Influence of Nitrosative Stress on Fatigue During Childhood Leukemia Treatment

Marilyn J. Hockenberry, PhD, RN, PPCNP-BC, FAAN¹,
Ida M. (Ki) Moore, PhD, RN, FAAN², Michael E. Scheurer, PhD, MPH^{3,4},
Mary C. Hooke, PhD, APRN, CNS-Pediatrics, CPON⁵,
Olga A. Taylor, MPH, BS³, Kari M. Koerner, MPH, CHES⁶,
Patricia M. Gundy, MS⁶, and Wei Pan, PhD¹

Abstract

The focus on a cure for childhood leukemia over the last three decades has resulted in survival rates of more than 80%. However, efforts to manage leukemia-treatment symptoms have not kept pace with new therapies. Symptom toxicity during treatment can result in complications, treatment delays, and therapy dose reductions. Compromise in therapy can negatively influence the quality of life and, even more notably, jeopardize chances for long-term survival. This study examined biologic mechanisms that influence fatigue caused by increased reactive oxidative species (ROS) or actual failure of the antioxidant defense system due to genetic variation by investigating reactive nitrosative species, a “downstream” consequence of ROS. The specific aims of this study were to characterize the trajectory of nitrosative stress during acute lymphoblastic leukemia treatment and evaluate the influence of nitrosative stress on fatigue. A repeated measures design was used to evaluate the fatigue experienced by 186 children and adolescents, 3–18 years of age, with a diagnosis of leukemia during the most intense phase of treatment. An established biomarker of nitrosative stress, protein 3-nitrotyrosine (3NT) residues in the cerebral spinal fluid, was evaluated at diagnosis, postinduction, and consolidation phases of treatment. Higher fatigue was associated with higher 3NT levels at the beginning of treatment. Two distinct groups of children experienced either consistently high or consistently low 3NT levels across the treatment trajectory, from diagnosis to 12 months postinduction. Findings from this study support continued exploration into the phenotypic biochemical mechanisms that influence a reactive response to childhood cancer treatment.

Keywords

nitrosative stress, fatigue, leukemia

Oxidative metabolism is the body’s first line of defense against environmental attack (Roberts et al., 2010). Reactive nitrosative species (RNS) act together with reactive oxidative species (ROS) to damage cells, causing nitrosative and oxidative stress. These two species, often collectively referred to as ROS/RNS, are part of the oxidative stress pathway and are constantly formed in the body as by-products of cellular metabolism and as cellular regulators. ROS/RNS are removed by antioxidant defenses, substances that have the ability to significantly delay or prevent ROS/RNS oxidation of substrates. Excessive production of ROS/RNS can lead to oxidative stress and disease. Production of ROS/RNS during tissue pathogenesis or in response to toxicants induces a complex series of downstream adaptive and reparative responses.

There is evidence that activation of the ROS/RNS pathway can induce numerous somatic symptoms in adults, including fatigue, depression, pain, muscle aches, flu-like malaise, and memory and concentration difficulties (Leonard & Maes, 2012; Maes, 2011). In depressed patients, there is a strong correlation

between the number of associated symptoms and ROS/RNS biomarkers; this association explains the co-occurrence of both depressive and fatigue and somatic symptoms (Maes, 2011). Researchers have reported that patients with neurodegenerative diseases have elevated levels of brain nitrosative stress and

¹ School of Nursing, Duke University, Durham, NC, USA

² Biobehavioral Health Science Division, College of Nursing, The University of Arizona, Tucson, AZ, USA

³ Department of Pediatrics, Texas Children’s Cancer and Hematology Centers, Baylor College of Medicine, Houston, TX, USA

⁴ Population Sciences Biorepository, Baylor College of Medicine, Houston, TX, USA

⁵ School of Nursing, University of Minnesota, Minneapolis, MN, USA

⁶ College of Nursing, The University of Arizona, Tucson, AZ, USA

Corresponding Author:

Marilyn J. Hockenberry, PhD, RN, PPCNP-BC, FAAN, 307 Trent Drive, DUMC 3322, Durham, NC 27710, USA.

Email: marilyn.hockenberry@duke.edu

protein nitration, which are thought to contribute to the disease processes in humans. These diseases include Parkinson's disease (Phani, Loike, & Przedborski, 2012), amyotrophic lateral sclerosis (Liu, Bao, Wen, & Liu, 2007), multiple sclerosis (Calabrese et al., 2003), stroke (Hardingham & Lipton, 2011), and traumatic brain injury (DeWitt & Prough, 2009).

Research has found variations in ROS/NOS biomarkers after chemotherapy for adult cancers (Atukeren et al., 2010). In a study evaluating ROS/RNS biomarkers in 30 breast cancer patients, Atukeren et al. (2010) found excessive amounts of DNA oxidation biomarkers in these patients after two cycles of chemotherapy compared to age-matched healthy controls. There is evidence that alkylating agents and antimetabolites, common pediatric leukemia chemotherapy drugs, increase the production of ROS/NOS (Al-Tonbary et al., 2011; Federico, Morgillo, Tuccillo, Ciardiello, & Loguercio, 2007; Fortunati et al., 2007; Kennedy, Ladas, Rheingold, Blumberg, & Kelly, 2005; Mazor, Abucoider, Meyerstein, & Kapelushnik, 2008; Papageorgiou et al., 2005). Other than our research team's previous work with oxidative stress biomarkers, we found only a few research studies that evaluated ROS in children with leukemia. Kennedy, Ladas, Rheingold, Blumberg, and Kelly (2005) evaluated an ROS biomarker, total antioxidant capacity (TAC), in 20 children with cancer at the time of diagnosis and during the first three cycles of chemotherapy and revealed significant decreases in the biomarker over time, suggesting an oxidative stress additive effect that could impact response to treatment, disease course, and prognosis. In a study of 13 children with leukemia or various solid tumors, TAC was impaired in both groups; however, higher oxidative stress occurred in the leukemia group (Papageorgiou et al., 2005). These studies showed that antioxidant status decreases in children with leukemia during chemotherapy treatment. Only one of these studies included a translational approach, demonstrating an association among antioxidant levels, ROS and complications of treatment, and improved quality of life (Kennedy et al., 2005). We found no studies evaluating the effects of both ROS and RNS pathways on fatigue experienced by children with cancer.

Dynamic models are needed to explain the complexities of symptoms during cancer treatment. The ROS and RNS pathways are dynamic and interactive and may result in significant cellular damage and toxicity (Federico et al., 2007; Fortunati et al., 2007). The conceptual framework for the present study identifies the brain's (cerebral spinal fluid [CSF] biomarkers) responses to the oxidative stress pathway that, when activated, influence the number and severity of symptoms during childhood cancer therapy. Chemotherapy agents commonly used for childhood leukemia (e.g., methotrexate) can increase ROS production as a result of altered folate metabolism and decreased 24 antioxidant enzyme production. Nitrosative stress is a downstream reaction to the ROS and RNS pathways' responses to stress. In the present study, we evaluated a unique phenotype (biochemical characteristics of ROS/RNS) to increase our understanding of fatigue associated with childhood leukemia treatment. The purposes of this study were to characterize the

trajectory of nitrosative stress during acute lymphoblastic leukemia (ALL) treatment; evaluate the effects of gender, age, and treatment intensity on nitrosative stress biomarkers; and determine the influence of nitrosative stress on fatigue.

Method

Prior to initiation of the study, we obtained institutional review board approval. We approached potential subjects and their parents about the study in the hospital setting or outpatient cancer clinic. We obtained consent from a parent or legal guardian, children 7 years and older, and adolescents provided assent. To evaluate treatment-related symptom associations experienced by 186 children and adolescents 3–18 years of age receiving leukemia treatment, we used a repeated measures research design. We explored fatigue over four time periods: initiation of postinduction therapy, 4 and 8 months postinduction therapy, and the beginning of maintenance/continuation therapy. For nitrosative stress analyses, we used CSF obtained by lumbar punctures as part of clinical treatment.

Sample

The initial sample size was 191. We did not obtain data on nitrosative stress measures (3-nitrotyrosine [3NT]) or fatigue measures from five of the children. The remaining 186 children provided data for at least one time point on 3NT and fatigue measures, including some who did not provide data for one or more of these longitudinal measures. We handled intermittent longitudinal missing data through techniques from latent class growth analysis (LCGA) and mixed modeling. In addition, those 186 children provided complete socio-demographic data and leukemia risk levels. The final sample size for this study was 186.

Leukemia Treatment

All participants were treated according to one of the following ALL protocols supported by the Children's Oncology Group: AALL0932, AALL1231, AALL1131, AALL0434, AALL1122, or AALL0031. ALL protocols treat children according to leukemia risk. Childhood ALL is classified into four risk groups: low, average, high, and very high. At diagnosis, children are placed into groups based on age and white blood cell count. Further risk stratification is based on genetic abnormalities and <0.01% presence of minimal risk disease at Day 29 of induction treatment. Specific treatment information for each protocol is available at the U.S. National Library of Medicine's <http://ClinicalTrials.gov> (<https://clinicaltrials.gov>) under the trial numbers NCT00022737, NCT00408005, NCT01190930, NCT01460160, NCT02883049, and NCT02112916. In general, treatment was provided as follows: induction therapy (1 month) and postinduction (6–8 months) followed by maintenance/continuation therapy beginning 6–8 months after the end of induction therapy. Time intervals for each phase of treatment were influenced by participants' side effects and recovery from low blood counts.

Data Collection

We obtained fatigue assessments using a standardized instrument during a routine follow-up clinic visit; time points were chosen to evaluate the most intensive phase of therapy post-induction. We used a tablet (iPad) for all survey data collection. Study measures took no longer than 35 min to complete. For consistency across fatigue-related symptoms, we used parent proxy for the 3- to 6-year-old age-group and symptom self-report for children ≥ 7 years of age. All symptom measures applied to the past week. We included both English- and Spanish-speaking children in the study and translated the instruments into Spanish versions to prevent language barriers.

CSF nitrosative stress measures. CSF obtained in conjunction with lumbar punctures as part of clinical treatment was available for the CSF nitrosative stress analyses. CSF samples were placed on ice and processed within 2 hr of collection to maintain stability. We centrifuged samples at 4°C to remove any particulates, aliquoted them, and stored them at -80°C until assay.

To measure 3NT, we used the Nitrotyrosine Assay Kit with chemiluminescence detection (Millipore Corp., U.S.), a competitive enzyme-linked immunosorbent assay based upon competition between plate-bound nitrated bovine serum albumin (BSA) and CSF-nitrated proteins for binding to an anti-3NT antibody. The assay is highly sensitive ($0.020\text{ }\mu\text{g/ml}$) and has a wide dynamic range (up to $1,000\text{ }\mu\text{g/ml}$) for nitrated protein. We ran each CSF sample in triplicate wells ($50\text{ }\mu\text{g/well}$) of a 96-well white plate. We produced a standard curve using nitrated BSA and blanks were run on each plate and processed the plates as instructed by the vendor. To measure luminescence emission intensity, expressed in relative luminescence units (RLU), we used the Perkin Elmer EnVision Multilabel Plate Reader in luminescence mode. Using a four-parameter curve-fitting method, we calculated the sample concentration of nitrated proteins from the standard curve, reporting results in nitro-BSA equivalents. As quality control, we determined the lower detection limit for each run and calculated inter- and intrarun variability.

Fatigue. We used the Childhood Fatigue Scale, a 10-item questionnaire, to assess the experience of fatigue-related symptoms during the past week in children 7–12 years of age (Hinds et al., 2010). Children were asked to rate how much they were bothered by fatigue using the 10 items on a 4-point Likert-type scale ranging from *not at all* to *a lot*. Total intensity ratings ranged from 10 (*no fatigue*) to 40 (*highest fatigue*). Analysis using Rasch methods identified a cut score of 31 for severe fatigue. In adolescents, 13–18 years of age, we used the Adolescent Fatigue Scale, a 13-item self-report scale developed to measure fatigue experienced in the past 72 hr (Hinds et al., 2007). Items describe the intensity of fatigue on a 4-point Likert-type scale. Intensity ratings range from 13 (*no fatigue symptoms*) to 65 (*highest fatigue*). Analysis using Rasch methods identified a cut score of 31 for severe fatigue (Mandrell et al., 2011). To

obtain proxy responses from parents of children aged 3–6 years, we used the Parent Fatigue Scale (PFS). The PFS is a 17-item scale that assesses parent perception of fatigue experienced by their child in the past 72 hr. Internal consistency of the 17-item PFS in a group of 147 parents was 0.88, and item-to-total correlations ranged from .34 to .69. Scores range from 0 to 68 with a cut score of ≥ 41 defining severe fatigue (Hockenberry et al., 2003).

Data Analysis

Descriptive statistics were computed to describe the sample characteristics as well as 3NT and fatigue measures over time. All analyses in this study were performed using SAS Version 9.4, and the significance level was set at $\alpha = .05$.

To characterize the trajectories of nitrosative stress (3NT), which demonstrated little change over time, LCGA with growth mixture modeling (i.e., group-based trajectory modeling) was conducted using SAS Proc Traj (Jones & Nagin, 2007; Jones, Nagin, & Roeder, 2001). This statistical technique helps identify distinct groups of participants following similar trajectories of change (or constantly high vs. constantly low in case of no change) over time for a variable of interest. The 3NT measures were continuous with approximately normally distributions and, thus, were modeled using the censored normal model in SAS Proc Traj. When comparing model fit to determine the number of distinct latent classes of 3NT trajectories over time, Bayesian information criterion (BIC; Schwartz, 1978) as well as minimum overlapping 95% confidence intervals of trajectories (Nagin, 2005) were considered. LCGA is a relatively new technique, and little is known about requirements in terms of sample size and the number of time points needed for good estimation and strong power (Muthén, 2004). Nagin (2005) only provided a general guideline that at least 100 cases are required for running LCGA. Thus, our sample size of 186 with 3NT measured at three time points would be sufficient for determining distinct latent classes of trajectories.

The characteristics of trajectories of 3NT, along with sociodemographic background and leukemia risk levels, were evaluated for their influence on fatigue in a mixed model. According to Raudenbush et al.'s (2011) Optimal Design program, a mixed model on a sample of 186 with four time points would have a power of .85 to detect a medium linear change over time ($\delta = .50$) at a significance level of .05, assuming that the intraclass correlation is .40, which is demonstrated in this sample.

Results

Descriptive Statistics

Table 1 shows that among 186 participants in the final sample, ages were not equally distributed, with 48.9%, 31.2%, and 19.9% comprising young children, children, and adolescents, respectively. Sex was fairly balanced with only a few more boys than girls (55.9% vs. 44.1%), whereas race/ethnicity was unbalanced with the majority being Hispanic (41.9%) or non-

Table 1. Sample Characteristics.

Characteristic	n (%)
Age-group	
Adolescent (13–18 years)	37 (19.9)
Child (7–12 years)	58 (31.2)
Young child (3–6 years)	91 (48.9)
Sex: Female	82 (44.1)
Race/ethnicity	
Hispanic	78 (41.9)
Non-Hispanic White	76 (40.9)
Non-Hispanic Black	16 (8.6)
Non-Hispanic other	16 (8.6)
Leukemia risk	
Low/average	82 (44.1)
High/very high	104 (55.9)

Note. *N* = 186.

Hispanic White (40.9%), and many fewer being non-Hispanic Black or non-Hispanic Other, which both comprised 8.6% of the sample. As for leukemia risk levels, 55.9% of the children in the study sample were at high or very high risk, and 44.1% at low or average risk. The means and standard deviations of 3NT and fatigue measures across time (Table 2) demonstrate that 3NT remained constant, whereas fatigue decreased over time. It is worth noting that we measured fatigue and 3NT at different time points. Specifically, we measured fatigue at each time point from Time 1 to Time 4, whereas we measured 3NT only at every other time point, Times 0, 2, and 4 (Table 2).

The Trajectory of Nitrosative Stress

LCGA revealed two distinct classes ($BIC = -958.82$ for a two-class solution vs. $BIC = -911.11$ for a three-class solution; $2\log_e[B_{10}] = 95.42$) of patients who shared the same trajectories of nitrosative stress across the three treatment phases: 0 = at diagnosis, 2 = 4-month postinduction, and 4 = 12-month postinduction or consolidation; although neither trajectories demonstrated an increased or decreased trend (Figure 1). We used the two classes or groups of patients (Group 1 = 41.1% with low 3NT around 25.5 million RLU, and Group 2 = 58.9% with high 3NT around 30.0 million RLU) to evaluate the influence of nitrosative stress on fatigue.

The Influence of Nitrosative Stress on Fatigue

We conducted a mixed model to evaluate the influence of nitrosative stress (3NT) on fatigue, controlling for sociodemographic characteristics (age-group, gender, and race) and leukemia risk levels. The results of fixed effects from the mixed model are displayed in Table 3. Overall, patients' fatigue significantly reduced by 1.46 points ($p < .01$) as time increased from one treatment phase to another. Initially, patients who had a higher 3NT level experienced more fatigue ($\beta = 4.28$, $p < .03$) than those who had a lower level of 3NT, whereas, as time increased from one treatment phase to another, fatigue symptoms for patients who had a higher 3NT level reduced faster at a rate of $\beta = -1.43$ ($p < .02$) compared with those who had a lower 3NT level. The interaction effect of 3NT with time on fatigue is also depicted in Figure 2.

Discussion

This study is the first to evaluate an established biomarker of nitrosative stress, 3NT residues in the CSF at diagnosis, post-induction, and consolidation phases of childhood leukemia treatment. We evaluated characteristics of the trajectories of 3NT along with sociodemographic characteristics and leukemia risk levels to determine their influence on fatigue during the first year of childhood ALL treatment.

Higher fatigue was associated with higher 3NT levels at the beginning of treatment. The early treatment phase of leukemia therapy is intense, and this finding documents the effect on the child's physical status. It is interesting that children with higher fatigue initially had a faster reduction in 3NT levels over time compared to those who had lower levels of initial fatigue. This finding is surprising and merits further investigation. Perhaps 3NT levels are influenced by the leukemia, itself, and so could also be influenced by a rapid response to therapy. We measured 3NT in CSF samples obtained in conjunction with intrathecal chemotherapy administration. CSF concentrations of 3NT more closely reflect the level of nitrosative stress in brain tissue than concentrations in serum or plasma; however, it is possible that fatigue levels were also influenced by systemic therapy-related ROS, detection of which would require measuring 3NT in serum or plasma. Measuring CSF and serum 3NT concentrations could provide a more complete understanding of the association between nitrosative stress and fatigue.

Table 2. Means (*M*) and Standard Deviations (*SDs*) of 3-Nitrotyrosine (3NT) and Fatigue Measures by Time.

	Time 0 (At Diagnosis)		Time 1 (Postinduction)		Time 2 (4 Months Postinduction)		Time 3 (8 Months Postinduction)		Time 4 (12 Months Postinduction)	
Measure	<i>n</i>	<i>M</i> (<i>SD</i>)	<i>n</i>	<i>M</i> (<i>SD</i>)	<i>n</i>	<i>M</i> (<i>SD</i>)	<i>n</i>	<i>M</i> (<i>SD</i>)	<i>n</i>	<i>M</i> (<i>SD</i>)
3NT ^a	134	28.12 (2.50)	—	—	180	28.20 (2.56)	—	—	170	28.28 (2.53)
Fatigue ^b	—	—	163	54.03 (10.54)	182	49.46 (10.44)	171	48.20 (9.08)	159	46.49 (8.33)

^aMillion relative luminescence units. ^bT score with a range of 20–80.

Figure 1. Trajectories of nitrosative stress during acute lymphoblastic leukemia treatment. 3NT = 3-nitrotyrosine.

Table 3. Solution for Fixed Effects From Mixed Model Evaluating Fatigue.

Effect	Estimate	Standard Error	df	t Value	Pr > t
Intercept	52.52	2.09	177	25.16	<.001
Time (treatment phase)	−1.46	0.57	179	−2.55	.012
Age-group (adolescent: 13–18 years)	1.19	1.59	307	0.75	.454
Age-group (child: 7–12 years)	0.57	1.34	307	0.42	.672
Age-group (young child: 3–6 years)	0.00	—	—	—	—
Gender (female)	−1.48	1.12	307	−1.33	.184
Gender (male)	0.00	—	—	—	—
Race (non-Hispanic Black)	3.36	2.15	307	1.56	.120
Race (non-Hispanic Other)	2.54	2.08	307	1.22	.223
Race (non-Hispanic White)	2.32	1.24	307	1.87	.063
Race (Hispanic)	0.00	—	—	—	—
Leukemia risk (high/very high)	−1.65	2.01	307	−0.82	.413
Leukemia risk (low/average)	0.00	—	—	—	—
3NT group (constantly high: ~30.0 million RLU)	4.28	1.96	307	2.18	.030
3NT group (constantly low: ~25.5 million RLU)	0.00	—	—	—	—
Time* (high/very high leukemia risk)	−0.07	0.59	307	−0.11	.911
Time* (low/average leukemia risk)	0.00	—	—	—	—
Time* (constantly high 3NT)	−1.43	0.60	307	−2.4	.017
Time* (constantly low 3NT)	0.00	—	—	—	—

Note. 3NT = 3-nitrotyrosine; RLU = relative luminescence units; df = degree of freedom.

* = <.05.

Two distinct groups of children experienced either consistently high 3NT or consistently low 3NT levels across the treatment trajectory, from diagnosis to 12 months postinduction. This finding of consistency in the 3NT levels across three treatment phases raises the question of the potential role that genetics might play in determining this biomarker's levels. Since there was very little variability in response to therapy, but differences nonetheless existed between these two groups of children, it may be that levels of 3NT are under genetic control; if so, this information could be harnessed to predict

those patients at risk of poor outcomes. Further exploration of the genotype–phenotype correlations with 3NT levels within this population and across time is needed to more completely understand this finding.

Leukemia risk, age, race, and sex had no impact on 3NT levels or fatigue throughout the treatment trajectory. These findings are consistent with research by Crabtree and colleagues (2015), which showed that fatigue did not differ by age-group, gender, or socioeconomic status in 170 children in early treatment for their cancer.

Figure 2. The interaction effect of 3-nitrotyrosine (3NT) with time on fatigue.

Conclusion

In the present study, we examined specific biologic mechanisms that influence fatigue caused by increased ROS. Specifically, we investigated RNS, a “downstream” consequence of ROS during childhood leukemia therapy. We found that fatigue, a common symptom experienced by children with leukemia, was most significant during the early phase of treatment and lessened over time. Findings from this study support continued exploration into the phenotypic biochemical mechanisms that influence a reactive response to childhood cancer treatment.

Author Contributions

Marilyn J. Hockenberry contributed to conception and design, acquisition, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Ida M. (Ki) Moore contributed to conception and design, acquisition, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Michael E. Scheurer contributed to conception and design, acquisition, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Mary C. Hooke contributed to interpretation, drafted the manuscript, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Olga A. Taylor contributed to acquisition, drafted the manuscript, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Kari M. Koerner contributed to acquisition, drafted the manuscript, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Patricia M. Gundy contributed to acquisition, drafted the manuscript, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Wei Pan contributed to conception and design, acquisition, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Conflicting Interests

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