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EFFECT OF INTRACEREBROVENTRICULAR METHOTREXATE ON BRAIN AMINES

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Abstract : Intrathecal methotrexate in children with leukemia is known to cause seizures, dementia, leukoencephalopathy and cognitive dysfunction. To investigate the role of brain amines in cognitive dysfunction, male Wistar rats were given multiple intracerebroventricular injections of methotrexate. Our earlier studies in this regard revealed disruption of brain monoamines in hippocampus with severe cytotoxic effect on CA4 hippocampal neurons. Further extending this study, the levels of brain monoamines in frontal cortex, hypothalamus and brainstem were estimated by HPLC method and histopathological study of the frontal cortex. The concentration of all three-brain amine (norepinephrine, dopamine and serotonin) levels was reduced in 2 mg/kg dose of methotrexate in frontal cortex and brain stem. Hypothalamus did not show any significant change in brain monoamine levels. No structural changes in the frontal cortex neurons were observed. Disruption of brain monoamines has been proposed as a cause of brain dysfunction from this chemotherapy. The outcome of the study may have therapeutic implications in the management of childhood lymphoblastic leukemia.

Key words : methotrexate norepinephrine dopamine serotonin frontal cortex hypothalamus brainstem

INTRODUCTION

Methotrexate, a folate antagonist is a mainstay treatment for childhood acute lymphoblastic leukemia. Intrathecal methotrexate alone or in combination is used widely for control of this leukemia and to treat meningeal infiltration. Methotrexate cannot cross blood brain barrier (1), hence it is a common practice to administer methotrexate directly into the CSF. The development of methotrexate induced neurotoxicity however has caused major clinical problems (2). The methotrexate induced neurotoxicity in the form of paresis (3); paraplegia (4), seizures (5), encephalopathy (6) and even death (7) were reported. Inspite of its neurotoxicity, methotrexate is extensively used in prevention and maintenance therapy of childhood lymphoblastic leukemia for its excellent results since last two decades.

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Children given CNS prophylactic therapy have significant reductions in overall intelligence quotient scores, impaired verbal and visual-spatial memory abilities (8, 9, 10, 11 & 12). However, studies conducted by Duller and Vandekerkhof (13) did not show any impairment of memory. Our earlier study (14) clearly demonstrated impairment of learning and memory abilities after methotrexate treatment. The mechanism of methotrexate-induced behavioral toxicity has not been studied extensively. The role of brain amines in methotrexate induced learning and memory deficit is poorly reported. Netter et al (15) proposed neurotoxicity by methotrexate is due to interference in supply of neurotransmitters. Our earlier study has confirmed the involvement of hippocampal neurotransmitters in methotrexate – induced learning and memory deficits (15). In which the level of dopamine and serotonin were significantly decreased ($P<0.001$) in both 1.5 and 2 mg/kg dose of methotrexate. Continuing our earlier study, brain amines (Norepinephrine, Dopamine and Serotonin) level in various regions of the brain were estimated after intracerebroventricular methotrexate.

METHODS

Animals

Four-month-old male Wistar rats bred in house were used in the present study. Animals were maintained under controlled conditions of light (10 h : light : 14 h : dark), temperature (22 ± 3°C), and humidity (approximately 50 ± 10%) in an air-conditioned animal house. All rats were maintained on the standard rat food and water *ad libitum*. The average weight of the rat was 226 g. All the experimental procedures were approved by the Institutional ethics committee.

Chemicals

Methotrexate was obtained from 'Biochem Pharmaceutical Industries' (Ahmedabad, India). All other chemicals and reagents were HPLC or analytical grade (Sigma, St. Louis, Mo.).

Animal groups

Four groups of rats were subjected to the biochemical analysis. Group 1 consisted of control rats, group 2 consisted of rats with a cannula implanted in the lateral ventricle (saline treated – vehicle control) and groups 3 and 4 consisted of rats treated with 1.5 and 2 mg methotrexate/kg, respectively.

Surgery and drug administration

Under general anesthesia (sodium pentobarbital, 40 mg/kg, i.p) rats were implanted with a 7-mm, 21-gauge stainless steel guide cannula anchored over the right (50% rats) or left (50% of rats) lateral ventricle (anteroposterior position : –0.8 mm posterior to bregma, ±2 mm lateral to bregma, and depth –3.2 mm below the skull surface at the point of entry) using stereotaxic apparatus. A stainless steel stylet, cut the same length as the cannula, was inserted to keep the cannula patent. Following surgery, the animals were housed separately in single cages until end of the experiment. At the end of the experiment cannula placements were verified by visual inspection by injecting 2% fast green dye solution (5 µl) through the cannula system.

The rats were observed till they recovered from anesthesia.

Methotrexate in physiological saline was administered for 3 alternative days at the rate of 1 µl every 60 seconds, through a Hamilton syringe via silastic tube (0.46). For group 2 rats, an equal volume of saline was infused instead of methotrexate. The doses of methotrexate selected were according to earlier studies (10 and 16). Ten rats from each group were selected randomly for brain amine studies and five rats for histopathological studies.

Acute toxicity test

Methotrexate (in physiological saline) was administered into the lateral ventricle at a dose of 3, 4, 5 or 6 mg/kg body weight to different groups of rats (n=6, in each group). During the first 4h after the drug administration, the freely moving rats were observed for gross behavioral changes. The parameters observed were hyperactivity, grooming, convulsion, sedation, increased respiration and hypothermia.

Biochemical Studies

Rats were sacrificed two weeks after the first dose of methotrexate by decapitation. Brains were rapidly removed and various regions of the brain (Frontal cortex, Hypothalamus and Brainstem) were separated on a ice slab and then transferred to icecold saline. The tissues were immediately homogenized in 5 ml 0.4N ice cold perchloric acid containing 0.1% Ethylene diamine tetraacetic acid (EDTA) disodium salt, and 0.05% sodium metabisulphite and internal standard 3,4-dihydroxy benzylamine (DHBA) using a teflon-glass homogenizer (Thomas Scientific, Philadelphia Penn). The homogenate was centrifuged at 12000 rpm (12 235 Xg) for 10 minutes at 40°C. The supernatant was removed and stored at –20°C until assayed. Brain monoamine levels were estimated by HPLC with electrochemical detector (VMD-101a, Yaagimoto manufacturing Co. Ltd. Kyoto, Japan).

The brain amines were isolated on a C18 reverse phase column of particle size 5 micron meter and 25 cm length using the isocratic elution method. The mobile phase was composed of 70 mM sodium acetate buffer (pH 4.5), containing 1 ml of 10% EDTA and 0.05 M hexane sulfonic acid in one litre of buffer. The buffer was filtered through a 0.45 micron millipore membrane filter and then mixed with methanol in 87:13 (v/v) ratio before use. The mobile phase was degassed using an on-line degasser (ERC-3310, Tokyo-Japan) before passing through the column. The flow rate was kept at 1.5 ml/min. The voltametric detector with a glassy carbon electrode was used for electrochemical detection of the amines. The detector potential was set at 0.8 V versus an Ag/AgCI reference electrode with a sensitivity of 1 nA. A volume of 20 microlitres of the filtered homogenate was injected without prior processing. Peak recording and quantitation was done using the chromatocorder 11 (Alphatech Corporation Limited, Tokyo, Japan). The actual concentration of amines was calculated by comparing the recovery of each standard amine from the crude homogenate with that of the internal standard. The amounts of brain amines were expressed in nanograms per gram wet weight of the tissue (17).

Histopathological studies

Perfusion : Each animal was deeply anesthetized with ether and secured on a dissection board, and its chest cavity was opened to expose the heart. About 15 ml of 0.9% saline was perfused through the left ventricle at the rate of 1 mL/min. This was followed by perfusion of 10% formalin at the same flow rate. The animal was decapitated and the brain was removed and kept in 10% formalin for 48 h (postfixation). Paraffin blocks were made in an embedding bath. Coronal sections of 3–5 µm thickness were cut in the frontal cortex (18) using a rotary microtome (Jung Biocutt 2035, Leica, Germany). Twenty-five sections from each animal were mounted serially on air-dried gelatinized slides.

Staining : The sections were stained with cresyl violet stain. One hundred milligrams of cresyl violet was dissolved in 100 mL of distilled water. To this 0.5 mL of acetic acid was added to give a pH of 3.5–3.8 The stain was filtered before use.

Scoring : Coronal sections at the level of the frontal cortex were screened for structural abnormalities (using ocular grid) under light microscope. Twentyfive sections from each rat (five rats in each group) were considered.

Statistical analysis

The data were expressed as mean ± SE. The significance of differences among the groups was assessed using one way analysis of variance (ANOVA) test followed by Bonferroni's multiple comparison test. P values less than 0.05 were considered as significant.

RESULTS

Acute toxicity test

A mortality of 16% was observed following 6 mg/kg dose of intraventricular methotrexate. In the remaining doses tested epileptic reactions started with "running of fits", developing into generalized tonic and clonic seizures immediately after the treatment.

Biochemical studies

Cannulation procedure did not affect brain amine levels as no significant change was observed when this group was compared with control group. The level of norepinephrine decreased significantly ($P<0.05$) in frontal cortex with both doses of methotrexate. However the hypothalamus and brainstem did not show any significant ($P>0.05$) change in the norepinephrine level (Table–I). The level of dopamine decreased significantly ($P<0.001$) in both frontal cortex and brainstem at both doses of methotrexate. The decrease in hypothalamus dopamine level is significant ($P<0.05$) (Table–II). The level of serotonin also decreased significantly ($P<0.01$) in frontal cortex and brainstem, only at 2 mg/kg dose of methotrexate. The serotonin level did not alter significantly in hypothalamus (Table–III). Except for the serotonin level, the remaining brain amine levels did not differ between the two doses of methotrexate.

Histopathological studies

No structural changes were observed in the frontal cortex of the rats treated with methotrexate ($F=2.05$).

TABLE I: Level of norepinephrine (ng/g wet weight of the tissue) in different regions of the brain after different doses of methotrexate in 4-month old male Wistar rats.

Animal groups	*Hypothalamus*	*Frontal cortex*	*Brainstem*
Group 1 (Control)	729.58±29.83	95.5±3.30	77.96±3.53
Group 2 (Sham operated)	698.44±38.0	95.61±3.10	74.20±3.12
Group 3 (1.5 mg/kg MTX)	685.21±29.20	81.76±1.96*	68.88±2.87
Group 4 (2 mg/kg MTX)	611.23±17.4	80.46±3.61*	66.54±3.43
Anova significance:			
F value	2.88	7.50	2.52
P value	0.049	0.0005	0.0731

Values are expressed as means ± SE (n = 10 animals in each group). Animals were sacrificed 2 weeks after methotrexate administration. Group 1, untreated controls; group 2, sham-operated control; group 3, 1. 5 mg methotrexate/kg; group 4, 2 mg methotrexate/kg. Group 1 versus group 3 & 4; *=P<0.05.

TABLE II: Level of dopamine (ng/g wet weight of the tissue) in different regions of the brain after different doses of methotrexate in 4-month old male Wistar rats.

Animal groups	*Hypothalamus*	*Frontal cortex*	*Brainstem*
Group 1 (Control)	192.82±2.63	394.22±9.66	111.34±3.40
Group 2 (Sham operated)	196.03±5.37	377.19±7.58	99.46±3.92
Group 3 (1.5 mg/kg MTX)	177.34±3.21*	310.53±4.57**	86.71±1.70**
Group 4 (2 mg/kg MTX)	175.46±2.52*	312.15±4.44**	84.28±2.76**
Anova significance:			
F value	8.44	39.57	16.76
P value	0.0002	<0.0001	<0.0001

Values are expressed as means ± SE (n = 10 animals in each group). Animals were sacrificed 2 weeks after methotrexate administration. Group 1, untreated controls; group 2, sham-operated control; group 3, 1. 5 mg methotrexate/kg; group 4, 2 mg methotrexate/kg. Group 1 versus group 3 and 4; *=P<0.05 & **=P<0.001.

TABLE III: Level of serotonin (ng/g wet weight of the tissue) in different regions of the brain after different doses of methotrexate in 4-month old male Wistar rats.

Animal groups	*Hypothalamus*	*Frontal cortex*	*Brainstem*
Group 1 (Control)	497.35±9.50	166.9±6.17	433.66±6.75
Group 2 (Sham operated)	490.80±13.70	169.52±3.30	412.81±7.46
Group 3 (1.5 mg/kg MTX)	487.03±8.04	156.94±4.46	390.86±5.87
Group 4 (2 mg/kg MTX)	458.93±6.67	137.52±6.32*	366.90±18.8*
Anova significance:			
F value	2.96	7.74	6.73
P value	0.0448	0.0004	0.0010

Values are expressed as means ± SE (n = 10 animals in each group). Animals were sacrificed 2 weeks after methotrexate administration. Group 1, untreated controls; group 2, sham-operated control; group 3, 1. 5 mg methotrexate/kg; group 4, 2 mg methotrexate/kg. Group 1 versus group 4; *=P<0.01.

DISCUSSION

Data generated in this study clearly show that intraventricular methotrexate alters brain amines (norepinephrine, dopamine and serotonin) in various regions (hypothalamus, brain stem and frontal cortex) of brain in addition to the hippocampus. We have reported earlier that methotrexate impairs learning and memory and significant decrease in the level of norepinephrine ($P<0.01$), dopamine ($P<0.001$) and serotonin ($P<0.001$) in hippocampus at both 1.5 and 2 mg/kg dose (14). Hence it is clear that the methotrexate-induced learning and memory dysfunction is not only due to the involvement of hippocampus but also involves brain amines in other areas of brain.

There are reports that brain amines especially norepinephrine, dopamine and serotonin play a role in memory processing (19 and 20). In the present study, the level of norepinephrine was significantly reduced in frontal cortex but not in hypothalamus and brain stem. From our earlier and present study, it is clear that hippocampal norepinephrine involvement is more specific in methotrexate induced cognitive deficit.

Dopamine is another amine implicated in cognitive function. Dopamine receptors of the hippocampus and prefrontal cortex have been reported to be involved in learning and memory (21, 22 and 23). In the present study, a highly significant decline in the dopamine level of both brainstem and frontal cortex was observed. The decline of dopamine level in hypothalamus is less significant. Earlier we observed similar decline in the level of dopamine in hippocampus (14). Thus it is clear that methotrexate induced cognitive dysfunction also involves brain dopamine.

Another important amine, namely serotonin has been implicated in a variety of behavioral patterns including sleep, perception of pain and social behavior (24). The role of serotonin in learning and memory continues to be a subject of behavioral research. Serotonin is crucial for maintaining hippocampal synapses and memory acquisition in rats (25, 26 and 27). Nowakowska and co workers (28) showed stress-induced memory dysfunction associated with decreased levels of serotonin in rat brain. Lesions of serotonergic neurons of rat brain resulted in reduced concentration of serotonin by 60% in frontoparietal cortex and 80% in hippocampus with severe memory deficit (29). From these available reports, it is evident that declined serotonin level in brain has inhibitory action on learning and memory. In the present study serotonin level fell significantly in brainstem and frontal cortex (serotonergic areas) except in hypothalamus. The above findings suggest the involvement of serotonergic systems in methotrexate-induced learning and memory deficit.

It is now clear that all the three major brain monoamines were involved in methotrexate induced cognitive dysfunction. Among the areas investigated in the present study, the frontal cortex showed involvement of all the three brain amines. The histopathological study of the frontal cortex did not show any structural changes in

neurons and neuroglial cells. In our earlier study we observed changes in both histological and brain amine level of hippocampus after methotrexate treatment. However, such histological changes are not observed in frontal cortex. Intracerebrally microperfused methotrexate will have quantitatively different spatial distributions (maximum at the cannula tip and declined sharply with radial distance from the tip) in rodents (30). Such different spatial distribution of methotrexate will account for more pronounced cytotoxic effect in hippocampus (hippocampus form the wall of the ventricle to which the methotrexate was administered directly) in our earlier study and not in the frontal cortex. Thus our present study claims that methotrexate induced neurotoxicity is due to impaired synthesis of amines, rather than cytotoxic effect.

Norepinephrine containing cell bodies are situated almost exclusively in a group of nuclei in brainstem. In the present study, methotrexate has not affected the norepinephrine level in brainstem but significantly affected frontal cortex. It could be due to the involvement of transport system of norepinephrine from brainstem. Interestingly the brainstem did not show any increase in norepinephrine level in such case. Hence decreased norepinephrine level may be due to impaired synthesis. The altered levels of brain amines could be due to impaired synthesis of these amines by methotrexate. Millot et al (31) and Netter et al. (15) supported this view, claiming that altered biopterine metabolism is blocking the synthesis of these amines in brain. In the last few years, the mechanism involved in methotrexate induced learning and memory dysfunction is not adequately reported. The other biochemical changes following methotrexate treatment is poorly reported. A clinical study by Chan et al (32) states that, children survived for long time with intrathecal methotrexate therapy had decreased N-acetylaspartate (NAA/Cr) and choline (Cho)/creatine (Cr) in the brain tissue, estimated by proton magnetic resonance spectroscopy. Another study by Quinn et al (33) claimed an elevation in the level of CSF homocysteine in children undergoing intrathecal methotrexate treatment.

To conclude, intracerebroventricular methotrexate, decreases major brain amines in different parts of the brain. This decreased brain amine could be one of the factors in inducing various types of neurotoxicity after intrathecal methotrexate in children with acute lymphoblastic leukemia. One has to consider both benefit and risk factors before selecting the dose, as the prevalent therapeutic dose has been proved to be neurotoxic in the present study. The procedure employed in the current work provides an animal model to aid in understanding methotrexate-induced central nervous system toxicity.

REFERENCES

1. Mallet LB. Physiochemical considerations and pharmacokinetic behavior in delivery of drugs to the central nervous system. *Cancer Treatment Reports* 1977; 61: 527.

2. Goldberg ID. Nervous system toxic effects of cancer therapy. *JAMA* 1982; 247: 1437–1441.

3. Yim YS, Mahoney DH Jr, Oshman DG. Hemiparesis, ischemic changes of the white matter after intrathecal therapy for children with acute lymphocytic leukemia. *Cancer* 1991; 67(8): 2058–2061.

4. Garcia-Tena J, Lopez-Andreu JA, Ferris J, Menor F, Mulas F, Millet. Intrathecal chemotherapy-related myeloencephalopathy in a young child with acute lymphoblastic leukemia. *Pediatr-Hematol-Oncol* 1995; 12(4): 377–385.

5. Genvresse I, Dietzmann A, Massenkeil G, Spath-Schwalbe E, Possinger K. Subacute encephalopathy after combination chemotherapy including moderate dose of methotrexate in a patient with gastric cancer. *Anticancer-Drugs* 1999; 10(3): 293–294.

6. Spencer MD. Leukoencephalopathy after CMS prophylaxis for acute lymphoblastic leukemia. *Pediatr Rehabil* 1998; 2(1): 33–39.

7. Colleoni M, Price KN, Castiglione Gertsch M. Mortality during adjuvant treatment of early breast cancer with cyclophosphamide, methotrexate and flurouracil. *Lancet* 1999; 354 (9173): 130–131.

8. Waber DP, Tarbell NJ, Fairclough D, Atmore K, Castro R, Isquith P, Lussier F, Romero I, Carpeneter PJ, Schiller M, Sallan SE. Cognitive sequelae of treatment in childhood acute lymphoblastic leukemia : Cranial radiation requires an accomplice. *Journal of Clinical Oncology* 1995; 13: 2490–2496.

9. Oztop S, Centengul N, Nisli G, Aydinok Y, Kantar M, Oniz H, Kavakli K, Yalman O, Erermis S, Celebisoy N, Akyurekli O. Neuropsychologic sequelae in the long-term survivors of childhood acute lymphoblastic leukemia. *Pediatric Hematology and* Oncology 1999. 16(3), 213–220.

10. Mullenix PJ, William J Kernan S, Melissa. Tassinari. Ann Schunior, Deborah, P. An animal model to study toxicity of CNS therapy for childhood acute lymphoblastic leukemia: Effects on behavior. *Cancer Research* 1990; 50(20): 6461–6465.

11. Giralt J, Ortega JJ, Olive T, Verges R, Forio, Salvador L. Long term neuropsychologic sequelae of childhood leukemia: comparison of two CNS prophylactic regimens. *Int J Radiat Oncol Biol Phys* 1992; 24(1): 49–53.

12. Kingma A, Mooyaart EL, Kamps WA, Nleuwenhulzen P, Wllmink JT. Magnetic resonance imaging of the brain and neuropsychological evaluation in children treated for acute lymphoblastic leukemia at young age. *Am J Pediatr Hematol Onco* 1993; 15(2): 231–238.

13. Duller P, Vandekerkhof PCM. The impact of methotrexate on psycho-organic functioning. *British Journal of Dermatol* 1980: 113: 503–504.

14. Madhyastha S, Somayaji SN, Rao MS, Nalini K, Bairy KL. Hippocampal brain amines in methotrexate-induced learning and memory deficit. *Canadian Journal of Physiol Pharmacol* 2002; 80: 1076–1084.

15. Netter JC, Dhondt F, Ranee F, Petrus M. Early neurotoxicity of high dose of methotrexate and tetrahydrobiopterin deficiency. *Arch Fr Pediatr* 1991; 48: 719–722.

16. Silverstein FS, Johnston MV. A model of methotrexate encephalopathy: neurotransmitters and pathologic abnormalities. *J Child Neurol* 1986; 1(4): 351–357.

17. Nalini K, Karanth KS, Rao A, Aroor AR. Effect of Celastrus paniculatus on passive avoidance performance and biogenic amine turn over in albino rats. *Journal of Ethnopharmacology* 1995; 47: 101–108.

18. Pelligrino LJ, Pelligrino AS, Cushman AJ. A stereotaxic atlas of the rat brain. Second edition. Plenum press, New York. 1981 plates 41–43.

19. Squire LR, Davis HP. The pharmacology of memory and neurobiological perspective. *Annu Rev Pharmacol Toxicol* 1981; 21: 323–356.

20. Kobayashi K, Noda Y, Matsushita N, Nishii K, Sawada H, Nagatsu T, Nakahara D, Fukabori R, Yasoshima Y, Yamamoto T, Miura M, Kano M, Mamiya T, Miyamoto Y, Nabeshima T. Modest neuropsychological deficits caused by reduced noradrenaline metabolism in mice heterozygous for a mutated tyrosine hydroxylase gene. *J Neurosci* 2000; 20: 2418–2426.

21. EI-Ghundi M, Fletcher PJ, Sibley DR, O'Dowd, George SR. Spatial learning deficit in dopamine receptor knockout mice. *Eur J Pharmacol* 1999; 383: 95–106.

22. Wilkerson A, Levin ED. Ventral hippocampal dopamine D1 and D2 systems and spatial working memory in rats. *Neuroscience* 1999; 9: 743–749.

23. Mizoguch K, Yuzurihara M, Ishige A, Sasaki H, DeHua Chui, Tabira T. Chronic stress includes impairment of spatial working memory because of prefrontal dopaminergic dysfunction. *J of Neuroscience* 2000; 20(4): 1568–1574.

24. Menneses A. Physiological, pathophysiological and therapeutic roles of 5HT systems in learning and memory. *Rev Neurosci* 1998; 9(4): 275–289.

25. Matsukawa M, Oquwa M, Nakadate K. Serotonin and acetylcholine are crucial to maintain hippocampal synapses and memory acquisition in rats. *Neurosci-Lett* 1997; 230(1): 13–6.

26. Molodtsova GF, Yuchenok RY. Pre and postsynaptic mechanisms of the involvement of amygdala complex serotonin in the reproduction of a conditioned passive avoidance response in rats. *Neurosci-Behav-Physiol* 1999; 29(4): 359–363.

27. Stancapiano R, Coccos. Cugusi C. Serotonin and acetylcholine release responses in the rat hippocampus during a spatial memory task. *Neuroscience* 1999; 89(4): 1135–1143.

28. Nowakowska E, Chodera A, Kus K, Nowak P, Szkilink R. Reversal of stress induced memory changes by moclobemide: the role of neurotransmitters. *Pol J Pharmacol* 2001; 53(3): 227–233.

29. Lehmann O, Jeltsch H, Lehnardt U. Combined lesions of cholinergic and serotonergic neurons in the rat brain using 192 IqG, saporin and 5, 7-dihydroxytryptamine. *Eur J Neurosci* 2000; 12(1): 67–79.

30. Sendelbeck SL, Urquhart J. Spatialdistribution of dopamine, methotrexate and antipyrne during continuous intracerebral microperfusion. *Brain Research* 1985; 4: 328(2); 251–258.

31. Millot, Dhondt, Mazingue F, Mechinaud P, Ingrand F, and Guilhot F. Changes of cerebral biopterin and biogenic amine metabolism in leukemic children receiving 5 g/m2 intravenous methotrexate. *Pediatr Res* 1995; 151–154.

32. Chan YL, Roebuck DJ, Yueng KW, Lau KY, LiCK, Chik KW. Long-term cerebral metabolite changes on proton magnetic resonance spectroscopy in patients cured of acute lymphoblastic leukemia with previous intrathecal methotrexate and cranial irradiation prophylaxis. *Int J Radiat Oncol Biol Phys* 2001; 50(3); 759–763.

33. Quinn C, Griener JC, Bottigeri. Elevation of homocysteine and excitatory amino acid neurotransmitters in the CSF of children who receive methotrexate for the treatment of cancer. *J Clin Oncol* 1997; 15(8); 2800–2806.