ABSTRACT

Background: Attention Deficit Hyperactivity Disorder (ADHD) is one of the most common neuropsychiatric disorders of childhood. Phthalates are industrial chemicals often used in personal care products and to soften plastics in toys and household items such as food containers, and medical devices. Animal studies have reported that the phthalate compound might cause hyperactivity and impulsivity in rats. However, the relation between phthalates and ADHD in human is still controversial. The aim of our study is to investigate the possible association between urinary phthalate metabolite levels and attention deficit disorder, learning disability in 6–12-years old children.

Methods: Urine samples were obtained from 80 children. All children were selected from the attendants of Abu Elreesh child psychiatry outpatient clinic, Cairo University. subjects were divided into two main groups Group A (patients) consisted of 50 children with Attention-deficit/hyperactivity disorder (ADHD) diagnosed by psychiatric consultant and Group B (control) consisted of 30 normal children. In urine, mono butyl phthalate (MBP) metabolite were measured with high performance liquid chromatography (HPLC).

Results: The mean concentration of MBP level was (15.539±8.316) for patients (group A) and (8.085 ±2.426) for controls (group B) with significant difference between groups. Also, there was significant correlation with processing milk, plastic containers, toys, cosmetics, wall, and floor materials containing phthalate exposure and higher level of (MBP) in urine of group A.

Conclusion: The present study showed association between phthalate metabolites in urine and symptoms of ADHD among school-age children.

Key Words: Attention deficit/hyperactivity disorder (ADHD), Learning disability, Phthalates, Plasticizers

INTRODUCTION

The attention deficit hyperactivity disorder (ADHD) is one of the most common neuropsychiatric disorders. Inappropriate patterns of inattention, impulsivity, and hyperactivity are the main behavioral symptoms of attention deficit hyperactivity disorder (ADHD). Disorder of attention-deficit / hyperactivity is a highly prevalent, early neurobehavioral disorder (Patil et al., 2016).

Phthalates are plasticizers which are used commonly nowadays. Vast amounts of phthalate plasticizers are manufactured all over the world. As they are used in many products (Haidy et al., 2018)

Over time, phthalates can leach into the surrounding environment from plastic products. This happens due to production of large quantities of phthalates as well as wide spread of use. Routes of intake include skin contact, inhalation, and ingestion (Talsness et al., 2009).

Children and infants are commonly exposed to phthalates through multiple routes that may be; oral via phthalate-
contaminated food (stored in plastic containers), water and other liquids and toys especially pacifiers also plastic feeding bottles, dermal (some personal care products) and inhalation (PVC-contaminated phthalates, hair spray, and other phthalate-containing products).

Also neonates are exposed in neonatal intensive care units via ryle tubes and I.V lines (Swan, 2008).

Animal studies in rats have shown that phthalate compounds could induce hyperactivity and impulsivity, similar to that happening to children. Studies have also shown that prolonged environmental exposure to phthalates causes injury to the developing brain (Kim et al., 2009).

AIM OF WORK

Assess and examine the potential correlation between exposure to phthalates and attention deficit hyperactivity disorder (ADHD) by evaluating mono-butyl phthalate in urine using high-performance liquid chromatography (HPLC) from October 2016 to April 2017.

SUBJECTS AND METHODS:

Patient selection was based on the following criteria:

Criteria for inclusion:

1. Children diagnosed as having ADHD by DSM 5 clinical psychiatric evaluation.
2. Children of both sexes aged between 6 and 12 years.
3. Children whose parents gave oral consent.

Criteria for exclusion:

1. Infants of mental retardation IQ score below 70.
2. Children with history of bipolar disorder and anxiety.
3. Children with organic brain disease, chronic medical condition (renal, endocrinial, liver ….. etc).
4. Children with any neurological disorder (epilepsy, cerebral palsy … etc).

The 80 children were divided into 2 groups:

Group A (study group): consisted of 50 children with Attention-deficit/hyperactivity disorder (ADHD) diagnosed by psychiatric consultant.

Group B (control group): consisted of 30 normal children who matched the study group concerning age, sex and socio-economic level.

For each child, the following was carried out:

1) Full history including age, residence, medical history and full psychiatric history.

2) General and Local examination.

3) Special psychiatric assessment:
   - The Arabic version of Conner's Parent Rating.
   - IQ assessment by using Stanford Binet test.

A questionnaire of potential exposure to phthalate sources:

1) Dietary exposure
   - Milk & Type of feeding
   - Beverages.
   - Fish.
   - Preservatives
   - Dietary containers & method of food preservation.

2) Environmental exposure
   - Household cleaners.
   - Cosmetics e.g., soaps, shampoos, lotions
   - Flooring material
   - Wall material
   - Plastic toys and pacifiers

3) Medical exposure (Esp. in neonatal period)
   - Chronic need for blood transfusion
   - Chronic use of any IV preparations
   - Hospitalization &admission to NICU
   - Operative history

4) Laboratory investigation:
   - Urine Samples analysis:

Sample:

Urine samples were collected in clean, glass containers marked with identification number of the subjects. Samples excluded were turbid or those containing blood. All samples were kept frozen below 20 degrees Celsius.
Urine samples were then analysed in laboratory by high performance liquid chromatography (HPLC) in the department of forensic medicine and clinical toxicology, Cairo University.

**Instruments:**

- **HPLC**
  A high-pressure isocratic system was used, consisting of a DionexUltiMate 3000 UHPLC; RS pump, auto-sampler, column compartment, and DIODE ARRAY detector. Chromatographic column used was Hypersil BDS; C18 column; particle size 5µm (reversed phase column).
- **Solid phase extraction:**
  Hypersep cnasses block 16 port vacuum manifolds and vacuum pump ROCKER 400 Thermoscientific. SPE columns were purchased from Thermo scientific. HYPERSEP C18500MG/3ML/50PKG.

**Methods:**

I. **Sample Pretreatment (solid phase extraction):**

Samples of urine were allowed to thaw and then become homogenously vortexed. 950µ urine sample was transferred into each glass. Add 50µ of sodium hydroxide and boiled to 100°C for 1 hour (alkaline hydrolysis.).

II. **Solid phase extraction:**

  **Conditioning:** was done with 1 mL of methanol, 1mL of acetonitrile, followed by 1mL of phosphate buffer. **Loading:** 5mL of urine sample (with 10 µl IS benzyl benzoate) dilution was done using 1 mL of phosphate buffer solution (pH 2.0) and then the mixture was added to column of solid phase extraction. **Wash:** wash was done by adding 2 mL of formic acid solution (0.1 M) and 1 mL of water. Lastly, drying of the cartridges was done under negative pressure.

  **Elution:** 1mL acetonitrile and 1 mL of ethyl acetate were added. The eluent was collected, concentrated, and evaporated. The dry residue was reconstituted with 1000 µL of mobile phase

III. **Chromatographic conditions:**

  **Mobile phase:**

  For preparing 1L of mobile phase; 1.0mL of acetic acid is added to 100mL of acetonitrile (HPLC) grade This solution is stored at room temperature in an amber jar. The column temperature had been set to 40 °C. The amount of injection was 20 µL, and a rate of flow was 0.3 mL / min. The average absorbance of UV was 240-280 nm to 254 nm.

**Figure (1):** Chromatogram of spiked urine sample containing 4µg/ml of monobutylphthalate (MBP) and benzyl benzoate (internal standard), concentrations 10µl.

  Retention time of MBP = 1.270 minute, Retention time of benzyl benzoate = 3.840 minute
Validation of method:
Validation of analytical method was done to demonstrate:

- Linearity
- Limit of detection (LOD) and limit of quantification (LOQ)
- Accuracy
- Precision

Linearity:
The presented analytical procedure proved to be linear (squared correlation coefficient $r^2 = 0.991$, $n = 7$).

Linear regression of calibration curve of seven concentrations of mono butylephalate (MBP). Blank urine samples spiked with a series of standard MBP concentrations (0.041, 0.8, 2.5, 10, 12.5, 25, 100 $\mu$g/mL), was prepared to investigate the linearity, LOD, and LOQ. Range 0.041 to 100 $\mu$g/mL.

- LOD = 3.3 * St dev/slope
- LOD = 0.006644 ug/ml

- LOQ = 10 * St dev/slope
- LOQ = 0.02 ug/ml

Extraction recovery:

Five quality control samples were used to test extraction recovery; consisting of blank urine added to the external standard (with three different concentrations) and 10 $\mu$L of internal standard benzyl benzoate.

Concentrations of external standard were 1000 $\mu$g/mL, 200 $\mu$g/mL and 25 $\mu$g/mL (low, medium, and high concentrations). Extraction recovery was calculated as a percentage ratio of area between external and internal standard.

The value of phthalate extracted from urine samples divided by those obtained by direct determination of the standard solutions (and internal standard) at the same concentration level. Extraction recovery for three QC samples of MBPs were more than 79%.

Precision:
Precision is relative standard deviation (RSD) of areas of three replicates at three different concentrations ($n = 5$). High 100 $\mu$g (44.52, 42.43, 46.6) 2.085%, low 0.8 $\mu$g (2.5, 3.2, 2) 0.6028%, medium 25 $\mu$g (13.3, 14.7, 14.33) 0.7255%.

RESULTS

The mean age was (7.6 ± 1.8 years) for cases (group A) and (7.4 ± 1.3 years) for controls (group B). Males were predominant though there was no significant difference in sex between both groups. Rural residences were more common in study group with no residence difference in the control group. Phthalate level in urine (MBP): the mean level was (15.539 ± 8.316) for cases (group A) and (8.085 ± 2.426) for controls (group B) with significant difference between groups as shown in table (1).
Regarding history of phthalate exposure in relation to MBP level in urine in study group A (cases). There was a significant difference in intake of milk, uses of pacifier and using of plastic dietary containers in relation to urinary phthalate level (p = 0.019), (p = 0.039) and (p = 0.048) and urinary phthalate level as shown in table (2).

**Dietary exposure**

**Table (2):** Relation between dietary exposure and urine phthalate level in group A (cases).

|                                  | Phthalate level | P value |
|----------------------------------|-----------------|---------|
|                                  | Median | Q1    | Q3     |
| Milk                             |        |       |        |
| No                               | 8.0    | 3.5   | 9.0    |
| Yes                              | 8.6    | 8.0   | 23.7   | 0.019
| Pacifier                         |        |       |        |
| No                               | 8.6    | 2.9   | 8.6    |
| Yes                              | 8.3    | 14.9  | 21.7   | 0.039
| Plastic dietary container        |        |       |        |
| high                             | 8.45   | 4.95  | 23.29  | 0.048
| low                              | 8.6    | 7     | 7.47   |

Regarding environmental exposure and MBP level in urine in study group A (cases): There was no significant difference concerning ceramic, plastic carpet and painted wall in relation to urinary phthalate (p = 0.061), (p = 0.525), (p = 0.004) and (p = 0.694) as shown in table (3).

**Table (3):** Relation between environmental exposure and urinary phthalate level in group A (cases).

| Flooring material | Phthalate level | P VALUE |
|-------------------|-----------------|---------|
|                   | median | Q1    | Q2     |
| ceramic            |        |       |        |
| no                 | 7.6    | 3.5   | 8.6    | 0.061
| yes                | 8.6    | 6     | 20.1   |
| Plastic carpet     |        |       |        |
| no                 | 8.5    | 2.9   | 23.9   | 0.525
| yes                | 8.5    | 6.9   | 8.6    |
| Painted wall       |        |       |        |
| no                 | 8.7    | 7     | 10.8   | 0.694
| yes                | 8.5    | 4.9   | 17.1   |

Regarding Developmental history and MBP level in urine of study group A (cases).

**Table (4):** shows a significant difference in group A who were complaining of learning disability and phthalate level in urine (P = 0.047) while there was no significant difference concerning developmental milestones and phthalate level in urine between group A (P = 0.065).
Table (4): Relation concerning learning disabilities, developmental milestones and urinary phthalate level in urine among group A (cases)

| Learning Disabilities | phthalates level | P value |
|-----------------------|------------------|---------|
|                       | Median | Q1    | Q3    |        |
| No                    | 8.6    | 3.2   | 8.9   | 0.047  |
| Yes                   | 8.34   | 8.945 | 22.695|        |

| Developmental milestones | phthalates level | P value |
|--------------------------|------------------|---------|
| Normal                   | 8.6              | 7.15   | 13.05  |        |
| Delayed                  | 4.44             | 1.35   | 13.92  | 0.065  |

Relation between ADHD cases classified by Conner’s test and MBP level in urine in study group A (cases): Table (5) shows no significant difference concerning correlation with results of phthalate level in urine and severity of disease in group A (p = 0.674).

Table (5): Relation between degree of severity of ADHD and urinary phthalate level in group A

| Conner’s Test classification | phthalate level | P value |
|-----------------------------|-----------------|---------|
|                            | Median | Q1    | Q3    |        |
| Mild                        | 17.6   | 0.8   | 46.8  | 0.674  |
| Moderate                    | 6.8    | 2.1   | 20.1  |        |
| Sever                       | 8.3    | 8.0   | 17.2  |        |

DISCUSSION

The current study shows that there was no significant difference in sex distribution and phthalate level in group A. This was shown also by (Miodovnik et al. 2011) who found that there were no sex specific differences between exposure to phthalate, social impairment in childhood and neuropsychiatric disorders at 7–9 years which is consistent with results of this study.

This study showed no significant difference in residence distribution and phthalate level between groups A&B. This was not in accordance with (Pant et al., 2008 and Růžičková et al., 2016) who found rural population shows statistically significant lower levels of phthalate due to difference in industrial activities. Also, the urban water system is an important sink for pollutants like phthalates (Björklund, 2009).

Results of the present study showed significant difference in group A, who were complaining of learning disability and phthalate level (P= 0.047). A cross-sectional study by (Cho et al. 2010) showed negative association between vocabulary sub scores on intelligence tests urine phthalate levels at 8–11 years of age which is in accordance with results of the present study.

In the current work, there was significant difference between studied groups (A&B) regarding exposure to plastic containers (p = 0.019). High degree of exposure represents 66 % in group A. Also, there was significant difference in using of plastic dietary containers and phthalate level between group A (p =0.048).

This was in accordance with (Gonzalez-Castro et al., 2011) who found the endocrine disruptors in analyzing of the plastic food containers, microwaveable containers and baby bottles that may be associated with hazardous effect. Also, this finding can be explained by (Bošnir et al., 2007) who found that leaching of phthalates from plastic containers to soft
drinks is significantly higher than (3 to 40 folds) leaching of phthalates from plastic containers to mineral water. This is due difference in pH between mineral water (pH 5) and soft drinks (pH 3). Phthalates tend to dissolve more in acidic medium and migrate from plastic containers.

The current study revealed a significant difference in urinary phthalate level among ADHD children (group A) depending on intake of milk which in agreement with Serrano et al., (2014) who considered milk & dairy products is the highest contributor to exposure to phthalates. (Fierens et al., 2013) explained this by mechanical milking of cattle as well as, feed containing phthalates given to cattle.

During industry and retail of milk, packaging and contact materials are additional sources of phthalate contamination to milk and dairy products.

There was a significant difference concerning plastic toys and phthalate level in group A (p = 0.004) and significant difference in uses of pacifier and phthalate level in group A (p = 0.039). The study which was done by (Barušić et al., 2015) showed that there was a high of exposure to phthalates in plastic toys bought in 2012 and 20213 this was before legalizations of EU concerning phthalate levels in products.

In the current study, there was a significant difference between studied groups (A&B) regarding using cosmetics containing phthalate (p = 0.019). This agreed with (Romero-Franco et al., 2011) who found association between some phthalates and learning difficulties as well as attention deficit disorders especially more in females than in males most probably due to higher use of personal care products; as perfumes, deodorants and lotins by females. But it was not in accordance with (Duty et al. 2005; Just et al. 2010) who believed that DBP exposures were not readily explained by personal care products use.

There was no significant difference concerning ceramic, painted wall, building and phthalate level in group A.

These results were approved by (Bornhag et al., 2005) who found no association with urinary metabolite levels of phthalates & housing characteristics or building materials & they returned this to the small sample size.

In contrast to the previous results (Peia et al., 2013) found higher phthalates in indoor air in newly decorated apartments. Also (Orecchio et al., 2014) reported that PVC floor materials, paper wall and wall paintings as sources of PAEs that damage the hormonal system. Also, because PAEs are not chemically, but only physically bound to the building materials, they, may be leached.

Regarding phthalate level, results of present study showed that the mean level was (15.539±8.316) for ADHD cases (group A) and (8.085 ±2.426) for controls (group B) with significant difference between groups A&B (p =0.046) and showed no significant difference concerning different types of ADHD and phthalate level in group A (p =0.836).

These results agreed with (kim et al., 2009) who reported that there was a significant correlation between level of phthalate metabolite concentrations and the poor attention. Also (Park et al., 2014) found that high urinary phthalate metabolite concentrations may have a role in neurotransmitter or neurodevelopmental system changes leading to increased risk of ADHD.

Also, the study conducted by (chopra, 2014) in the USA showed there was an association between phthalate exposure and developmental disorders of childhood such as ADD AND LD.

CONCLUSION & RECOMMENDATIONS
From the current study, it was concluded that:

- There was statistically significant correlation regarding childhood...
developmental disorders, specifically ADHD and learning disabilities and levels of Mono Butyl Phthalate.

Also, significant correlation with processing milk, plastic containers, toys, cosmetics, wall and floor material containing phthalate.

A significantly higher level of mono butyl phthalate in exposed cases.

Limitations of our current study
It must be taken into consideration that these results are only presented as a preliminary study and carry a risk of statistical error due to the small sample size and only one phthalate metabolite was measured.

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الملخص العربي

ان مرض فرط الحركة و تشتبه الانتباه لدى الأطفال يعد من أكثر الأمراض العصبية شيوعًا لدى الأطفال. وتعد أيضاً مادة الفثالات من المواد المستخدمة في صناعة البلاستيك ما يجعلها موجودة في أواني الطعام والبلاستيك وأيضاً في الألعاب المستخدمة من قبل الأطفال. كما أنها أيضًا موجودة في بعض المستحضرات الطبية مثل الحقن الطبي و أكياس الدم. عند اختبار مادة الفثالات على الفئران أثبتت أنها تؤدي إلى فرط الحركة والإندفاع.

في هذا البحث قمنا بتحليل مادة الفثالات في البول لدى الأطفال الذين يعانون من مرض فرط الحركة و تشتبه الانتباه و صعوبات التعلم في سن ما بين 6 إلى 12 عام. عينات البول المجمعه من الأطفال المصابين بمرض فرط الحركة و تشتبه الانتباه المتدرين على مستشفى أبو الريش، تم اختبارها لمعرفة نسبة مادة الفثالات فيها.

و قد تبين في هذا البحث أن هناك زيادة في نسبة الفثالات في البول للفئران المصابين بمرض فرط الحركة، و تشتبه الانتباه وذلك مقارنة بأقرانهم الذين لا يعانون بنفس المرض، مما يعني وجود صلة بين زيادة مادة الفثالات في البول و زيادة الاصابة بذلك المرض.