RESEARCH PAPER

Pesticide (Endosulfan) Levels in the Bone Marrow of Children with Hematological Malignancies

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Objectives: (1) To confirm the presence of Pesticide (Endosulfan) residues in the bone marrow (BM) of children with acute hematological malignancies and compare them with controls. (2) To ascertain if children with Endosulfan in their marrow reside in areas sprayed with Endosulfan.

Study design: Case control study

Setting: Pediatric oncology unit of a medical college teaching hospital in Dakshina Kannada district of Karnataka.

Subjects: 26 patients with proven hematological malignancy and 26 age matched controls suffering from benign hematological disease.

Methods: Endosulfan residues in the BM were estimated by gas chromatography – mass spectrometry (Minimum detection limit 10ng/mL). The subject's geographical area of location (residence) was determined to see whether they belong to sprayed area or not. The Chi-square test was applied to see an association between exposure status and hematological malignancy.

Results: A total of 52 children were enrolled of which 26 were study cases and 26 were controls. Of the study and control groups, 84.7% and 73.1%, respectively were from exposed areas. The major (88.4%) illness in the study group was ALL, while ITP (50%) occurred most frequently in the control group. Six out of 26 study cases tested positive for endosulfan in the BM, against 1 out of 26 controls (P = 0.042). The Odds ratio was 7.5. All children who had endosulfan in the bone marrow originated from areas, where endosulfan is still being used.

Conclusions: Children with hematological malignancy had raised levels of endosulfan in the bone marrow compared to those without. All the children with raised bone marrow Endosulfan levels were found to be from areas exposed to the pesticide.

Key words: *Endosulfan, bone marrow, hematological malignancy, India.*

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A pesticide is a substance or mixture of substances intended to prevent, destroy, repel, or lessen the damage caused by a pest [1]. Most pesticides are poisonous to humans and if released into the environment, have significant adverse ecological effects. Endosulfan is one such compound extensively used in agriculture around the world, belonging to the organochlorine group of pesticides.

In India, more endosulfan is produced than any other pesticide except mancozeb and monocrotophos [2]. The advantage of endosulfan over other safer pesticides is its low cost, easy availability and extreme efficacy against the Tea mosquito bug (*H. antonii*), a dreaded pest in cashew and cash crop plantations. The US Environmental Protection Agency and the European Union classifies endosulfan under Category 1b – highly hazardous. Aerial spraying of endosulfan was banned by court order in 2001 [3]. However, there are reports of the continued use of endosulfan in areas where cashew and other cash crop plantations are aplenty.

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A number of studies have highlighted various adverse effects of Endosulfan but its role in leukemogenesis has not been conclusively established [4]. Pandey, *et al*. [5] demonstrated the genotoxic potential of endosulfan by demonstrating DNA damage following experimental

exposure in fish. Being a lipophilic compound, we postulated that endosulfan is likely to concentrate in the fatty bone marrow, affect the progenitor cells and subsequently adversely influence the maturation of all hemopoietic cell lines. The study by Olea, *et al*. [6] revealed that isomers and metabolites of endosulfan were present in the fatty tissues of 30-40% of hospitalized children in the agricultural regions of Spain.

We undertook this study to assess if children with hematological malignancies have increased amounts of endosulfan in their bone marrow in comparison to matched controls without hematological malignancies, and further determine if these children resided in area where Endosulfan had been or is being used.

METHODS

This was a case control study, involving children in the age group of 1-15 years conducted in the constituent hospitals of a medical college in Dakshina Kannada district of Karnataka, over an 18 month period from September 2006 to March 2008.

26 cases of proven hematological malignancy and 26 age matched controls who presented serially to our hospital during the study were recruited. Case criteria being children in the age group 1-15 years, with proven hematological malignancies by bone marrow study. Controls were age matched children with proven absence of hematological malignancies by bone marrow aspiration analysis, but who required the bone marrow aspiration for diagnosis. The study period was 18 months.

The patients were interviewed to record relevant clinical history and clinical findings in a structured proforma. Details about family and other pre-existing illness were elicited with special reference to other important diseases reported in the area where the patients usually resided. Parents were explained the objective of the study and after written consent of one of the parents, the children were examined and a bone marrow aspiration done under short dissociative anaesthesia. In addition to relevant studies for diagnosis, Endosulfan residues in the bone marrow were estimated. In those patients where Endosulfan was detected, we determined their geographical location to determine the source of exposure. The Institutional Ethics Committee approved this study.

Methodology for Endosulfan Analysis

Detection of low levels of endosulfan involves extraction of samples with organic solvents, a cleanup step to remove lipids and other materials that may interfere with analysis, High resolution gas chromatography (HRGC) to separate endosulfan from other compounds in the extract, and confirmation of endosulfan by Electron capture detector (ECD) or Mass spectroscopy (MS).

Extraction of endosulfan from bone marrow: 150µL of ethyl acetate was added to 250µL bone marrow sample followed by 600µL of cold 60% H_2SO_4. The sample was vortexed for 1 minute and 3 mL of n-hexane-acetone mixture (90:10, v/v) was added for drug extraction. The mixture was vortexed again and centrifuged at –10° C at 2500 × *g* for 25 minutes. The organic phase was collected and evaporated to dryness in a water bath at 50° C under a gentle stream of nitrogen. The dried residue was reconstituted with 250µL of ethyl acetate and vortexed for 1 minute. The supernatant was transferred entirely into a 250µL vial-insert and a volume of 2µL was injected into the gas chromatography mass spectrometry (GC-MS) system.

Validity of the method was studied and the method was found to be precise and accurate with a linearity range from 10 to 100ng/mL (r^2 >0.998). The quantification limit (LOQ) was found to be 10 ng/mL. The levels of all three forms of endosulfan i.e. alpha, beta and endosulfan sulfate (a metabolite) were estimated.

Instrument conditions: The GC-MS system consisted of a Shimadzu QP 5000 mass spectrometer and a Shimadzu GC 17A gas chromatograph equipped with an AOC 14000 autosampler and a GCMS solution (version 1.10) software (Shimadzu, Courtaboeuf, France). The capillary column used was a DB-5 ms (30 m × 0.25 mm (id), 0.25 µm film thickness. The injector was set at a temperature of 240° C and used in splitless mode. The carrier gas was grade N55 helium and its flow rate was 2.1 mL/min. Column initial temperature was 60° C held for 2 min, then increased at 10ºC min^{-1} to 280° C. interface was set at 290°C. Selective ion mode (SIM) was used for quantification. The specific fragment ions in SIM mode were: alpha-endosulfan: 195, 237, 339; beta-endosulfan 195, 229, 341; endosulfan sulfate: 229, 272,385. Retention times were alpha-endosulfan: 20.3 min, beta-endosulfan: 21.5 min and endosulfan sulfate: 22.5 min. Ionization was performed in electron impact mode at 70 eV. The minimum detection limits of α–endosulfan, β endosulfan and endosulfan sulphate were 10ng/mL.

Standard reference material: α–endosulfan: detection purity - 98.5%; β-endosulfan: detection purity - 98%. The spectrochromatograms of the various samples showed specific molecular ion peaks when detectable quantities of the Endosulfan isomers were noted. The peak obtained in the study subjects was compared with those of the

controls. Recovery tests were performed to check the efficiency of the extraction procedure by spiking known quantities of standard endosulfan to the samples and determining the quantities by the method developed.

The data obtained was entered in an appropriate format and analyzed using SPSS version 11.0. Chi-square test was applied on the data and a *P*-value of less than 0.05 was taken to be statistically significant. The Odds ratio was calculated on appropriate data to determine the disease risk associated with the exposure.

RESULTS

A total of 52 children were enrolled for the study of which 26 were study cases (23 acute lymphoblastic leukemia, 3 myeloid leukemia) and the remaining controls. In the control group, 50% cases were immune thrombocytopenic purpura, 23% refractory anemias, 15% had pyrexia of unknown origin, and 12% had atypical juvenile rheumatoid arthritis. Both groups were comparable statistically (***Table* I**). A total of 36 children in the study hailed from sprayed areas of which 21 (58.3%) and 15 (41.7%) belonged to the study and control groups, respectively. Parental occupation revealed no relation on analysis of either mother's or father's occupation; but was significant when considering fathers occupation, wherein, a significant number of fathers in the study group were found to be farmers or in occupations involving exposure to pesticides, as compared to the control group.

Endosulfan estimation in the bone marrow revealed that of the seven children (6 study, 1 control) who had detectable levels of alpha and beta endosulfan, six were also found to be positive for endosulfan sulfate, a degradation metabolic product of both alpha and beta isomers. All 6 of them belonged to study group. The odds ratio was 7.5 (95% CI: 1.34, 176.43), indicating a 7.5 times higher risk of developing hematological malignancy in children with detectable endosulfan levels compared to those with undetectable endosulfan in the bone marrow. ***Fig.* 1** shows the peak detectable concentration of alpha,beta and sulphate isomers of endosulfan in a positive bone marrow sample.

All the children who had raised endosulfan levels in the bone marrow originated from the areas, which were or are still exposed to endosulfan.

DISCUSSION

Exposure to endosulphan in the environment results in bioaccumulation in humans and other animals, concentrating particularly in fatty tissues [7].

Zahm and Ward [8] found that children who live on or whose parents work on a farm, had higher levels of pesticides in the immediate environment of their homes, compared to children who do not. Shu, *et al*. [9] reported

TABLE I SOCIO-DEMOGRAPHIC PROFILE OF STUDY SUBJECTS

	Cases (*n*= 26) No (%)	Controls (*n*=26) No (%)	*P* value
Age (Yrs)			
< 5	13 (50)	7 (26.9)	0.19
5 to 10	9 (34.6)	11 (42.3)	
>10	4 (15.4)	8 (30.8)	
Male Sex	15 (57.7)	14 (53.8)	0.78
Socio economic status			
Upper	1 (3.8)	1 (3.8)	0.33
Upper Middle	5 (19.2)	1 (3.8)	
Lower Middle	4 (15.4)	9 (34.6)	
Upper Lower	14 (53.8)	13 (50)	
Lower	2 (7.7)	2 (7.7)	
Place of Residence			
Dakshina Kannada (including Mangalore)*	15 (57.6)	11 (42.3)	0.639
Kasargod*	6 (23.1)	4 (15.4)	
Kannur	2 (7.7)	5 (19.2)	
Others	3 (11.5)	6 (23.0)	
Father's Occupation			
Agriculture	6 (23.1)	4 (15.2)	0.07
Business	8 (30.7)	12 (45.6)	
Coolie	11 (42.3)	7 (26.6)	
Others	1 (3.8)	10 (38.4)	
Mother's Occupation			
Housewife	17 (65.4)	19 (73.1)	0.57
Beedi roller	8 (30.8)	5 (19.2)	
Others	1 (3.8)	2 (7.7)	

**Areas sprayed with endosulfan.*

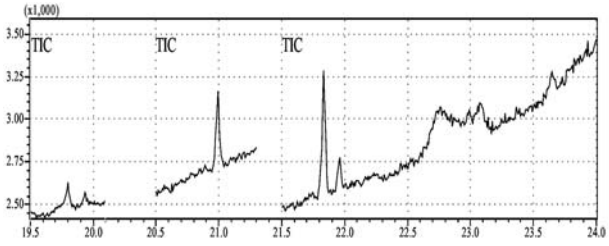

FIG.1 *Endosulfan isomers in chromatograph of a positive bone marrow sample.*

WHAT IS ALREADY KNOWN?

- Endosulfan is a pesticide with a wide variety of adverse effects on all living beings.

WHAT THIS STUDY ADDS?

- Endosulfan was detected in the bone marrow of children with hematologic malignancies.

that those with acute lymphoblastic leukemia (ALL) were 3-5 times more likely to have mothers who had been occupationally exposed to pesticides during pregnancy, compared to healthy children. Infante- Rivard, *et al*. [10] found that children with ALL were 3-9 times as likely to have parents who were exposed to pesticides during pregnancy or lactation. In a more recent study, Ma, *et al*. [11] found that the use of professional pest control services at home, at any time from 1 year before birth to 3 years after, was also associated with a 2.8 fold increase in the likelihood of developing childhood leukemia. A study conducted by the Children Cancer Study Group found that children with acute non-lymphocytic leukemia (ANLL) were more than 2.5 times as likely as children without the disease to have fathers who had used pesticides occupationally for more than 1000 days [12]. However, in this study, in both groups, a number of fathers were found to be residing away from home due to demands of the job, whereby the chance of exposure due to father's occupation was minimal.

We found seven children with detectable bone marrow endosulfan levels and all seven resided in areas which were and are reportedly still exposed to endosulfan. These children had more than 10ng/mL of endosulfan in their bone marrow, as the lowest detectable limit of endosulfan by our methods was 10ng/mL. These seven children may represent only the tip of the iceberg, as we have no knowledge of the baseline bone marrow endosulfan levels in apparently normal children nor do we have age based normograms to this effect. Six of these children were also found to be positive for endosulfan sulfate, a degradation metabolic product of both alpha and beta isomers, which is more persistent in the body than the parent compound and indicates chronic exposure to the pesticide [13]. In a study done by the National Institute of Occupational Health in 2002, Kasargod district (North Kerala), it was found that the serum endosulfan levels were significantly higher in the study population (from areas exposed to endosulfan) as compared to the reference group (hailing from areas which had never been exposed). Endosulfan residues were found in 85% and 78% of female and male subjects, respectively hailing from the study area compared to 34% and 29% of female and male subjects in the reference group [14]. Cerrillo, *et al*. [15] also found evidence of significant accumulation of endosulfan in humans exposed to it in South Spain. There are no earlier studies in literature to support our finding. Although there are no human studies relating to the carcinogenicity of endosulfan to date, a study done by National Cancer Institute, USA in 1978 had shown that endosulfan can induce lymphosarcomas and neoplasms of the reproductive system in rats. Reuber, *et al*. [16] found endosulfan to be carcinogenic in the liver of female mice.

This study does not in any way proves that endosulfan is a cause of leukemia, rather it affirms the fact that Endosulfan, a lipophilic compound, has the potential to accumulate in the bone marrow, the site of fundamental cell biological processes that control cell differentiation and maturation and can hence affect the outcome and functioning of various organ systems subsequently. To the best of our knowledge, this study is the first of its kind where bone marrow samples of children has been subjected to analysis for pesticide levels and this may serve as the basis for future larger studies. The small sample size, other confounding factors like genetic susceptibility, exposure to other kinds of pesticides and carcinogens and their effects, are the limitations of the study.

Greater awareness of the toxic effects and improper use of pesticides needs to be created among the public. Siblings of children with leukemia may need to be screened for pesticide levels to prevent chronic long term exposure in the future. Large scale, multidisciplinary, prospective studies along with cytogenetic analysis are recommended to evaluate the leukemogenic potential of endosulfan.

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Contributors: ATK: conceived, designed and authored the first manuscript of the study and subsequently revised it for important intellectual content. He is the guarantor of the study; ATK, AC, KSA: recruited the subjects and collected the data; Hematological investigations were carried out by AR; ATK, AC, KSA and AR: wrote the final manuscript with inputs from RW who also monitored the progress of the study. All authors reviewed and approved the final manuscript.

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