Changes in Oxidant Defense, Apoptosis, and Cognitive Abilities During Treatment for Childhood Leukemia

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Abstract

Aggressive central nervous system (CNS)-directed treatment for acute lymphoblastic leukemia (ALL), the most prevalent cancer among children and adolescents, prevents metastasis of leukemia cells into the brain. Up to 60% of survivors experience cognitive problems, but knowledge about risk factors for and mechanisms of neurologic injury is lacking. Objectives of the present study were to (1) quantify changes in oxidant defense and apoptosis over the course of ALL therapy and (2) elucidate risk factors for long-term cognitive problems. The sample included 71 children with ALL. Cerebrospinal fluid (CSF) samples were collected at diagnosis and during intrathecal chemotherapy administration. Oxidant defense was measured by reduced glutathione (GSH), oxidized glutathione (GSSG), and the ratio of GSH:GSSG. Apoptosis was measured by activity of several cysteine-dependent aspartate-specific protease (abbreviated as caspase) enzymes that initiate (caspases 8 and 9) or execute (caspases 3/7) apoptosis. Cognitive abilities were assessed by standardized measures of short-term memory, visual-motor integration, and attention 3 years after ALL diagnosis. GSH and GSSG concentration increased significantly during ALL therapy, and a low GSH:GSSG ratio was indicative of an oxidized extracellular environment. Caspase enzyme activity increased significantly, and caspases 3/7 activity was significantly and negatively associated with performance on measures of cognitive abilities. Younger age at time of ALL diagnosis was associated with some measures of attention. Efflux of glutathione into CSF maintains oxidant defense by scavenging free radicals and other reactive oxygen species and is an early event in apoptosis. These mechanisms may be involved in neurologic injury associated with CNS-directed treatment and subsequent cognitive problems.

Keywords

oxidant defense, glutathione, apoptosis, caspase enzymes, cognitive abilities

Acute lymphoblastic leukemia (ALL) is the most prevalent cancer in the United States among children and adolescents less than 15 years of age (Li, Thompson, Miller, Pollack, & Stewart, 2008; Winick, Carroll, & Hunger, 2004). Aggressive central nervous system (CNS)-directed treatment is necessary to prevent metastasis of leukemia cells into brain tissue, but up to 60% of ALL survivors experience problems with memory, attention, processing speed, and visual spatial skills (Buizer, De Sonnevile, van den Heuvel-Eibrink, Njiokiktjien, & Veerman, 2005; Carey et al., 2007; Cheung & Krull, 2015; Hockenberry et al., 2007; Kanellopoulos et al., 2016; Krull, Brinkman, et al., 2013; B. D. Moore, 2005). Despite substantial evidence that CNS-directed treatment with chemotherapy is associated with cognitive problems, knowledge about risk factors for and mechanisms of neurologic injury is lacking. Such knowledge is greatly needed in order to develop novel neuroprotective interventions (Ahles & Saykin, 2007). There is some evidence that children

who are less than 5 years of age at the time of diagnosis and/or female are at greater risk for cognitive problems, but findings across studies are inconclusive (Insel et al., 2017; Jacola et al., 2016; Kanellopoulos et al., 2016; Waber et al., 2012). Knowledge about individual risk factors for cognitive problems is also needed to identify children who are at greater risk for cognitive problems and most likely to benefit from early interventions.

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Even under normal conditions, the brain is particularly vulnerable to an imbalance in oxidant defense because of high levels of reactive oxygen species (ROS) and low-to-moderate antioxidant mechanisms (Aoyama & Nakaki, 2013; Floyd, 1999). Further, accumulation of ROS activates a cascade of cysteine-dependent aspartate-specific protease (abbreviated as caspase) enzymes, which regulate apoptosis and stimulate inflammation in the nervous system (Aquilano, Baldelli, & Ciriolo, 2014; Nicotera, 2000; Shah et al., 2008; Yuan & Yankner, 2000). Unique among caspase enzymes, caspases 3 and 7 (caspases 3/7) activation has a central role in apoptosis during brain development and neurodegeneration as well as in the progressive dismantling of neuronal circuits in brain regions that mediate memory functions (D'Amelio, Sheng, & Cecconi, 2012; Robertson, Crocker, Nicholson, & Schulz, 2000).

Building on this evidence, we sought in the present study to explore changes in oxidant defense and apoptosis as possible mechanisms involved in neurologic injury associated with CNS-directed treatment and to identify risk factors for long-term cognitive problems. Our objectives were to:

1. quantify changes in oxidant defense and apoptosis biomarkers over the course of ALL therapy. Oxidant defense (glutathione) and apoptosis (caspase enzyme activity) biomarkers were measured in cerebrospinal fluid samples obtained during diagnostic and therapeutic lumbar punctures (LPs).
2. elucidate risk factors for long-term cognitive problems. Risk factors included caspases 3/7 activity, age at the time of ALL diagnosis, and sex. Cognitive abilities were assessed by standardized measures of short-term memory, visual-motor integration, and attention.

Background

ALL is a malignancy of lymphoid progenitor cells (lymphoblasts) that originate in the bone marrow and quickly enter the blood. Once in the blood, lymphoblasts can cross the blood-brain barrier and metastasize into brain parenchyma (Pui, 2003). Aggressive CNS-directed treatment with systemic and intrathecal chemotherapy, primarily methotrexate, is essential to prevent disease relapse in the brain and for long-term disease-free survival, which, with contemporary therapy, is 85–90% (Hunger et al., 2012; Tasian, Loh, & Hunger, 2015; Winick et al., 2004). Declines in cognitive abilities, particularly in the areas of working memory, processing speed, attention, visual spatial abilities, and motor speed (Ashford et al., 2010; Bisen-Hersh, Hineline, & Walker, 2011; Buizer et al., 2005; Buizer, de Sonnevill, & Veerman, 2009; Espy et al., 2001; Hockenberry et al., 2007; Insel et al., 2017) are among the most challenging treatment-related sequelae ALL survivors experience. Compared to children with tumors not requiring CNS-directed therapy, healthy controls, and/or normative data, children with ALL perform significantly worse on visual-motor tasks, motor skills, and motor proficiency for up to 5 years after completion of ALL therapy (Waber et al., 2012). Researchers

initially attributed cognitive and academic problems to the effects of cranial radiation, but more recent evidence also implicates CNS-directed chemotherapy (Krull, Hockenberry, Miketova, Carey, & Moore, 2013; I. M. Moore et al., 2000, 2015). Further, we found a greater decline in verbal working memory among children who were less than 5 years of age when diagnosed with ALL compared to those diagnosed at age 5 years or older (Insel et al., 2017).

Glutathione (GSH), the most important antioxidant synthesized by cells, plays a major role in regulating the redox status of all tissues (Aoyama & Nakaki, 2013; Franklin et al., 2002). Glutathione is present in either the reduced (GSH) or oxidized (GSSG) form. Under physiologic conditions, reduced GSH is the predominant form with a concentration from 10- to 100-fold higher than that of the oxidized species (Aquilano et al., 2014). The ratio of GSH:GSSG is normally maintained in excess of 100:1 but can significantly decrease to less than 4:1 during conditions of oxidative stress (Circu & Aw, 2008). The biologically active form of glutathione (GSH) is considered to be the major brain antioxidant (Circu & Aw, 2012). Deficiencies in GSH can sensitize the brain to injury, and a wide range of human diseases, including neurodegenerative diseases, are linked to either decreased GSH levels or abnormal levels of enzymes involved in GSH metabolism (Ribas, Garcia-Ruiz, & Fernandez-Checa, 2014). Observations that CNS dysfunction occurs in diseases due to inborn errors of GSH metabolism offer additional evidence that the brain is particularly susceptible to alterations in GSH production (Aoyama & Nakaki, 2013). A decrease in GSH concentration to below a threshold level due to efflux from cells or GSH oxidation constitutes an apoptotic signal that initiates death receptor activation or mitochondria apoptotic signaling, including activation of caspase enzymes (Aquilano et al., 2014; Circu & Aw, 2008).

Mitochondrial ROS are increased during many pathophysiological conditions and drug treatment. Upon reaction with ROS, GSH becomes oxidized to GSSG (Ribas et al., 2014). Rouse, Nwokedi, Woodliff, Epstein, and Klimberg (1995) were among the first to report that tumor tissue obtained from rats treated with methotrexate (a drug commonly used to treat children with ALL) was depleted of the crucial antioxidant GSH. Cellular levels of GSH were reduced by 70% in cultured HeLa cells treated with methotrexate at concentrations equivalent to plasma levels achieved in patients receiving high-dose treatment with this drug. Examination of cellular enzyme activities revealed that several enzymes involved in GSH synthesis, including glutathione reductase, were inhibited. Glutathione reductase is essential for maintaining homeostasis of the intracellular redox state, and methotrexate can significantly decrease the effectiveness of this antioxidant defense system. Compared to other regions of the brain, the hippocampus and cortex of rat brains treated with a relatively low dose (0.2 mg/kg/day) of methotrexate had the greatest decrease in GSH and increase in lipid peroxidation after treatment (Rajamani, Muthuvel, Senthilvelan, & Sheeladevi, 2006).

Caspase is an acronym for cysteine-dependent aspartate-specific protease. To date, investigators have identified

14 caspase enzymes and numbered consecutively in the order of their discovery (Eldadah & Faden, 2000). These enzymes initiate and/or execute apoptosis (McIlwain, Berger, & Mak, 2013), a fundamental biological process that removes unwanted cells during development, differentiation, and disease prevention. However, excessive or premature caspase activation is implicated in the pathogenesis of tissue injury, including acute and chronic CNS injury (Robertson et al., 2000). Oxidative stress is one process that can result in excessive caspase activation, and in the brain, it plays a critical role in cell death associated with traumatic brain injury, spinal cord injury, cerebral ischemia, and chronic neurodegenerative conditions (Eldadah & Faden, 2000; Porter & Janicke, 1999; Robertson et al., 2000).

Caspase enzymes are categorized by their role in apoptosis. “Initiator” caspases mediate the signal for apoptosis at a death receptor or at the mitochondria. Caspases 8 (extrinsic-mediated pathway) and 9 (intrinsic-mediated pathway) play a role in acute and chronic CNS injury (Eldadah & Faden, 2000). The extrinsic-mediated pathway is triggered by extracellular signals that bind to plasma membrane death receptors, and the intrinsic-mediated pathway is activated through various cellular stresses that lead to cytochrome *c* release from the mitochondria (Cirru, 2008; McIlwain et al., 2013). Caspase 9 directly cleaves and activates “effector” caspases 3 and 7. Effector caspases execute cell death by processes including cytoskeleton disintegration, DNA fragmentation, nuclear membrane dissolution, and plasma membrane blebbing. Caspase 3 is activated in traumatic brain injury, ischemic stroke, and drug toxicity (Porter & Janicke, 1999; Uzan et al., 2006; Yoneyama, Seko, Kawada, Sugiyama, & Ogita, 2009).

Material and Method

We used a within-subject repeated measures design to investigate changes in oxidant defense, apoptosis, and cognitive abilities among children with ALL who were receiving CNS-directed treatment with chemotherapy. Eligible children were between 2.3 and 14.7 years of age at the time of ALL diagnosis, recruited from two pediatric oncology treatment centers in the southwestern United States and treated according to Children Oncology Group protocols. We obtained consent at the time of ALL diagnosis from parents and assent from children aged 7 years or older. Exclusion criteria included prior history of other causes of neurologic injury (i.e., seizures, traumatic brain injury, or developmental disabilities such as Down syndrome or attention deficit disorder).

We measured antioxidant and apoptosis biomarkers in cerebral spinal fluid (CSF) samples obtained at the time of ALL diagnosis and then in conjunction with LPs for administration of intrathecal chemotherapy at standard time points during ALL treatment: induction (first 29 days of therapy, average 2.9 LPs), postinduction (aggressive phase of CNS-directed treatment, average 7.9 LPs), and continuation (last phase of therapy, average 8.0 LPs). We took special precautions because antioxidant results can be influenced by sample handling, and some auto-oxidation of GSH will occur spontaneously in the

presence of oxygen. We placed all CSF samples on ice immediately to slow/prevent reactions, kept samples cold during the entire process, and stored them at -80°C to further minimize auto-oxidation. Before analysis, we thawed all samples on ice. Assessments of cognitive abilities were completed approximately 3 years after ALL diagnosis.

Oxidant Defense

We measured antioxidant levels with the Promega GSH Glo™ glutathione assay and reported them as the concentration of GSH, concentration of GSSG, and the GSH:GSSG ratio. The Promega GSH Glo assay is a luminescence-based assay that requires 100 μl of CSF for analysis. The luminescence assay utilizes a luciferin derivative that is converted to luciferin by the glutathione S-transferase enzyme in the presence of GSH. A stable luminescent signal is then generated when firefly luciferase is added; signal intensity is directly proportional to the amount of GSH in the sample. Duplicate wells on one plate measure existing GSH and on a second plate contain the reducing agent added to the sample to convert any oxidized GSSG to reduced GSH, thus measuring total reduced GSH. Each well contains 25 μl . The reducing agent is a thiol-free compound, tris(2-carboxyethyl)phosphine hydrochloride (0.5 mM), to reduce disulfide bonds without any deleterious effects on the assay itself. GSSG was then calculated as (total reduced [GSH] – initial reduced [GSH]) divided by 2 ($2\text{ GSH} = 1\text{ GSSG}$).

Apoptosis

Promega Caspase-Glo luminescence assay kits were used to measure caspases 3/7, 8, and 9 activity as instructed by the manufacturer. These homogenous single-step assays measure the enzymatic activities of caspases 3/7 (simultaneously in same assay), 8, and 9. Each sample (25 μl /well) was measured in duplicate in 96-well, half-area plates. Plates were read by the Perkin-Elmer Envision Xcite multilabel plate reader with a high-sensitivity luminometer. Caspase activity is determined by comparison to a standard curve for each purified caspase enzyme using regression analysis and data expressed as units of caspase activity.

Cognitive Abilities

We assessed cognitive abilities as soon as the child was in disease remission and medically stable (baseline) and then annually for 3 years (Years 1, 2, and 3). Assessments included measures of visual-motor integration, short-term memory, working memory, and attention, as these domains are particularly vulnerable to CNS-directed treatment (Ashford et al., 2010; Buizer et al., 2009; Insel et al., 2017; Kanellopoulos et al., 2016; I. M. Moore et al., 2015). For these measures, we report the child's performance as an age-adjusted score based on standardized norms. Researchers have previously reported changes over time in these cognitive abilities in children treated for ALL (Insel et al., 2017; I. M. Moore et al., 2015). For the present study, we

sought to determine if changes in oxidant defense and apoptosis biomarkers during ALL treatment were associated with Year 3 cognitive outcomes assessed at the end of therapy.

We assessed visual-motor integration with the Beery Visual-Motor Integration (VMI), sixth edition. The VMI assesses the ability to copy designs of increasing difficulty and requires coordination of visual perception and finger and hand movements. The test has been normed for children ages 2–18 years; performance is reported as a standard age-adjusted score with a mean of 100 and standard deviation (*SD*) of 15; higher scores indicate better visual-motor integration skills.

We measured short-term memory with the Memory for Sentences and Bead Memory subtests from the Stanford–Binet Intelligence Scale, fourth edition (Thorndike, Hagen, & Sattler, 1986). In the Bead Memory test, participants are shown either beads in the examiner's hand (easy task) or a picture of the beads on a stick (difficult task). They must then either recall which beads they were shown (easy task) or recreate the picture they were shown (difficult task). The Memory for Sentences task requires the participant to listen to sentences and repeat them back. Both tests become more difficult as the testing progresses. Performance on Bead Memory and Memory for Sentences is reported as standard age-adjusted scores with a mean of 50 and *SD* of 8; higher scores reflect better performance.

We measured working memory using subtests from the attention/concentration index from the Wide Range Assessment of Memory and Learning, second edition (WRAML2; Adams & Sheslow, 2003). The WRAML2 index is composed of the Finger Windows and Number Letter subtests. Children must recall a series of visual cues (Finger Windows) or verbal cues (Number Letter) that lengthen as the task becomes more difficult (Kane & Engle, 2003). Performance on Finger Windows and Number Letter is reported as a scaled score with a mean of 10 and *SD* of 3; the WRAML2 composite has a mean score of 100 and *SD* of 15.

We measured attention using the Conners' Continuous Performance Test second edition (CPT II) for children ages 6 and older, and the Kiddie Continuous Performance Test first edition (K-CPT) for children ages 4 and 5. Both are computer-based tests that assess several dimensions of attention including inattention, impulsivity, and vigilance. The test for older children uses letters, while the test for younger children uses simple pictures (Conners, 2014). Children are instructed to touch the space bar every time they see a stimulus except for when they see the letter X (CPT II) or a soccer ball (K-CPT). Subscale scores are *T* scores with a mean of 50 and *SD* of 10.

Data Analysis

We analyzed data using SPSS version 20. To summarize sample characteristics, we used descriptive statistics. We compared mean scores on cognitive measures to age-adjusted standardized norms using one sample *t* tests and calculated percentage of subjects performing 1.0 or 1.5 *SD* below the norms. To test for change over time in the oxidant defense and apoptosis biomarkers, we used a general linear model (GLM) for repeated measures analysis of variance (ANOVA).

Table 1. Sample Demographic and Clinical Characteristics.

Variable	<i>N</i>	%
Gender		
Female	39	54.9
Race/ethnicity		
Caucasian	31	43.7
Hispanic	32	45.1
African American	2	2.8
Native American	2	2.8
Other	4	5.6
ALL risk group		
Low	6	8.5
Standard	40	56.3
High	22	31.0
Very high	3	4.2

Note. *N* = 71.

To summarize levels of antioxidant and apoptosis biomarkers, we used descriptive statistics. Because the number of CSF samples obtained during induction, postinduction, and continuation varied across subjects depending on the specific ALL treatment protocol, we calculated the mean GSH and caspase value for each child and used it for the Generalized Linear Model (GLM) ANOVA.

Caspases 3/7 executes apoptosis, and concentrations were highest during induction and postinduction. Therefore, we tested associations among caspases 3/7 during induction and postinduction, age at the time of ALL diagnosis, and cognitive abilities using Pearson product correlation. We used *t* test to determine if there were significant sex differences in cognitive abilities. We set statistical significance at $\alpha \leq .05$.

Results

Demographic Characteristics

Table 1 summarizes demographic and clinical characteristics of the sample. Participants included 71 children with ALL. Mean age at the time of diagnosis was 6.5 (2.3–14.7; *SD* = 3.2) years. There were 32 males and 39 females; the majority were Hispanic (45.1%) or Caucasian (43.7%). Most children were diagnosed with standard-risk (*n* = 40, 56.3%) or high-risk (*n* = 22, 31.0%) ALL. Mean age at the time of the cognitive assessment was 9.3 years (6.3–17.8; *SD* = 2.7).

Oxidant Defense Biomarkers

Figure 1 summarizes changes in the total GSH, initial reduced GSH and GSSG concentration over time. Results from the GLM analysis showed a significant increase in total GSH peptide concentration over time, $F(3, 147) = 24.7, p < .001$. Mean total GSH peptide concentration was 620.8 nM (Standard Error of the Mean (SEM) = 42.3) at diagnosis and was significantly increased compared to baseline during the induction ($M = 832.4, SEM = 50.0, F = 34.00, p < .001$), postinduction ($M = 820.8, SEM = 46.6, F = 34.6, p < .001$), and continuation

Figure 1. Changes in glutathione (GSH) over time. Changes in the total GSH peptide, initial reduced GSH, and mean oxidized glutathione (GSSG) at diagnosis and during the induction, postinduction, and continuation phases of treatment (mean with standard error bars) are illustrated. *Indicates significant difference from diagnosis.

($M = 805.3$, $SEM = 44.9$, $F = 32.3$, $p < .001$) treatment phases. The initial reduced GSH concentration also increased significantly over time, $F(3, 147) = 10.7$, $p < .001$. Mean initial reduced GSH concentration was 115.7 nM ($SEM = 11.3$) at diagnosis and significantly increased at all treatment phases with the highest concentration occurring during the continuation treatment phase ($M = 171$, $SEM = 10.4$, $F = 18.3$, $p < .001$). GSSG (oxidized form) also increased significantly over the course of ALL therapy, $F(3, 147) = 21.0$, $p < .001$. Mean GSSG in the diagnostic CSF sample was 253.2 nM ($SEM = 18.4$); there was a significant increase over baseline in mean concentrations during induction ($M = 336.7$, $SEM = 20.5$, $F = 35.3$, $p < .001$) and postinduction ($M = 325.4$, $SEM = 19.4$, $F = 27.5$, $p < .001$). The GSH:GSSG ratio was low at diagnosis ($M = 0.5$, $SEM = 0.1$) and remained low across all phases of treatment (0.5–0.6). The change in GSH/GSSG over time was not significant, $F(3, 147) = 2.3$, $p = .1$.

Apoptosis Biomarkers

Changes over time in caspases 3/7, 8, and 9 activity are summarized in Figure 2. The change over time in caspases 3/7 was statistically significant, $F(3, 177) = 27.3$, $p < .001$. Caspases 3/7 activity increased from diagnosis ($M = 18.9$, $SEM = 2.0$) to the induction ($M = 45.6$, $SEM = 3.7$, $F = 45.0$, $p < .001$) and postinduction ($M = 46.9$, $SEM = 3.7$, $F = 47.4$, $p < .001$) treatment phases followed by a decrease during continuation that left levels still significantly higher than at diagnosis ($M = 29.9$, $SEM = 1.4$, $F = 22.3$, $p < .001$). We found a similar pattern of change over the course of ALL therapy for caspase 8, $F(3, 132) = 31.6$, $p < .001$, and caspase 9, $F(3, 153) = 28.2$, $p < .001$. However, concentrations were only significantly higher than at diagnosis during the induction and postinduction treatment phases.

Figure 2. Changes in caspase enzyme activity over time. Changes over time in caspase 3/7, caspase 8, and caspase 9 activity are summarized (mean and standard error) by treatment phase. *Indicates significant difference from diagnosis.

Cognitive Abilities

Performance on the cognitive abilities and results from the one sample t test comparing mean scores with standardized norms are summarized in Table 2. Participants' scores were significantly lower than what is expected for healthy peers on visual-motor integration ($p < .001$) and short-term verbal memory ($p = .03$). Participants also had significantly worse scores on several measures of attention including inattention (omissions, $p = .008$), impulsivity (hit reaction time, $p = .046$), and vigilance (hit reaction time block change, $p = .001$). Mean scores did not differ significantly from norm references for visual memory (BEAD MEMORY), verbal recall (NUMBER LETTER), commission errors, or detectability. Participants had a higher score than the norm reference value in a composite measure of visual and verbal attention/concentration ($p = .02$) which was due to higher scores in visual memory (FINGER WINDOWS, $p = .001$).

Based on normative data, 15.86% of children would be expected to perform 1 SD below the mean score, and 6.68% 1.5 SD below the mean. As shown in Figure 3, more than 15.86% of children scored 1 SD below the mean on measures of visual-motor integration, inattention, vigilance, and detectability, and more than 6.68% of children scored greater than 1.5 SD below the mean on visual-motor integration, inattention, and vigilance.

Associations Between Caspases 3/7 Activity, Gender, and Age at Diagnosis and Cognitive Abilities

Associations between caspases 3/7 activity during induction and postinduction with cognitive abilities 3 years after ALL

Table 2. Results of One Sample *t* Test Comparing Cognitive Abilities With Standardized Norms.

Measure	Mean	SD	Range	Difference From Norm		
				<i>t</i>	<i>df</i>	<i>p</i>
Age at diagnosis	6.5	3.2	2.3–14.7			
Age at evaluation	9.3	2.7	6.3–17.8			
Beery Visual-Motor Integration	92.8	12.8	61–126	–4.0	49	<.001
Bead Memory	52.5	9.3	31–71	1.9	48	.066
Memory for Sentences	47.4	8.3	26–74	–2.2	48	.030
WRAML2 Number Letter	10.2	2.5	5–16	0.6	49	.577
WRAML2 Finger Windows	11.6	3.3	5–19	3.5	49	.001
WRAML2 attention/concentration composite	104.7	13.7	73–131	2.4	49	.020
CPT omissions ^a	55.6	14.2	40.6–107.7	2.8	48	.008
CPT commissions ^a	49.9	8.0	32.1–63.7	–0.1	48	.902
CPT hit reaction time ^a	53.5	11.8	29.0–72.9	2.0	48	.046
CPT hit reaction time block change ^a	56.1	11.8	36.6–86.4	3.6	48	.001
CPT detectability ^a	52.1	8.8	34.3–67.3	1.7	48	.101

Note. CPT = Conners' Continuous Performance Test; WRAML2 = Wide Range Assessment of Memory and Learning, second edition; SD = standard deviation.

^aCPT measure, type of attention, and interpretation are summarized below:

Measure	Type of Attention	Interpretation
Omissions	Inattention	High scores indicate poor performance, many missed targets
Commissions	Inattention/impulsivity	High scores indicate poor performance, incorrect responses to the target
Hit reaction time	Inattention/impulsivity	Higher scores indicate inattention, lower scores indicate impulsivity
Hit reaction time block change	Vigilance	Higher scores indicate poor performance, loss of vigilance during the test
Detectability	Inattention	Higher scores indicate poor performance, inability to distinguish target from nontarget

Figure 3. Percentage of subjects performing 1 or 1.5 standard deviations (SDs) below norm on cognitive tests once children were in disease remission. The percentage of subjects who performed 1 or 1.5 SDs below the norm are summarized for each cognitive measure. Based on normative data, 15.86% of children would be expected to perform 1 SD below the mean and 6.68% expected to perform 1.5 SD below the mean score. CPT = Conners' Continuous Performance Test; WRAML2 = Wide Range Assessment of Memory and Learning, second edition.

Table 3. Associations Between Caspases 3/7 Activity During Induction and Postinduction and Cognitive Abilities 3 Years After Acute Lymphoblastic Leukemia Diagnosis.

Mean Caspase 3/7	Year 3 Cognitive Measures															
	Beery VMI		Bead Memory		Memory for Sentences		WRAML2 Finger Windows		WRAML2 Number Letter		WRAML2 Attention/Concentration Composite		CPT Commissions		CPT Detectability	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>R</i>	<i>p</i>
Induction	-.4	<.01	-.4	<.01	-.4	<.01	.1	ns	-.3	.02	-.3	.01	-.3	.03	-.3	.03
Postinduction	-.4	<.01	-.2	ns	-.3	.02	-.3	.04	-.2	ns	-.3	.01	-.2	ns	-.2	ns

Note. CPT = Conners' Continuous Performance Test; ns = not significant; WRAML2 = Wide Range Assessment of Memory and Learning, second edition; VMI = Beery Visual-Motor Integration.

diagnosis are summarized in Table 3. Caspases 3/7 activity during induction was significantly and negatively associated with performance on measures of visual-motor integration, short-term memory, working memory, commissions, and detectability ($r = -.03$ to $-.4$, $p < .01$ to $p = .03$). Caspases 3/7 concentration during postinduction was significantly and negatively associated with visual-motor integration ($r = -.4$, $p < .01$), Memory for Sentences ($r = -.3$, $p < .01$), WRAML2 Finger Windows ($r = -.3$, $p = .04$), and the WRAML2 attention concentration composite ($r = -.3$, $p = .01$). There were no significant sex differences on any of the cognitive measures. Age at the time of ALL diagnosis was not associated with performance on measures of visual-motor integration or short-term or working memory. However, children who were younger at the time of diagnosis had poorer performance on inattention/impulsivity ($r = -.3$, $p = .02$) and vigilance ($r = -.3$, $p = .04$).

Discussion

In CSF samples obtained from children diagnosed with ALL during the induction and postinduction treatment phases, we found significant changes in oxidant defense biomarkers (GSH and GSSG), a low GSH:GSSG ratio, and significant increases in caspase enzyme (3/7, 8, and 9) activity. We also found that, 3 years after ALL diagnosis, the children's performance on visual-motor integration, Memory for Sentences, and working memory measures and some measures of attention/vigilance was significantly below normative values of healthy peers and was associated with caspases 3/7 activity during induction or postinduction.

The biologically active (reduced) form of GSH protects against exogenous and endogenous toxins including ROS (Aquilano et al., 2014; Circu & Aw, 2012). GSH is only synthesized intracellularly (primarily in the cytosol), and degradation occurs exclusively in extracellular spaces. The significant increase in GSH concentration during CNS-directed treatment for ALL suggests an efflux of the biologically active form of GSH into the CSF to maintain oxidant defense by scavenging free radicals and other ROS (Aquilano et al., 2014). The significant increase in GSSG concentration and the very low

GSH:GSSG ratio in CSF is also indicative of a highly oxidized extracellular environment. Circu and Aw (2012) found that apoptosis induced by oxidant defense was consistently associated with an imbalance between GSH and GSSG, specifically a rise in GSSG. Grunwell and colleagues (2015) reported an increase in GSSG and a more oxidized GSH:GSSG ratio in the serum of critically ill children compared to that of healthy children. The investigators proposed that GSSG and the GSH:GSSG ratio may serve as potential biomarkers of oxidative stress; however, there were no strong associations with measures of illness severity, days of mechanical ventilation, or clinical outcomes.

Active GSH export from the cell is an early event in apoptosis through either the intrinsic or extrinsic pathways (Aquilano et al., 2014). We found significant increases in caspase 8 (extrinsic pathway) and caspase 9 (intrinsic pathway) activity during the induction and postinduction treatment phases. It is possible that the observed increase in extracellular GSH during these treatment phases was the initiating event triggering the increase in caspases 8 and 9 activity by reducing intracellular antioxidant levels. The intrinsic and extrinsic pathways converge at caspase 3, which, together with other effector caspases, executes apoptotic cell death (D'Amelio et al., 2012). We found that the activity of caspases 8 and 9 was significantly associated with caspases 3/7 activity during induction ($r = .4$, $p = .001$ and $r = .6$, $p < .001$, respectively) and postinduction ($r = .6$, $p < .001$ and $r = .6$, $p < .001$, respectively), which suggests that caspases 8 and 9 could activate caspases 3/7.

Caspase 3 activation is a crucial event in neuronal cell death associated with chronic neurodegenerative conditions. We found that caspases 3/7 activity during induction and postinduction was associated with short-term memory, working memory, and visual-motor integration abilities 3 years after ALL diagnosis. This finding could be explained by the fact that caspases 3 and 7 activity executes cell death. The strength of the correlations was moderate which suggests that other potentially "upstream" (i.e., epigenetic) mechanisms may be important. Future studies of epigenetic modifications involved in oxidant defense and apoptosis may yield further insights into underlying mechanisms of neurologic injury and resulting cognitive problems.

Younger age at the time of diagnosis was associated with poorer performance on inattention/impulsivity and vigilance measures and could reflect greater vulnerability in attention mechanisms when treatment occurs at younger ages. This finding is consistent with that of a previous study showing an interaction between age and omission errors such that younger age at diagnosis was associated with increased omission errors 2 years after the end of therapy (Jacola et al., 2016). We found no other clinical studies reporting associations among CNS-directed treatment with methotrexate, antioxidant level, apoptosis, and cognitive abilities.

The hippocampus is an important brain region for learning and memory (Eichenbaum, 2001, 2004; Fortin, Agster, & Eichenbaum, 2002) and highly vulnerable to oxidative stress (Liang et al., 2008; Marino, Aksenov, & Kelly, 2004; Rodriguez-Martinez, Martinez, Espinosa-Garcia, Maldonado, & Rivas-Arancibia, 2013). Compared to controls, animals treated with intraventricular chemotherapy (methotrexate) had a greater percentage of degenerating neurons in the dentate gyrus and CA3 regions of the hippocampus and decreased expression of manganese superoxide dismutase (Sod2), an enzyme that reduces damage by superoxide (I. M. Moore et al., 2016). The hippocampus as well as other brain regions may be particularly vulnerable to chemotherapy-induced oxidative stress, an initiating event leading to a decrease in intracellular GSH and activation of caspase enzymes regulating apoptosis.

Conclusion

Findings from the present study suggest that GSH is actively transported into CSF in response to the highly oxidized extracellular environment present during treatment with methotrexate and that GSH extracellular transport activates the intracellular intrinsic (caspase 9) and extrinsic (caspase 8) apoptosis pathways. Caspases 8 and 9 then activate caspase 3/7, which executes cell death. Early (induction and postinduction treatment phases) increases in caspase 3/7 were negatively correlated with Year 3 cognitive outcomes, which suggests a possible role for apoptosis in cognitive problems experienced by many of the children in this study.

The present study has several implications for future studies and nursing more generally. First, future studies are needed to further examine the role of GSH metabolism and apoptosis in neurologic injury and neuroprotective strategies. There is a paucity of information on neuroprotective strategies to minimize CNS treatment-related neurologic injury and cognitive problems. Antioxidant and apoptosis biomarkers could be used to monitor children for methotrexate toxicity, who may benefit from treatment with the drug leucovorin (rescues cells from methotrexate damage; Shuper et al., 2002) and careful assessment of potentially diminished cognitive abilities. Finally, parents could be educated regarding the potential for cognitive effects and helped to seek additional academic resources at school for their children.

Limitations

Although we implemented measures to minimize auto-oxidation of our CSF samples, it is possible that the concentration of GSSG and the GSH:GSSG ratio were influenced in part by freezer storage. However, GSH:GSSG ratios were also very low (10 or less) in CSF samples that were assayed prior to freezing. We attempted to complete cognitive assessments as soon as possible after each child was 3 years from ALL diagnosis; however, there was variability in this time interval due to the fact that we scheduled assessments to coincide with clinic appointments with the oncology team.

Author Contributions

Ida M. (Ki) Moore contributed to conception, 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. Kari M. Koerner contributed to conception, design, acquisition, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; and gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy. Patricia M. Gundy contributed to conception, 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. David W. Montgomery contributed to conception, design, analysis, and interpretation; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Kathleen C. Insel contributed to conception, design, acquisition, analysis, and interpretation; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Lynnette L. Harris contributed to acquisition and interpretation; 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; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Marilyn J. Hockenberry contributed to conception, design, acquisition, analysis, and interpretation; 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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