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F2-IsoProstanes: A Measure of Oxidative Stress in Children Receiving Treatment for Leukemia

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Abstract

Acute lymphoblastic leukemia (ALL) is the most prevalent and curable cancer among children and adolescents less than 15 years of age in the United States. Essential for cure of childhood ALL is prophylactic treatment of the central nervous system (CNS), with methotrexate (MTX) being the most widely used drug in this treatment. While CNS treatment has contributed to long-term disease-free survival, resulting declines in academic abilities have been reported. There is growing evidence that CNS treatment with MTX increases oxidative stress, a potential mechanism of CNS injury. This article reports changes in oxidative stress, measured by the biomarker F2-IsoProstane (F2-IsoP), in the cerebrospinal fluid (CSF) in 47 children with ALL during the first 18 months of treatment. The number of CSF samples ranged from 5 to 14 during postinduction and from 1 to 9 during continuation. Total doses of intrathecal MTX during postinduction were significantly correlated with the mean and highest concentrations of F2-IsoP during postinduction and the mean concentration of F2-IsoP during continuation. F2-IsoP concentrations during postinduction and continuation were higher in children who received more than six doses of intrathecal MTX. New therapies for a highly curable disease such as childhood leukemia have the potential to be individualized in the future, requiring reliable molecular and biochemical markers, such as oxidative stress indicators. Innovative use of biomarkers has the potential to increase our understanding of treatment-related toxicities and associated symptoms and to inform future therapeutic approaches for optimizing cure and quality of life among children with leukemia.

Introduction

Acute lymphoblastic leukemia (ALL) is the most prevalent and curable cancer among children and adolescents less than 15 years of age in the United States. Treatment for children with ALL is typically divided into induction (at the time of diagnosis), postinduction (after achieving complete remission), and continuation phases of therapy. The intensity of postinduction chemotherapy varies considerably depending on risk-group assignment, but all children receive some form of intensification following achievement of remission and before beginning continuation therapy. Children with high-risk ALL usually receive more aggressive treatment than children with standard-risk ALL. Aggressive postinduction chemotherapy comes with costs in terms of potential short- and long-term side effects, making it imperative to balance improvements in survival against these risks.

Prophylactic treatment of the central nervous system (CNS) with a combination of intrathecal (IT) and systemic chemotherapy are critical components of post-induction

treatment. IT methotrexate (MTX) is administered in age-titrated doses directly to the CNS through the cerebral spinal fluid (CSF; Moe & Holen, 2000). Previous studies have confirmed long-term effects in children of CNS treatment with radiation and/or chemotherapy on general intelligence, memory, attention, and academic abilities that become apparent after completion of ALL treatment (Anderson, Smibert, Ekert, & Godber, 1994; Brown et al., 1996; Buizer, de Sonnevile, van den Heuvel-Eibrink, & Veerman, 2006; Espy et al., 2001; Halberg, Kramer, Moore, Wara, Matthay, & Ablin, 1992; Kaemingk, Carey, Moore, Herzer, & Hutter, 2004; Mennes et al., 2005; Montour-Proulx et al., 2005; Moore, Copeland, Ried, & Levy, 1992; Moore, et al., 2000; Raymond-Speden, Tripp, Lawrence, & Holdaway, 2000; Reddick et al., 2005a; Reddick et al., 2005b; Reddick et al., 2006; Reddick et al., 2003; Waber et al., 1995). Recent research shows declines in non-verbal cognitive abilities during the first 2 years of CNS treatment (Carey et al. 2007; Hockenberry, Krull, Moore, Gregurich, & Kaemingk, 2007). Academic problems, especially in math and reading, emerge within 3 years of ALL diagnosis and treatment (Espy et al., 2001; Kaemingk et al., 2004; Moore et al., 2000). MTX therapy is associated with long-term academic problems among ALL survivors, with difficulties in visual-perceptual and attention skills being among the most commonly reported (Brown et al., 1996; Buizer, De Sonnevile, Van Den Heuvel-Eibrink, Njiokiktjien, & Veerman, 2005; Espy et al., 2001; Halberg et al., 1992; Hockenberry et al., 2007; Kaemingk et al., 2004; Lehtinen et al., 2002; Moore et al., 1992; Raymond-Speden et al., 2000; Waber & Tarbell, 1997).

There is evidence that MTX increases oxidative stress, cellular injury, and degeneration caused by reactive oxygen species (ROS), a potential mechanism of CNS injury (Babiak, Campello, Carnieri, & Oliveira, 1998; Girotti, 1998; Jahovic, Cevik, Sehirli, Yegen, & Sener, 2003; Miketova et al., 2005; Rajamani, Muthuvel, Senthilvelan, & Sheeladevi, 2006; Rouse, Nwokedi, Woodliffe, Epstein, & Klimberg, 1995). ROS include oxygen ions, free radicals, and peroxides that are highly reactive due to the presence of an oxygen molecule's unpaired electron. Researchers are investigating specific markers associated with MTX-induced oxidative stress to evaluate treatment-related cellular injury within the CNS. In one study, neurochemical markers of CNS morbidity involving brain specific proteins (neuron-specific enolase, glial fibrillary acidic protein, and neurofilament protein light chain) increased during induction in a group of 121 children with ALL; IT MTX was identified as the most likely noxious agent (Osterlundh et al., 2008). Authors identified IT MTX as the most likely noxious agent. In another study, increased oxidation of phosphatidylcholine, the predominant phospholipid in CSF, was associated with poorer executive function at the end of chemotherapy in 88 children with ALL (Caron et al., 2009).

One consequence of oxidative stress is oxidation of lipids, referred to as lipid peroxidation (Milne et al., 2005; Roberts and Morrow, 2000). Membrane phospholipids and lipids have high concentrations of polyunsaturated fatty acids that are the source of lipid peroxidation. Lipid peroxidation is different from other forms of free radical injury because it is self-propagating and will proceed until the substrate is consumed or termination occurs. F₂-IsoProstanes (F₂-IsoPs) are stable prostaglandin-like compounds formed from the peroxidation of arachidonic acid (a polyunsaturated free fatty acid found in high concentrations in membrane lipids) and are present in detectable quantities in biological tissues and fluids (Milne et al., 2005; Roberts and Morrow, 2000). F₂-IsoPs are found in

adults in CSF, with 23 ± 1.0 pg/ml concentration basal levels reported (Fam & Morrow, 2003). F₂-IsoPs levels have been shown to increase substantially in animal models of oxidant injury, are modulated by endogenous antioxidant status, and unaffected by the lipid content in diet. The ability to quantify F₂-IsoPs has allowed exploration of the role free radicals play in the pathophysiology of numerous human diseases (Fam & Morrow, 2003; Grosso et al., 2011; Il'yasova et al., 2011; Il'yasova et al., 2010; Roberts & Morrow, 2000). There is a significant body of evidence that F₂-IsoPs are important transducers of the effects of oxidative stress involved with increased cardiovascular risk as well as a growing body of evidence associated with cancer (Barocas et al., 2011; Hopkins, Fedirko, Jones, Terry, & Bostick, 2010). Il'yasova et al., (2010, 2011) examined urinary F₂-IsoPs in 23 breast cancer patients receiving doxorubicin. In their studies, the clinical model of oxidative assault revealed that F₂-IsoP is a reliable biomarker to monitor oxidative status during chemotherapy treatment. F₂-IsoP may thus serve as a sensitive index of oxidative stress in vivo. However, we were able to find only one study evaluating 8- F₂-IsoP concentrations in children with cancer. Protas, Muszynska-Roslan, Holownia, Krawczuk-Rybak, and Braszko (2010) found elevated IsoPs in 38 children receiving chemotherapy for leukemia, indicating that neurotoxicity during treatment may be related to oxidative stress.

Methods

Children with newly diagnosed ALL who were between 2 years 9 months and 15 years of age at diagnosis were enrolled in the study. Parent informed consent and child assent was obtained. We obtained CSF samples during scheduled lumbar punctures (LPs) from 47 children who had completed the first 18 months of ALL therapy. Throughout therapy, study participants receive CNS prophylaxis with standardized doses of IT MTX based on age: 8 mg for children aged 1–1.99 years, 10 mg for children aged 2–2.99 years, 12 mg for children aged 3–8.99 years, and 15 mg for children aged 9 years. Induction therapy (1 month) included weekly treatment with vincristine and daunomycin (for high-risk ALL), a corticosteroid and a dose of Peg-asparaginase, and two IT MTX treatments (Days 1 and 29). Postinduction therapy (6–8 months) involved several courses of treatment that included asparaginase, high- or intermediate-dose IV MTX (depending on ALL protocol assignment), vincristine, doxorubicin, corticosteroid, cytarabine, and mercaptopurine. During postinduction IT MTX was given on Day 1 of each 12-week cycle. Maintenance therapy (2–3 years) consisted of daily mercaptopurine and weekly oral MTX, with monthly pulses of vincristine and a corticosteroid, and an IT MTX treatment every 12 weeks.

CSF was obtained with the diagnostic LP (referred to as diagnosis) and subsequently at the first LP prior to IT MTX treatment (referred to as Day 8 or Day 15). The remaining CSF samples were obtained during induction (referred to as end of induction), aggressive CNS and systemic treatment (referred to as postinduction), and the continuation (referred to as continuation) phases of therapy. The number of CSF samples obtained in conjunction with IT MTX administration ranged from 5 to 14 during postinduction and from 1 to 9 during continuation. The participants were divided into two groups to represent a median split in CNS treatment intensity and to determine whether changes in F₂-IsoPs across treatment phases differed by treatment intensity. Children who received five or six doses of IT MTX during postinduction were assigned to Group 1 and those who received more than six doses

of IT MTX during postinduction were assigned to Group 2. Table 1 summarizes the range and frequency of IT MTX doses received during postinduction and continuation.

CSF samples were placed on ice immediately after collection, and centrifuged for 10 min at 3000 rpm to remove any cellular debris. Processed CSF was stored at -80°C for no more than 3 months to ensure that the samples would not degrade. F_2 -IsoPs were measured by a competitive enzyme-linked immunoassay (ELISA) kit that according to instructions (Cayman Chemical, Ann Arbor, MI). The kit is designed for extraction-free analysis of bodily fluids including CSF, and has been validated for detection of 8- F_2 -IsoPs in urine, plasma, and other sample matrices. Briefly, 150 μl of standard or CSF samples were added to each well. Then 50 μl of 8- F_2 -IsoP AchE Tracer was added to each well, except the Total Activity (TA) and Reaction Blank (RB); and 50 μl of 8-Isoprostane EIA Antiserum was added except to the TA, RB, and Non-Specific Binding (NSB) wells. Plates were incubated for 18 hours at 4°C . Wells were then washed five times with 200 ml of wash buffer. Ellman's reagent (200 ml) was added to each well. A total activity well contained only Ellman's reagent and tracer added just before the second incubation. A reaction blank contained only Ellman's reagent, and a nonspecific binding well contained only tracer, added before incubation, and Ellman's reagent. Plates were covered and placed on an orbital shaker at 400 rpm for 90–120 min. The plates were then read on a Perkin Elmer Envision Multi-label plate reader at 405 nm. Average replicates of each standard (0.08–500 pg/ml) were used to generate the standard curve. CSF samples were analyzed in triplicate to confirm reproducibility.

Descriptive statistics were used to summarize sample characteristics. General linear model (GLM) for repeated measures analysis of variance (ANOVA) was used to test for significant differences in F_2 -IsoPs by treatment phase and for Group-byTime interactions. For these analyses the within-subject factor was treatment phase (diagnosis, Day 8 or 15, end of induction, postinduction, continuation) and the between-subject factor was group (Group 1 or Group 2).

Results

The sample included 47 children (20 females and 27 males) with ALL. Mean (M) age at diagnosis was 6.7 years (range = 2.9 to 13.4 years; SD = 2.86). Parental years of education ranged from 7 to 20 years (M = 12 years; SD = 2.4) for mothers, and from 5 to 20 years for fathers (M = 12.9 years; SD = 3.0). Racial categories included Caucasian (n = 21), Hispanic (n = 21), Native American (n = 1) and other (n = 4).

Table 2 summarizes the mean and highest concentration of F_2 -IsoPs at diagnosis, day 8 or 15, end of induction, post-induction and continuation in Groups 1 and 2. Mean concentration of f_2 isoprostanes was higher in Group 1 at diagnosis (8.83; SEM = 0.71) and at day 8 or 15 (9.28, SEM = 0.85) than Group 2 (diagnosis mean = 6.80; SEM = 0.86; day 8 or 15 mean = 8.15, SEM = 1.03). However, F_2 -IsoP concentrations during post-induction and continuation were higher in Group 2 (children who received more than six doses of IT MTX) compared to Group 1 (children who received six or less doses of IT MTX).

Results of the repeated measures ANOVA on mean concentration of F₂-IsoPs showed a significant effect for time (df = 4; F = 3.25; p = 0.032) and for the group-by-time interaction (F = 4.96; p = 0.005). Figure 1 summarizes mean CSF concentrations of F₂-IsoPs at diagnosis, day 8 or 15, end of induction, post-induction, and continuation. Results of the repeated measures ANOVA on the highest concentration of F₂-IsoPs also showed a significant effect for time (df = 4, F = 16.88; p < 0.001) and for the group by time interaction (F = 5.157, p = 0.001). Figure 2 summarizes the highest concentration of CSF F₂-IsoPs during end of induction, post-induction and continuation.

To further determine whether IT MTX was associated with oxidative stress in the CNS, we correlated the number of IT MTX doses received during postinduction with the mean and highest concentrations of F₂-IsoP during the postinduction and continuation phases of therapy. Total doses of IT MTX received during postinduction were significantly correlated with the mean (r = .424, p = .003) and highest (r = .479, p = .001) concentrations of F₂-IsoP during postinduction. Total doses of IT MTX received during postinduction were also significantly correlated with the mean (r = .410, p = .004) concentration of F₂-IsoP during continuation.

Discussion

Childhood leukemia survival rates have greatly increased over the last three decades. However, toxicities experienced during treatment often result in complications, that may compromise therapy, negatively influence quality of life, and even more notably, jeopardize chances for long-term survival. In the present study, we examined the oxidative stress pathway, an indicator of cellular injury and degeneration caused by ROS, by measuring CSF F₂-IsoP levels during the first 18 months of childhood leukemia treatment. Total doses of IT MTX during post-induction were significantly correlated with the mean and highest concentrations of F₂-IsoPs during post-induction and continuation therapy. Acute increases in oxidative stress measures were highest among children who received more than six doses of IT MTX during the post-induction phase of treatment. These findings are similar to those found in the Protas and colleagues (2010) study where 8- F₂-IsoP concentrations were elevated during leukemia treatment.

In the present study, we found higher levels of oxidative stress at baseline in the group receiving less than six doses of IT MTX during postinduction. Variations in oxidative status in individuals have been documented (Block et al., 2002; Halliwell & Whiteman, 2004). Il'yasova and colleagues (2011) found interindividual differences in oxidative stress levels measured by F₂-IsoP in the urine of 23 newly diagnosed breast cancer patients; 10 of these patients had initial high systemic levels of oxidative stress, similar to our findings at baseline. Interestingly, in Il'yasova's study, individuals with lower pretreatment levels experienced the sharpest increase in oxidative stress after chemotherapy. Two potential explanations should be considered for the higher initial levels of F₂-IsoPs. First, this finding adds to the evidence indicating individual variation in pretreatment oxidative (defense) status. Second, we should consider the possibility that there are individual differences in redox homeostatic mechanisms and the ability to mobilize an antioxidant defense. If the latter explanation were true, it may be due to differences in redox phenotypes characterized

by levels of systemic oxidative status and corresponding reactions to oxidative stressors over time.

Individual susceptibility to oxidative stress can contribute to clinical outcomes and symptom severity. CNS treatment with MTX is associated with significant declines in academic abilities, especially math abilities, compared to children with other cancers that do not require CNS treatment, healthy siblings, and healthy children (Brown et al., 1996; Raymond-Speden et al., 2000; Waber & Tarbell, 1997; Waber et al., 1995). Our findings, along with those of other researchers, support the need for future research to address why interindividual differences in oxidative stress levels exist to allow us to identify cancer patients who are most susceptible to drug toxicity and resistance in the future.

The oxidative stress pathway is known to be interactive and may result in significant cellular damage and toxicity. Chemotherapy agents, such as MTX, can trigger ROS production as byproducts of cellular destruction. Research has shown that MTX increases oxidative stress in several organs, including the brain (Caron et al., 2009; Moore et al., 2008; Stenzel et al., 2010). White matter and the hippocampus are known to be vulnerable to oxidative stress. These structures are important for cognitive abilities, such as working memory, learning, visual-spatial and visual-motor skills. The brain is particularly vulnerable to oxidative stress for several reasons. First, cells of the human brain generate more ROS than other tissues because of their high rate of oxidative metabolism. Second, the brain is rich in lipids that consist of unsaturated fatty acids, making them a target for lipid peroxidation. Finally, the brain has low to moderate antioxidant systems compared to other organs such as the kidney or liver (Illingworth & Glover, 1971; Margail, Plotkine, & Lerouet, 2005; Qin, Goswami, Balabanov, & Dawson, 2007). White matter has a high lipid content (lipid: protein, 70:30) compared to other membranes making it a preferential target for oxidative stress and lipid peroxidation (Verstraeten, Golul, Keen, & Oteiza, 1997). While there are a number of CSF biomarkers of neurodegeneration (e.g., Tau, amyloid β , S100, NSE), these biomarkers are not specific to oxidative stress. We chose to measure F₂-IsoP because MTX inhibits synthesis of new proteins including antioxidant enzymes.

Personalized therapy for a highly curable disease such as childhood leukemia that uses molecular and biochemical tools for clinical diagnosis will become reality as biomedical technology continues to evolve (Pui, Mullighan, Evans, & Relling, 2012). Exploration of biological markers that identify therapeutic toxicities will pave the way for pharmacologic-based individualization of treatment. This study is among the first to increase understanding of oxidative stress biomarkers related to childhood leukemia therapy and may lead to knowledge of treatment-related symptom associations and their ultimate impact on childhood leukemia therapy. Findings suggest that IT MTX is associated with an increase in oxidative stress as measured by the concentration of F₂-IsoP during the postinduction and continuation phases of treatment. These findings may inform future therapeutic approaches for optimizing cure and quality of life among children with leukemia.

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Figure 1.

Mean cerebral spinal fluid (CSF) concentration of F2-isoprostanes at diagnosis and treatment phase by group. Group 1 received six doses of intrathecal (IT) methotrexate (MTX) during the postinduction phase. Group 2 received > six doses of IT MTX during the postinduction phase.

Figure 2.

Highest concentration of F2-isoprostanes in cerebral spinal fluid (CSF) at diagnosis and treatment phase by group. Group 1 received six doses of intrathecal (IT) methotrexate (MTX) during the postinduction phase. Group 2 received > six doses of IT MTX during the postinduction phase.

Table 1
Numbers of Doses of Intrathecal Methotrexate (IT MTX) Received During Treatment (n=47)

		Number of doses of IT MTX												
Treatment Phase	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Postinduction	---	---	---	---	1	23	3	2	7	1	4	2	1	3
Continuation	3	2	3	11	14	4	8	---	2	---	---	---	---	---

Table 2
CSF F₂ Isoprostanes Concentrations at Diagnosis and Treatment Phase by Group

	Diagnosis	Day 8 or 15	End of Induction	Post-Induction	Continuation
Group 1 *					
mean (SEM)	8.83 (0.71)	9.28 (0.85)	7.72 (0.59)	6.26 (0.34)	8.52 (0.44)
Highest	8.83 (0.71)	9.28 (0.85)	8.70 (0.70)	9.31 (0.53)	12.66 (0.66)
Group 2 **					
mean (SEM)	6.80 (0.86)	8.15 (1.03)	8.74 (0.72)	8.47 (0.41)	9.88 (0.53)
Highest	6.80 (0.86)	8.15 (1.03)	10.38 (0.85)	12.82 (0.64)	13.07 (0.80)
Missing	n = 4	n = 4	n = 3	n = 0	n = 0

* Children who received 6 doses of intrathecal methotrexate, n=24

** Children who received > 6 doses of intrathecal methotrexate, n=23