/ml as measured in saline injected rats (control group), and did not change in the course of the experiment. Also, chemo-treatment did not significantly affect TNF α in the blood at any time point. These negative results indicate that the mechanisms of the drug action may not be mediated by peripheral cytokines. Therefore, before proceeding to Specific Aim 2 to assess changes in brain cytokine expression we decided to determine whether our chemo-treatment paradigm affects brain function.

We performed several behavioral tests to assess the effects of chemo-treatment on cognitive function of the rats. We tested the animals for locomotive activity using the open field test. No differences between control and treated rats at the beginning or at the end of the experiment were detected. Also, chemo-treatment did not change the exploratory activity of the rats as detected by the novelty test. After establishing this we assessed the memory function of the animals using the passive avoidance test. As shown in Fig. 1 this test revealed a significant impairment of cognitive function.

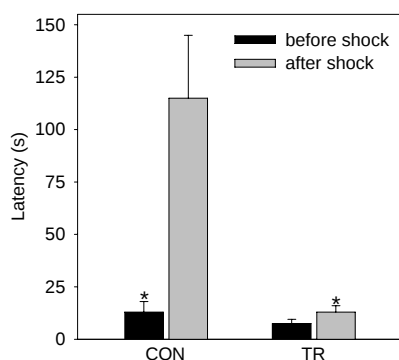


Fig. 1. Performance of chemo-treated (TR) and control (CON) rats in passive avoidance test. The animals were tested one week after the last dosing. Briefly, a rat was placed in illuminated chamber, and the latency of entering into dark chamber was recorded. Upon entering dark chamber electric shock was administered. Twenty four hours later, the rat was placed again in illuminated chamber, and the latency of entering the dark chamber was recorded for up to 180 s. Results are means $\pm$ SEs.

* values significantly different from electrically shocked control rats ($p \leq 0.05$)

No significant difference in the latency of entering in the dark chamber between control and treated groups was evident before the electric shock was applied. However, the latency after the electric shock increased profoundly in the control group, but not in the chemo-treated group. Because there were no locomotor and no exploratory deficiencies in the treated animals, this lack of increased latency demonstrated chemotherapy impaired memory function as the animals did not remember the electric shock experience and did not avoid entering the dark chamber. Therefore, our chemo-treatment paradigm models cognitive dysfunction in patients undergoing chemotherapy. This experimental model provides a convenient system for further studies of the mechanisms of brain damage elicited by chemotherapy.

The study showed that chemotherapeutic treatment severely impaired memory function of rats as measured by a passive avoidance test. This memory deficiency was fully prevented by 200 mg/kg of N-acetyl cysteine (NAC) injected subcutaneously three times a week in the course of chemotherapeutic treatment. These results indicate that chemotherapeutic agents alone, i.e., in the absence of malignancy, damage the brain resulting in memory dysfunction. Moreover, the results strongly indicate that the damaging effect is mediated by oxidative stress, as memory dysfunction is preventable by the co-administration of NAC.

Key Research Accomplishments (Task 5)

To further characterize the mechanisms of this cognitive dysfunction by molecular genomics approach, we analyzed the global gene expression in the hippocampi from treated vs control rats using the microarray methodology. This was done in order to identify genes whose expression is altered by the chemotherapeutic treatment. Expression of these genes was analyzed by real time RT-PCR to confirm the microarray results, to precisely quantify the differential expression of these genes, and to further streamline the selection of putative genes that underscore the memory impairment.

Animal Methods

We used ten month old Sprague-Dawley female rats (retired breeders) injected intraperitoneally with a combination of adriamycin and cytoxan (1:10 w/w) as an experimental system. The control group was injected with saline only. The dosage of 2.5 mg/kg of adriamycin and 25mg/kg of cytoxan was used in the experiments.

Array Methods

Gene expression in rat brain samples from chemotherapy-treated and control animals were evaluated by the Affymetrix GeneChip Rat Genome 230 2.0 Array. This array was comprised of over 31,000 probe sets representing approximately 28,700 well substantiated rat genes. Eleven pairs of oligo probes are used to measure the level of transcription of each sequence represented on the array.

Data

The data consisted of 31099 probes and 3 samples in the control group and 31099 probes and 3 samples in treatment group which were based on Affymetrix Rat 230 2.0 array.

Gene expression profiles

The gene expression data was extracted from the analytic (CEL) files using the RMA method, available under Bioconductor (www.bioconductor.org). The raw microarray data was transformed into gene expression data using the RMA (Robust Multichip Average) expression measure described in Irizarry et al (Biostat 2003;4:249-64).

Raw microarray data processing using RMA method

The expression data was normalized using quantile normalization, background correction was done using RMA background correction (the back ground used was similar to pure R rma background given in Affymetrix version 1.1 and above). The expression measure is given in a log base 2 scale.

Differentiation gene expression analysis using *t*-tests

Unpaired *t* test available in R software (<http://sekhon.berkeley.edu/stats/html/t.test.html>) was done between control group (C1,C3 & C5) and treatment group (T1,T3 & T5) for each of the probes. Only genes with significant differential expression ($p < 0.05$) were selected from the *t*-tests. Based on the fold ratio of gene expression (Average Treatment/Average Control), a total of five unique genes (6 probes) were selected that had fold ratio greater than 2. Among the five identified genes, Eif3s8, RGD1560479, and an unknown gene (probe ID: 1376430_at) were under expressed in the treated group, whereas Hba-a1 and LOC689064 were over expressed in the treated group. The details are as follows:

Affymetrix ID	Gene Symbol	Description	Control mean (a)	Treatment mean (b)	Difference (b-a)	Fold change $2^{(b-a)}$	P-value
1394620_at	Eif3s8	eukaryotic translation initiation factor 3, subunit C	8.063759	5.821998	-2.241761	0.211428	0.006379
1379929_at	RGD1560479	sine oculis-binding protein homolog-like	6.383473	4.666209	-1.717264	0.304125	0.000803
1376430_at		Transcribed locus	7.957087	6.750017	-1.207071	0.433147	0.027054

1370240_x_at	Hba-a1	hemoglobin alpha, adult chain 1	8.317422	11.08952	2.772099	6.83101	0.043777
1370239_at	Hba-a1	hemoglobin alpha, adult chain 1	8.184865	11.15775	2.972885	7.851048	0.038162
1371245_a_at	LOC689064	beta-globin	6.225699	9.868509	3.64281	12.49094	0.033944

Similarly, based on the fold ratio of gene expression (Average Treatment/Average Control), a total of ten unique genes were selected that had fold ratio between 1.5 and 1.9. Over expression was observed for Ube2, Gtf3c1, Cxc112, Gpd1 and 3 unidentified genes; whereas all of the under expressed genes were not identifiable. The details are as follows:

Probe ID	Gene Symbol	Gene description	Control mean (a)	Treatment mean (b)	Difference (b-a)	Fold change $2^{(b-a)}$	P-value
1384219_at	Ube2z	Ubiquitin-conjugating enzyme E2Z (putative)	6.956671	7.542888	0.586217	1.501305	0.002925
1395486_at	Gtf3c1	general transcription factor III C 1	4.946533	5.540813	0.59428	1.509719	0.001013
1380773_at			5.249612	5.865549	0.615937	1.532553	0.018933
1380704_a_at			4.944658	5.581742	0.637084	1.555183	0.037645
1388583_at	Cxcl12	chemokine (C-X-C motif) ligand 12	9.197871	9.839368	0.641497	1.559947	0.043175
1371363_at	Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)	9.476731	10.12074	0.644011	1.562667	0.037855
1391418_at			8.327846	9.188949	0.861103	1.816426	0.018577

1383189_at			5.277536	4.686337	-0.5912	1/1.506499	0.019535
1392736_at			7.799038	7.200038	-0.5990	1/1.514667	0.024304
1381736_at			8.385827	7.460143	-0.925684	1/1.899584	0.009424

The functions of several of these genes are intriguing for potential relevance to chemotherapy-induced cognitive dysfunction. Gtf3c1 was found to be upregulated by 1.5 fold in the chemotherapy-treated animals. Previous studies of this gene have found it is over expressed in the hippocampus of offspring of rodents who are exposed to cocaine in utero. The mechanism of such is thought to be epigenetic as methylation of the Gtf3c1 promoter was observed (Novikova SI, et al. PLoS ONE 2008;3:e1919.) The Cxcl12 gene was upregulated by 1.6 fold in the chemotherapy group. Interestingly, levels of Cxcl12 in the cerebrospinal fluid have been previously shown to be associated with disruption of the blood brain barrier (Pashenkov M, et al. J Neuroimmunol 2003;135:154-60.) Expression of Cxcl12 at the blood brain barrier has also been demonstrated in patients with multiple sclerosis (McCandless EE, et al. Neurobiol 2008;172:799-808.)

Reportable Outcomes

Presentation

Invited Speaker, "Use of PET Scanning in Assessing the Pathophysiology of Cognitive Dysfunction: The Future of Supportive Therapy in Oncology an International Congress," Hamilton, Bermuda, March 13, 2003.

Presentation

"Cognitive Dysfunction in Adjuvant Breast Cancer Treatment," Mary Babb Randolph Cancer Center Research Retreat, Stonewall Jackson Resort, July 13-15 2005.

Poster Presentation

"The Effects of Adjuvant Chemotherapy for Breast Cancer on Cerebral White Matter and Cognitive Function: A Diffusion Tensor Imaging Pilot Study," American Society of Clinical Oncology Annual Meeting, May 15-18, 2005.

Presentation

"Chemotherapy-induced cognitive dysfunction in rats" by Kraszpulski M, James I, Zhang H-T, Krasowska A, Abraham J and Konat G., Nemacolin Woodlands Resort, September 25-26, 2006.

Presentation

"Experimental model of chemotherapy induced cognitive dysfunction" by Konat G.W., Kraszpulski M, James I, Zhang H-T, and Abraham J, 21st Biennial International Society for Neurochemistry Meeting, August 19-24, 2007; J. Neurochem. 102(Suppl 1):54.

Presentation

Konat GW, Kraszpulski M, James I, Zhang H-T and Abraham J (2008) Experimental model of chemotherapy induced cognitive dysfunction. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting, Baltimore, June 25-28, 2008.

Publication

Konat GW, Kraszpulski M, James I, Zhang H-T and Abraham J (2008) Memory impairment induced by chronic administration of common chemotherapeutics in rats. *Met Brain Dis* 23:325-333.

Abraham, J., Haut, M.W., Moran, M.T., Filburn, S., Iannetti, M.P., Lemieux, S., and Kuwabara, H. The Effect of Chemotherapy for Breast Cancer on Cerebral White Matter: A Diffusion Tensor Imaging Study;; *Clinical Breast Cancer*, 8 (1): 88-91, February 2008

The following individuals received grant funding for their work on this project:

- Jame Abraham
- Marc Haut
- Gregory Konat
- Alicia Krasowska

Conclusions

Our original proposal of a clinical trial with [¹⁵O] water PET scans was completely changed with permission from DOD to pursue animal experiments. These animal experiments have paid off tremendously in expanding the understanding of the pathophysiology of chemotherapy induced cognitive impairment(as explained below). Our goal is to use this information and plan for future clinical trials.

Summary of key research accomplishments:

1. Developed an animal model for cognitive dysfunction.
2. Identified limited or no role of cytokines in chemotherapy induced cognitive dysfunction.
3. Identified oxidative damage to the brain as a potential cause of chemotherapy induced cognitive dysfunction.
4. N-acetyl cysteine (NAC) an anti-oxidant is found to reverse or prevent the chemotherapy induced cognitive dysfunction.
5. Other clinical observation and studies with diffusion tensor imaging (DTI-MRI) in patients who received chemotherapy and developed cognitive impairment showed changes in white matter, especially in the genu of the internal capsule

These areas of the brain will be of interest when we design future clinical trials (not funded by DOD but carried out by the same research team).

6. Gene expression studies identified several genes that may be relevant in understanding the pathophysiology of cognitive dysfunction in animal models who are treated with chemotherapy.
7. We intend to utilize the data from the gene expression analyses to develop a translational project to test evidence and hypotheses derived from these animal experiments in a clinical scenario.