Association of blood lead level with neurobehavior and neurotransmitter expressions in Indian children

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A R T I C L E  I N F O

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A B S T R A C T

Present study aimed to assess the alterations in neurotransmitter expression and its association with Blood Lead Level (BLL) and neurobehavioral pattern in children. 72 school going children were recruited. Blood lead levels were determined by Atomic Absorption Spectrophotometer. Neurobehavioral state was assessed by means of population specific scale i.e. CPMS (Childhood Psychopathological measurement Schedule). Serum serotonin and dopamine were estimated by ELISA, receptor and transporter gene expressions were assessed by quantitative real time PCR. Significant positive correlation was observed between Total CPMS score (i.e. adverse neurobehaviour) and BLL. Further, serum serotonin levels and dopamine receptor expression showed a negative and positive association with BLL, respectively. In similarity, serum serotonin levels showed a negative correlation and dopamine receptor expression had a significant positive correlation with total CPMS score. Environmental exposure to Lead (Pb) may result in significant alterations in the neurotransmitter levels which may be associated with neurobehavioral changes in the children exposed to Pb.

1. Introduction

Lead (Pb), is a heavy metal that is commonly used in industries manufacturing paints, dyes, batteries, toys, jewellery etc. Irrespective of the advantage offered by Pb for industrial use, its adverse effects on health has led to tremendous reduction in its use. Occupational exposure remains to be the major source for Pb exposure in adults, whereas, ingestion of Pb from contaminated food, water and inhalation of Pb in dusts are chief source of Pb in children. Further, use of ceramic cookware and unfiltered water have also been reported as potential sources for Pb. Pb in water could be a result of environmental pollution from anthropogenic activities or from erosion of pipes used in water distribution system. In addition, certain lifestyle habits such as consumption of roadside food, owing pets, use of cosmetics, residing in old houses or residing close to vehicular traffic may also increase the risk of Pb exposure [1–4].

Pb is a multi-system toxicant that can cause acute encephalopathy at very high levels (>40 μg/dL), while increase incidence of hypertension, renal disorder, and neurodevelopmental defects at lower concentrations [5]. 63 % of idiopathic intellectual development failure and 10 % global burden of hypertensive heart disease have been attributed to Pb toxicity [37]. Children, in particular, are vulnerable to neurotoxic effects of Pb. Early studies have reported neurotoxicity in children at very high Blood Lead Levels (BLLs) (>40 μg/dL), however, later studies have identified Pb to be neurotoxic at much lower concentrations (<10 μg/dL) [6]. The US Centre for Disease Control and Prevention (CDC) defines BLL >5 μg/dL as toxic cut off level for children, with no threshold being safe ([7]). About 74 % of children residing in low- and middle-income countries of South East Asia and 64 % of children in Kathmandu valley of Nepal have BLL >5 μg/dL [8]. Childhood Pb toxicity is of major concern due to the irreversible nature of neurological damage incurred on the developing nervous system. Various aspects of neuronal function, such as, cognition, behaviour, and motor functions, are affected by Pb neurotoxicity [9]. A decline in overall IQ, in addition to, poor reading, math and spelling capabilities have been reported in children with high BLL [10]. Pb causes nearly 6 lakh (600,000) new cases of childhood intellectual disability every year with substantial proportion residing in developing countries [11]. Further, longitudinal studies report poor...
executive function and decision making in adults with high childhood BLL, suggestive of irreversible effect on cognitive development [12]. Behavioural changes of both internalising (depression, anxiety) and externalising (aggressiveness, impulsivity) nature have been observed in children with high BLL [15]. However, a characteristic neuropathological signature associated with Pb neurotoxicity is yet undefined.

Mechanisms such as oxidative stress, direct neural damage, imbalance in calcium and neurotransmitter homeostasis have been implicated in Pb neurotoxicity [14–16]. The chief neurotransmitter systems involved in Pb neurotoxicity include monoaminergic, cholinergic, and GABAergic systems. Serotonin (5 H T) and dopamine (DA) monoaminergic neurotransmitters mediate cognition, memory, learning, attention, mood, and reward behaviour. Pb is proposed to affect neurotransmitter availability by altering its presynaptic and post-synaptic factors such as synaptosome formation, release, uptake, receptors, and transporters, in addition to altering levels of second and third messengers such as PKC, Ca, transcription factors etc. [17]. Studies have reported a decrease in DA levels, with increased expression of Dopamine Transporter (DAT) and a possible compensatory increase in Dopamine Receptor (DR) levels. However, these changes have been region specific and varied with duration and concentration of Pb exposure. Likewise, changes in 5 H T levels, its receptor, and, transporter expression have also been reported. However, majority of these studies have been based on animal models and lack verification on human subjects. Irrespective of extensive studies on mechanism underlying Pb neurotoxicity, precise understanding of its pathogenesis remains unclear. Further, the infeasibility to conduct CNS related studies in live subjects adds to the deficiency in human studies. However, mRNA expression in peripheral lymphocytes and serum levels of neurotransmitters have shown to be an effective representatives of CNS levels [18]. Therefore, the present study has been designed to assess neurotransmitter expression in relation to BLL and neurobehavioral pattern in children.

2. Materials and methods

2.1. Study population

Study subjects consisted of school going children of Jodhpur, between 6–15 years of age (N = 72). The sample size for present study was calculated to be 73, based on 5% prevalence of High BLL (>5 μg/dl) in Jodhpur, as reported previously [19]. The power of study estimated to be 90 with the present sample size of 72. Ethical consent from Institutional Ethics Committee (Reference no. AIIMS/IEC/2018/640) and signed consent from parent/guardian was obtained prior to sample recruitment. Demographic details of the study participants were obtained by means of self-made questionnaire. Additionally, information regarding potential sources for Pb exposure as reported in previous studies, such as distance of residence from vehicular traffic, use of cosmetics, consumption of roadside food etc. were also included in the questionnaire [20]. In addition to demographic details, neurobehavioral assessment and venous blood sample were collected at the time of sample recruitment.

2.2. Sample collection

Venous blood sample was collected from each subject by means of venepuncture under aseptic conditions. 2 ml of whole blood sample was used for blood lead and mRNA expression analysis and 2 ml of serum sample was used for ELISA.

2.3. Estimation of blood lead level (BLL)

Blood lead levels (BLL) were estimated by Graphite Furnace Atomic Absorption Spectrometry (GFAAS) using Zeeman correction in an ICE 3000 system (Thermo Fisher scientific, Waltham, MA) ensuring appropriate quality control. Analysis was carried out by a five-step programme (130 c/20 s, 200 c/30 s, 600 c/10 s, 2000 c/3 s, 2450 c/ 3 s) followed by measurement of absorbance at 282.3 nm. A 5-point calibration curve was obtained using standard solutions of Pb (Pb concentrations: 2.5 ppb, 5 ppb, 10 ppb, 20 ppb and 40 ppb) prepared from serial dilutions of 1000 ppm stock Pb solution. Samples and standards were prepared in 1:20 dilution using matrix modifier (0.1% dmannitol hydrogen phosphate, 0.5% Triton X-100 and 0.2% HNO3) as diluent. Clin check whole blood control (Recipe, Munich, Germany) were used for quality check. Results are expressed in μg/dL.

2.4. Peripheral blood neurotransmitter levels

Serum Serotonin (5 H T) and Dopamine (DA) levels were estimated by commercially available ELISA kits (Elab sciences) following manufacturer instructions. Absorbance was measured at 450 nm. Standard curve was obtained using serial dilution of known concentrate. The results for 5 H T and DA are expressed as ng/mL and pg/mL respectively.

2.5. RNA isolation and relative mRNA expression

RNA was isolated from 750 μl whole blood sample by Trizol method using Trizol LS reagent (Sigma-Aldrich, Merck, Germany) as per manufacturer’s instructions. Quality and quantity of isolated RNA were determined using Thermo Scientific nanodrop One®. Isolated RNA was stored at ~80°C till further use. Subsequently, relative mRNA expression was performed in two-step reverse transcriptase PCR. Firstly, CDNA was synthesized from isolated RNA in 20 μl volume reactions using thermo scientific reverse cDNA synthesis kit (Waltham, Massachusetts, USA) following manufacturer instructions. Following cDNA synthesis, quantitative real time PCR (qPCR) was carried out in Bio-Rad CFX96 real time thermal cycler (Bio-Rad, USA) using SYBR green master mix (Thermo Scientific, Massachusetts, USA). Pre-validated primer sequences from Sigma-Aldrich for Serotonin Receptor (5 H TR2A, 5 H TR3A), Serotonin Transporter (ST), Dopamine Receptor (DRD2, DRD3, DRD4) and Dopamine Transporter (DT) gene were used. GAPDH was used as housekeeping gene for normalization of target gene expressions. The Ct of the target genes were normalized to Ct of the GAPDH gene.

2.6. Neurobehavioral assessment

Neurobehavioral state of the children was evaluated by means of Childhood Psychopathological Measurement Schedule (CPMS). CPMS is an adaptation of Achenbach Child Behaviour Check List (CBCL) standardized for Indian population [21]. It is a widely used screening tool for assessing psychopathological abnormalities in children between 4–14 years in both boys and girls. It is a one-time assessment questionnaire comprising of 75 questions assessing eight factors i.e. Factor I- low intelligence with behavioural problems, Factor II- Conduct disorder, Factor III- Anxiety, Factor IV- Depression, Factor V- Psychotic symptoms, Factor VI- Special symptoms, Factor VII -Physical illness with emotional problems and Factor VIII- Somatization. Each question evokes a ‘Yes’ or ‘No’ response carrying 1 (in presence of symptom) or 0 (in absence of symptom) score, respectively. A score of more than 10 indicates a high probability of psychopathological disorder in children. Questions were directed to the parent/guardian of the child and responses were recorded.

2.7. Statistical analysis

The data collected was tabulated and analysed by Microsoft Excel 2013. Statistical tests were performed using GraphPad Prism 8.3 and SPSS version 23. Descriptive statistics were carried out to determine mean, standard deviation (SD), median and range. To assess normal distribution of the data, Shapiro Wilk Test was carried out. Parametric
data was compared using t-test and non-parametric data using Mann Whitney U test. P-value < 0.05 was considered statistically significant. To assess the correlation, Pearson or Spearman correlation test was performed.

3. Results

3.1. Demographic data

The median age of children was 14 years with a male to female ratio of 0.84. BLL was found to be significantly greater in older age group and female children. Children living in older houses had significantly higher BLL than children living in newly constructed houses i.e. houses constructed in the recent 5 years. BLL did not differ significantly between children living in houses constructed within last 5 years and those in newly constructed houses. Details of socio-demographic and lifestyle characteristics are provided in Table 1.

3.2. Correlation of BLL and 5 H T

Serum serotonin levels showed significant negative association with BLL. Whereas serotonin receptor or transporter gene expression did not have any significant association with BLL (Table 2).

3.3. Correlation of BLL and DA

Unlike serum 5 H T levels, serum DA did not have any significant association with BLL. However, dopamine receptor (DRD2 and DRD3) gene expression had significant mild positive correlation with BLL (Table 3).

3.4. Neurotransmitter and BLL groups

When the study participants were divided into High and Low BLL groups based on median BLL of 4.9 μg/dL, serum 5 H T levels and serotonin receptor (5 H TR3A) expression were found to be significantly different between the groups. Serum 5 H T levels were significantly lower with increased expression of serotonin receptor (5 H TR3A) in the High BLL group (Table 4). Further, dopamine receptor (DRD2 and DRD3) expression was also significantly higher in the High BLL group (Table 4). Additionally, a correlation matrix of BLL, serum 5 H T, its receptor and transporter expression showed a significant positive relation of 5 H TR2A (P < 0.05, r = 0.30) and 5 H TR3A (P < 0.01, r = 0.36) with ST gene expression, respectively (data not shown).

3.5. BLL and neurobehavior

Spearman correlation analysis revealed a significant positive correlation between BLL and total CPMS score. Further, except for Factor II (Conduct Disorder), all other factors had significant association with BLL, independently (Table 5). Between the High and Low BLL groups, significant difference was observed with Total CPMS score, as well as Factors I, III, IV, V and VII (Table 6). A total of 16 children had CPMS score more than 10 (Data not shown) among which 15 children belong High BLL group and only 1 child in Low BLL group.

3.6. Neurotransmitter levels and neurobehavior

Serum 5 H T levels had significant negative association with Total CPMS score, whereas serotonin receptor or transporter expression showed no significant association with total CPMS score. Additionally, individual factors such as Factor I, II, V and VIII showed mild negative association with serum 5 H T. Although ST did not show any significant association with total CPMS score, there was mild association between ST and Factor VIII.

Unlike 5 H T, serum DA did not show any significant association with total CPMS score or with scores of individual factors and only dopamine receptor (DRD3) expression had significant positive association with Total CPMS score, as well as with Factor III. IV, V and VI (Table 7).

4. Discussion

Pb is one of the most studied heavy metal toxicants known to cause adverse effects in children [22]. Recent studies report toxic effects of Pb at concentrations lower than present cut off value (<5 μg/dL) [6]. The median BLL of children in present study was 4.95 μg/dL, with 50 % of

### Table 1

| Risk factor                              | N | BLL (μg/dL) Mean ±SD | P value |
|-----------------------------------------|---|----------------------|---------|
| Age                                     |   |                      | 0.01    |
| 9 – 13 years                            | 20| 3.8 ± 1.83           |         |
| 13 – 15 years                           | 52| 7.41 ± 7.55          |         |
| Sex                                     |   |                      | 0.03    |
| Male                                    | 33| 6.22 ± 3.71          |         |
| Female                                  | 39| 6.55 ± 8.46          |         |
| Habit of washing hands before food      |   |                      | 0.88    |
| No                                      | 16| 6.06 ± 4.77          |         |
| Yes                                     | 56| 6.5 ± 7.15           |         |
| Habit of eating roadside food frequently (> twice in a week) |   |                      | 0.56    |
| No                                      | 44| 6.88 ± 7.72          |         |
| Yes                                     | 28| 5.66 ± 4.61          |         |
| Habit of using kohl/surma routinely (>5 days in a week) |   |                      | 0.22    |
| No                                      | 60| 6.7 ± 11             |         |
| Yes                                     | 12| 4.49 ± 3.36          |         |
| Habit of using traditional medicine     |   |                      | 0.99    |
| No                                      | 44| 5.3 ± 3.23           |         |
| Yes                                     | 28| 8 ± 9.79             |         |
| Maternal education                      |   |                      | 0.02    |
| No formal education                     | 30| 4.22 ± 2.4           |         |
| Any formal education                    | 42| 8.06 ± 8.36          |         |
| Paternal education                      |   |                      | 0.29    |
| No formal education                     | 8 | 6.9 ± 5.14           |         |
| Any formal education                    | 64| 6.37 ± 6.93          |         |
| Residing in new house (constructed within last 5 years) |   |                      | 0.03    |
| No                                      | 56| 6.8 ± 7.35           |         |
| Yes                                     | 16| 4.66 ± 3.1           |         |
| History of recent painting (painted atleast once in last 2 years) |   |                      | 0.36    |
| No                                      | 43| 5.79 ± 7.71          |         |
| Yes                                     | 29| 7.29 ± 5.14          |         |
| Residence close to traffic (located <2 km from vehicular traffic prone areas) |   |                      | 0.47    |
| No                                      | 15| 6.07 ± 3.69          |         |
| Yes                                     | 57| 6.5 ± 7.41           |         |

P value < 0.05 was considered significant.
Table 3
Association between BLL and DA in study population (N = 72).

| Parameter | High BLL (>4.9 μg/dL) (N = 36) | Low BLL (<4.9 μg/dL) (N = 36) | P value | r  |
|-----------|---------------------------------|-------------------------------|---------|---|
| Serum DA (μg/mL) | 0.11 ± 0.35 | 0.11 ± 0.35 | >0.05 | 0.001 |
| DRD2 (2^dct) | 0.33 ± 0.05 | 0.09 ± 0.52 | >0.05 | 0.001 |
| DRD3 (2^dct) | 0.26 ± 0.02 | 0.02 ± 0.46 | >0.05 | 0.001 |
| DRD4 (2^dct) | 0.05 ± 0.67 | >0.19 ± 0.28 | >0.05 | 0.001 |
| DT (2^dct) | 0.11 ± 0.37 | >0.13 ± 0.34 | >0.05 | 0.001 |

P value < 0.05 was considered significant.

Table 4
Difference in neurotransmitter protein and gene expression between High BLL and Low BLL groups.

| Parameter | High BLL (>4.9 μg/dL) | Low BLL (<4.9 μg/dL) | P value | r  |
|-----------|-----------------------|----------------------|---------|---|
| Serum Serotonin (μg/mL) | 123.3 (162.6) | 303.1 (239.7) | >0.05 | 0.0008 |
| 5 H TR2A (2^dct) | 0.06 (0.1) | 0.11 (0.4) | >0.05 | 0.49 |
| 5 H TR3A (2^dct) | 13.15 (15.8) | 0.17 (4.1) | >0.05 | 0.0001 |
| ST (2^dct) | 0.81 (0.5) | 0.69 (1.0) | >0.05 | 0.66 |
| Serum Dopamine (μg/mL) | 770.2 (65.6) | 774.6 (64.6) | >0.05 | 0.77 |
| DRD2 (2^dct) | 2.53 (7.5) | 0.32 (3.1) | >0.05 | 0.003 |
| DRD3 (2^dct) | 13.1 (15.9) | 6.85 (6.3) | >0.05 | 0.0036 |
| DRD4 (2^dct) | 8.70 (12.0) | 5.57 (20.7) | >0.05 | 0.56 |
| DT (2^dct) | 0.1 (0.3) | 0.08 (0.2) | >0.05 | 0.14 |

P value < 0.05 was considered significant.

Table 5
Association between BLL and CPMS scores in total study subjects (N = 72).

| Factor | Low intelligence with behaviour problems | P value | r  |
|--------|----------------------------------------|---------|---|
| Factor 1 | 0.35 | 0.02 | 0.12 ± 0.54 |
| Factor 2 | 0.20 | 0.13 | >0.06 ± 0.39 |
| Factor 3 | 0.37 | 0.0001 | 0.21 ± 0.60 |
| Factor 4 | 0.49 | 0.007 | 0.26 ± 0.64 |
| Factor 5 | 0.37 | 0.01 | 0.14 ± 0.56 |
| Factor 6 | 0.34 | 0.009 | 0.07 ± 0.50 |
| Factor 7 | 0.44 | 0.0004 | 0.17 ± 0.58 |
| Factor 8 | 0.23 | 0.01 | 0.06 ± 0.45 |
| Total CPMS | 0.68 | 0.001 | 0.51 ± 0.78 |

Data is expressed as r i.e. spearman’s correlation coefficient; P value < 0.05 was considered significant.

Table 6
Comparison of CPMS score between High and Low BLL groups.

| CPMS Factors | High BLL (>4.9 μg/dL) (N = 36) | Low BLL (<4.9 μg/dL) (N = 36) | P value | Mean ± SD | Median (IQR) |
|--------------|-------------------------------|-------------------------------|---------|-----------|-------------|
| Factor 1     | 2.58 ± 1.48 (3 (3)) | 1.47 ± 1.38 (1.5 (3)) | >0.05 | 0.002 |
| Factor 2     | 1.70 ± 2.36 (1.5 (2)) | 0.75 ± 1.02 (0 (1.75)) | >0.05 | 0.060 |
| Factor 3     | 0.97 ± 1.05 (1.2) | 0.3 ± 0.70 (0 (0)) | >0.05 | 0.001 |
| Factor 4     | 1.91 ± 1.81 (2.3) | 0.55 ± 1.05 (0 (0.75)) | >0.05 | 0.001 |
| Factor 5     | 0.26 ± 0.51 (0 (0.75)) | 0.02 ± 0.16 (0) | >0.05 | 0.012 |
| Factor 6     | 0.17 ± 0.45 (0) | 0.02 ± 0.16 (0) | >0.05 | 0.092 |
| Factor 7     | 0.52 ± 0.66 (0) | 0.27 ± 0.51 (0 (0.75)) | >0.05 | 0.042 |
| Factor 8     | 1.14 ± 1.25 (1.2) | 0.75 ± 1.02 (0 (1)) | >0.05 | 0.109 |
| Total        | 9.29 ± 4.3 (6.75) | 4.16 ± 2.19 (5 (4.75)) | >0.05 | 0.001 |

P value < 0.05 was considered significant.
In conclusion, studies on Pb neurotoxicity and alteration in neurotransmitter expression have been conducted mainly on animal models, lacking verification by human studies, further, existing humans studies have also been limited to one neurotransmitter. Therefore, the results from our study may add to the existing knowledge on Pb neuropathogenesis by affirming the findings of animal studies, as well as, by depicting the gene expression of receptors and transporters of both serotinergic and dopaminergic system. However, the probability of long term Pb exposure since early childhood in our study population should be kept in mind, as exposure to lead (even at low concentration) as early as 2 years of age could critically impair intellectual development. Since our study is a cross-sectional study, the effect of early childhood lead exposure could not be evaluated in this study, nevertheless, there was substantial finding indicative of definitive psychopathological changes with increasing BLL. Additionally, the alterations in serotoninergic and dopaminergic system could underlie the neurobehavioral changes induced by Pb.

**Conflict of Interest**

The authors declare no conflict of interest.

**Compliance with ethical standards**

This study involves participations of human subjects with the approval from Institutional Ethical Committee (IEC), All India Institute of Medical Sciences, Jodhpur, Rajasthan India. The study was performed in accordance with down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

**Informed consent**

Prior written informed consent was obtained from all subjects recruited in this study.

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**Author's contributions**

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