Comparing Levels of Urinary Phthalate Metabolites in Egyptian Children with Autism Spectrum Disorders and Healthy Control Children: Referring to Sources of Phthalate Exposure

Manal Shehata, Ebtissam Salah, Mai M. Youssef, Mones Mahmoud Abu Shady, Inas El-Alameey, Engy Ashaat, Amr S. Gouda, Walaa Nazim

Introduction

Autism spectrum disorder (ASD) is a complex group of neurodevelopmental disorders affecting young children and identified by persistent deficits in social communication and interaction across diverse situations, accompanied with restricted and repetitive behaviors [1].

Autism prevalence has shown a progressive increase over the past 30 years. In 2018, the CDC determined that approximately 1 in 59 children has the diagnosis of autism [2]. There is no prevalence data for Autism in Egypt [3] except for forthcoming unpublished data (Personal communication) which coincide with data from Asia, Europe, and North America reported an average prevalence between 1% and 2% [2].

The most widely accepted hypothesis that may explain the rapid increase in ASD prevalence is the combination between genetic susceptibility and environmental factors [4]. While numerous studies have been conducted to investigate the effect of genetic, environmental, and immunological aspects on the etiology of ASD, there is still much to be done to realize the exact etiology [5], [6], [7].

Exposure to environmental chemicals during the early stages of brain development has been referred to as a possible etiological factor for neurodevelopmental disorders [8], [9]. Currently, considerable concern has developed regarding a group of chemical compounds (called endocrine-disrupting agents), namely, phthalates. Phthalates with a diester component are additive polymers used as plasticizers to synthesize high volumes of chemical products widely used in daily

Abstract

BACKGROUND: Evidence supporting environmental risk factors of autism spectrum disorder (ASD) is rising. Phthalates are assumed to contribute to this risk due to their extensive use in daily life as plasticizers and additives in numerous customer products. Phthalates are also accused as a neurotoxic agent affecting brain development.

AIM: The main objective of this study is to compare the concentrations of urinary phthalate metabolites as biomarkers of phthalate exposure in children with autism to that of a healthy control group and to compare their exposure to suspected environmental sources of phthalate.

METHODS: It was a case-control study; conducted over a period of 1 year. Thirty-eight children with ASD and 99 apparently healthy children comprised the control group, were enrolled in the study. Urinary concentrations of four phthalate metabolites were measured, using a combination of solid-phase extraction, high-pressure liquid chromatography, and tandem mass spectrometry.

RESULTS: Children with ASD comprised 38 children (32 boys and 6 girls), their mean age was 8.95 ± 4.17 years. There were significant higher levels of urinary Mono (2-ethylhexyl) phthalate, mono benzyl, and mono butyl phthalates in cases versus. controls with p value equals (0.006, 0.017, and <0.001), respectively. Regression analysis revealed that male gender and the level of mono butyl are the main predictors of ASD (p < 0.001).

CONCLUSION: This study suggested a link between phthalates and ASD with higher urinary levels of phthalate metabolites in children with ASD. These high levels are either due to increased exposure or defective metabolism in children with ASD. The study declined any relationship of the studied sources of phthalate exposure to ASD except the exposure to wall painting with plastic.

Keywords:
Environmental risk factors; Phthalate exposure


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life [10]. Human exposure to these chemical compounds occurs essentially through food and drink. Otherwise, dermal, and indoor air exposures are major sources as well [11], [12]. The main commercial products of phthalate include indoor residential environments such as polyvinyl chloride flooring and plastics, vinyl tiles, and children’s toys [13]. The most plentiful phthalates in food and surroundings is di-(2-ethylhexyl)-phthalate and its major metabolite is mono-(2-ethylhexyl)-phthalate (MEHP) [11], [12]. Urinary concentrations of phthalate metabolites are considered substantially adequate biomarkers of exposure [14]. Meanwhile, it is worth noting that metabolites of some phthalate compounds were found in several human specimens [15], [16], [17].

Human exposure to phthalate through several routes was proved in literature with the referral to a higher probability of exposure in children than adults, due to their lower body weight, mouthing behavior, and exposure to toys and tinned food containing phthalate [18]. Nevertheless, the association between this exposure and adverse health effects needs further investigations [19].

It was hypothesized that the anti-androgenic activity of phthalate might interfere with hormone-sensitive intervals of neuronal development [20]. Moreover, a previous study detected an association between prenatal phthalate exposure and disturbed mental and motor development in early childhood, indicating an evidence of adverse effects of these compounds on the brain [21]. Few studies were conducted to investigate the relation between phthalate exposure and autism, but no sufficient data are present to support this association [22]. Therefore, the objectives of this study were: First, to compare the concentrations of urinary phthalate metabolites as biomarkers of phthalate exposure in children with autism to that of a healthy control group. Second, to compare exposure to suspected environmental sources of phthalate in children with ASD and healthy control. If the results of this study agree with the hypothesis, it will help in demonstrating the causes behind the unusual rise in the cases of ASD owing to the ubiquitous use of phthalate-containing products.

Methods

It was a case-control study; conducted over a period of 1 year starting from October 2018 till October 2019. This study was a part of a local project was held in the National Research Center (NRC) under the title of Evaluation of the potential effects of phthalate on children health in Egypt. Its number was 11010147.

In the current study, subjects with ASD were recruited from the Clinical Genetics Clinic, Medical Research Center of Excellence, National Research Centre, Cairo, Egypt. Children matching the diagnostic criteria of ASD within an age ranged from 6 to 16 years were included. Exclusion criteria were the presence of signs suggestive of syndromic involvement, microcephaly, neuromuscular disorders, and motor handicapped children. The included children with ASD were 38. Ninety-nine healthy children, age and sex-matched with cases, acted as a control group, were randomly selected from the school health records of primary, and preparatory schools nearby the NRC in Giza. They were included if they were free of any genetic, neurodevelopmental, or chronic medical disorder and if their parents accepted to participate in this project.

Sample size

Group sample sizes of 38 ASD patients and 96 control achieve 80% power to detect a difference of –0.6 between the null hypothesis that both groups adjusted means of urinary metabolite (MEHP) are 6.8 and the alternative hypothesis that the mean of ASD group is 7.4 with estimated group standard deviations of 1.1 and 1.2 as reported in previous literature [23], and with a significance level (alpha) of 0.05000 using a two-sided two-sample t-test [24], [25].

Ethical approval of The Research and Ethical Committee of the NRC was obtained. A written consent was obtained from the mothers or caregivers of the children.

Each child enrolled in the study was subjected to the following:

Structured Questionnaire to collect data about:
- History of the child’s medical and developmental trajectory
- Socio-demographic variables including parental education and occupation
- Sources of exposure to phthalate:
  - Assessment of housing characteristics in the form of yes/no questions about plastic/vinyl flooring and plastic/oil painting of walls. The age of residential building and housing type, that was divided into four groups: 10 years or less, 11–20 years, 21–30 years, and over 30 years. Exposure to phthalates is proved to be augmented by aging of phthalate-containing materials [26].
  - Assessment of certain hazardous habits: included the storage of food and water in plastic boxes or jars, the usage of plastic microwave utensils, the reuse of plastic water bottles, and drinking water from plastic tanks.
- Exposure of the child to passive smoking
- Thorough clinical examination: which included meticulous general and specific systemic examination, and assessment of the control subjects to exclude any pathological symptoms or signs
- Diagnosis of autism was established according to the criteria of Diagnostic and Statistical Manual of mental disorders [1]
• Assessment of severity of autistic symptoms using childhood autism rating scale (CARS) [27]. The CARS is a 15-item behavioral rating scale developed to identify autism as well as to quantitatively describe the severity of the disorder. Accordingly, patients with ASDs were classified into three groups.

1. Children with mild symptoms: They had a score ranging from 30 to 33
2. Children with moderate symptoms: They had a score ranging from 33 to 37
3. Children with severe symptoms: They had a score equal to or above 37

**Laboratory investigations**

Determination of urinary phthalate metabolites

Exposure to phthalates in nearly all presented studies was dependent on definite biomarkers of exposure mostly in urine [28], [29]. As phthalates are metabolized to their monoesters within a few hours or days urinary phthalate monoesters are considered good biomarkers for assessing phthalate exposure in human [30]. A single spot urine sample is sufficiently representative of exposure over a 6-month period to warrant its use as an exposure estimate in epidemiological studies [31]. Phthalate metabolites were measured instead of their parent compounds to lower the potential for exposure misclassification. A combination of solid-phase extraction, high-pressure liquid chromatography, and tandem mass spectrometry were used to measure phthalate metabolite levels using methods described by Koch et al. elsewhere [32]. After a urine sample was thawed and sonicated for 10–15 min, the urine sample (100 μl) was loaded into a glass vial (2 ml) which contained ammonium acetate (AA, 20 μl, >98%, Sigma Aldrich Lab., Inc., St. Louis, MO, USA), β-glucuronidase (10 μl, Escherichia coli K12, Roche Biomedical, Mannheim, Germany), and a mixture of ten isotopic 13C4 phthalate metabolite standards (100 μl. Cambridge Isotope Lab., Inc, Andover, MA, USA). After the sample was incubated (37°C, 90 min), a 270 μl solution (5% ACN), Merck, Darmstadt, Germany) with 0.1% formic acid (FA, Merck, Darmstadt, Germany) was added and sealed with the PTEF cap for analysis.

We used two columns in our online system. One C18 column (Inertsil ODS-3, 33 × 4.6 mm, 5 μm, GL Science, Tokyo, Japan) was used to extract and clean our sample, and an analytical column (Inertsil Ph, 150 4.6 mm, 5 μm, GL Science, Tokyo, Japan) used to separate different phthalate metabolites. The gradient program of the clean-up column was listed as follows: 100% solution A (5% ACN + 0.1% FA) (0–7 min), 100% solution B (90% ACN + 0.1% FA) (7–9 min), 100% solution A (9–10 min) and continued to 12 min. The flow rate was set at 1000 μl/min. The analytical column gradient program was listed as follows: 100% solution C (50% ACN + 10 mmole AA) (0–3.6 min), 100% solution D (95% ACN + 10 mmole AA) (3.6–8.6 min), 100% solution C (8.6–9 min) and continued to 12 min. We used a negative multiple reaction monitoring models for mass spectroscopy detection. One blank, repeat, and quality control (QC) sample included in each batch of analyzed samples. The concentration of blank samples should be below 2 times the detection limit. The QC sample was spiked in a pooled urine sample with a mixture of phthalate metabolite standards (20–50 ng/ml) in each sample. We used the value (micrograms per liter)/creatinine (grams per liter) for dilution correction in the analyses.

**Statistical analysis**

The data were collected and analyzed on personal computer using the Statistical Package for the Social Science (SPSS) version number 22. Description of quantitative (numerical) variables was in the form of mean ± standard deviation. Qualitative (nominal) variables were in the form of number and percentage. Chi-square and the independent sample t-test were used to study the association of the studied variables and to analyze differences between the cases and control groups. Multiple logistic regression analysis was used to assess the main risk factors of autism. p<0.05 were considered statistically significant.

**Results**

Demographic characteristics of the participants are shown in Table 1. The Autism group comprised 38 children (32 boys and six girls), their mean age was 8.95 ± 4.17 years. They were classified according to CARS score to children with mild symptoms (56.3%) and children with moderate to severe symptoms (43.8%). The control group comprised 99 apparently healthy children (79 boys and 20 girls), and mean age (9.45 ± 2.91 years).

**Table 1: Demographic characteristics of the studied subjects**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Autism (n = 38)</th>
<th>Control (n = 99)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (mean ± SD)</td>
<td>8.95 ± 4.17</td>
<td>9.45 ± 2.91</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>32 (84.2%)</td>
<td>79 (79.8%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Female</td>
<td>6 (15.8%)</td>
<td>20 (20.2%)</td>
<td></td>
</tr>
<tr>
<td>CARS category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild ASD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate and severe ASD</td>
<td>21 (50.3%)</td>
<td>17 (44.7%)</td>
<td></td>
</tr>
</tbody>
</table>

*p = 0.05 is significant, ASD: Autism spectrum disorder, CARS: Childhood autism rating scale, SD: Standard deviation.

Table 2 shows the comparison between the mean urinary concentrations of phthalate metabolites of patients with autism vs. controls, there were significant higher levels of MEHP, monobenzyl, and monobutyl in cases versus controls with p value equals (0.008, 0.017 and <0.001), respectively.

The correlation analysis revealed insignificant relations between score of CARS and...
Table 2: Comparison of urinary concentrations of phthalate metabolites in children with ASD versus control group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Autism (n = 38)</th>
<th>Control (n = 99)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEHP (ng/ml)</td>
<td>69.04 ± 6.9</td>
<td>59.92 ± 20.65</td>
<td>0.008*</td>
</tr>
<tr>
<td>Monomethyl (ng/ml)</td>
<td>566.05 ± 74.60</td>
<td>526.23 ± 224.15</td>
<td>0.196</td>
</tr>
<tr>
<td>Monobenzyl (ng/ml)</td>
<td>1.85 ± 0.76</td>
<td>1.60 ± 0.83</td>
<td>0.017*</td>
</tr>
<tr>
<td>Monobutyl (ng/ml)</td>
<td>38.13 ± 8.15</td>
<td>30.39 ± 6.32</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*p < 0.05 is significant, SD: Standard deviation. MEHP: Mono (2-ethylhexyl) phthalate, ASD: Autism spectrum disorder.

Some risk factors that are hypothesized to increase exposure to phthalate have been studied and compared in the two groups. These factors included duration of house building, type of house floor, type of wall painting, reuse of water tanks, using plastic utensils in microwave, using plastic boxes for food storage, and exposure to passive smoking. Results are presented in (Tables 5 and 6).

Table 3: Correlation between CARS score and studied phthalates urinary concentrations

<table>
<thead>
<tr>
<th>Variables</th>
<th>CARS score</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEHP</td>
<td>0.149</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Monomethyl</td>
<td>0.153</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Monobenzyl</td>
<td>0.195</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Monobutyl</td>
<td>0.109</td>
<td>0.54</td>
<td></td>
</tr>
</tbody>
</table>

CARS: Childhood autism rating scale, MEHP: Mono (2-ethylhexyl) phthalate.

Regression analysis in (Table 4) revealed that autism was significantly associated with younger ages, with male gender, and with a high level of monobutyl (p < 0.005).

Table 4: Predictors of ASD in the studied children - multivariate analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>B</th>
<th>SE</th>
<th>Wald df</th>
<th>Sig</th>
<th>OR Lower</th>
<th>OR Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 21–30</td>
<td>0.377</td>
<td>0.105</td>
<td>12.846</td>
<td>0.001*</td>
<td>0.688</td>
<td>0.558</td>
</tr>
<tr>
<td>Sex Male</td>
<td>3.000</td>
<td>0.766</td>
<td>15.343</td>
<td>0.000*</td>
<td>0.050</td>
<td>0.011</td>
</tr>
<tr>
<td>MEHP 0.040</td>
<td>0.024</td>
<td>2.793</td>
<td>0.095</td>
<td>1.041</td>
<td>0.993</td>
<td>1.091</td>
</tr>
<tr>
<td>Monomethyl -0.001</td>
<td>0.002</td>
<td>0.328</td>
<td>0.567</td>
<td>0.999</td>
<td>0.999</td>
<td>1.003</td>
</tr>
<tr>
<td>Monobenzyl -0.062</td>
<td>0.036</td>
<td>0.032</td>
<td>0.857</td>
<td>0.396</td>
<td>0.457</td>
<td>1.919</td>
</tr>
<tr>
<td>Monobutyl 0.121</td>
<td>0.044</td>
<td>1.110</td>
<td>0.001</td>
<td>1.158</td>
<td>1.062</td>
<td>1.263</td>
</tr>
</tbody>
</table>

*p < 0.05 is significant, ASD: Autism spectrum disorder, MEHP: Mono (2-ethylhexyl) phthalate, df: Degree of freedom, CI: Confidence interval, OR: Odds ratio, SE: Standard error.

Discussion

In this study, the authors tried to explore the association between phthalate as a ubiquitous multifunctional plasticizer and ASD in children. The secondary metabolites of phthalates measured in urine have been used as biomarkers of exposure. Urinary levels of MEHP (mono- (2-ethylhexenyl) phthalate), Monobutyl phthalate, Monomethyl phthalate, and Monobenzyl phthalate were assessed.

To the limit of our knowledge, this is the first Egyptian study investigating phthalates exposure in children with ASD.

Data analysis of the current study demonstrated an association between phthalates and ASD. There were statistically significant higher levels of MEHP, Monobutyl, and mono benzyl phthalates in urinary samples of the ASD group when compared to healthy controls and P value equals (0.006, <0.001, and 0.017), respectively. In addition, regression analysis revealed that urinary metabolites as Monobutyl, followed by MEHP are the main predictors of ASD in the studied sample. This is in the agreement with Testa et al. [33], who recorded that the urinary concentrations of oxidized MEHP metabolites were significantly higher in autistic children vs healthy control. In another study, Stein et al. [23], showed that free MEHP levels were significantly higher in autistic group than healthy controls.

According to scores of CARS, more than half of the studied sample had mild autism (56.3%) and the rest of children had moderate-to-severe autism (43.8%). It seems that the level of phthalate did not play a role in the severity of symptoms. No significant correlation could be detected between total CARS

No significant difference could be detected between autism and control group as regard exposure to passive smoking or using plastic utensils (Table 5).
scores and urinary excretion of the different metabolites of phthalates (Monobenzyl, MEHP, Monobutyl, and Monomethyl levels).

This is in distinction to Testa et al., [33] who reported a positive correlation between CARS scores and urinary MEHP levels \( r = 0.429, p = 0.003 \), whereas no significant relationships with the levels of the other examined metabolites were found.

In our study, a questionnaire was applied to the parents of the control and autistic children to better understand the possible environmental exposure routes to phthalates. These factors included duration of house building, type of house floor, type of wall painting, reuse of water tanks, using plastic utensils in microwave, using plastic boxes for food storage, and exposure to passive smoking. There was highly significant association between painting the wall with plastic in children with ASD versus controls \( p = 0.006 \). It was reported that phthalate compounds present in various consumer products, including, vinyl flooring and wall coverings, can easily leach or vaporize out into surrounding environment under minor environmental changes [e.g. temperature and irradiation (ultraviolet, sunlight)] [34]. This means a higher risk of human exposure via several routes as ingestion, inhalation, and dermal contact [35].

Exposure to passive smoking is linked with adverse birth outcomes, such as fetal growth restriction and low birth weight, which are in turn related to higher risk of ASD [36], [37]. Nicotine’s adverse effects are assumed to develop through its action at nicotinic acetylcholine receptors, which mediate neural structural alterations that, in turn, can have substantial consequences for the offspring in later life [38]. In the current study, no significant difference could be detected between autism and control group as regard exposure to passive smoking or plastic utensils.

Based upon the above-mentioned findings, no considerable association was noticed between ASD and the studied sources of environmental exposure to phthalate, in spite of, the presence of possible relationship between exposure to phthalate and the development of ASD. These results generate the assumption that either current exposure is higher in children with ASD or, more likely children with ASD differ in their ability to metabolize phthalate.

Hence, it is worth mentioning that previous literature suggested the presence of defective detoxification of environmental pollutants for children with ASD. For example, a study by Stein et al. [23], evidenced a compromised detoxification pathway of phthalate metabolism in children with ASD. Nevertheless, the study stated that the association between compromised detoxification and ASD may possibly be to any compound metabolized by the same pathway, so the direct link between phthalate and ASD cannot be inferred.

On the other side, it was hypothesized that high phthalate metabolites urinary excretion does not correspond to the environmental exposure, but rather phthalate metabolites could have an endocrine-disrupting property [33]. It was proved that environmental chemical compounds could impact mothers’ thyroid function or decrease the production of androgen hormones during gestation, both of which are essential for babies’ brain development [39]. Phthalates could also reduce testosterone in baby boys, which could also elucidate the difference, the researchers observed, between the sexes [13]. This is in agreement with Lai et al. [40], and Schaafsma and Pfaff [41], who reported that a male gender is one of the strongest predictors of being diagnosed with autism, especially higher functioning autism.

Other mechanisms by which phthalate can produce developmental disorders were assessed previously to demonstrate the association between phthalate exposure and variable neurodevelopmental outcomes, such as; ASD, attention-deficit hyperactivity disorder, affected cognition, and retarded psychomotor development [42]. This association is supported by animal studies that indicated the role of phthalate-induced hypothyroidism in reduced intellectual abilities and development of ASD [10].

In a recent study, phthalates have been associated with variations in gene expression and DNA function, which is thought to be another mechanism for the chemical compounds to affect children’s brain [43]. Moreover, oxidative damage was evidenced to play a critical role in the mode of action by which phthalate can induce neurotoxicity [44].

In addition, it was reported that phthalates can cross the placental barriers, affecting the developing brain. Higher gestational concentrations of some phthalate metabolites were associated with higher scores of autistic traits in 3–4 years old children [13]. Accordingly, prenatal plus postnatal phthalate exposures were assumed to have accumulative actions affecting brain development, so, a prenatal/postnatal screening for phthalate metabolites in populations at higher risk for ASD could have a vital influence [33].

**Study limitations**

In contrast to cohort studies, a causal relationship cannot be proved by cross-sectional studies which is a main limitation of the current study. Limited budget and high cost of analysis hindered estimation of urinary phthalate metabolites in normal siblings of children with ASD which may help to define increased exposure. However, a strength of this study is being the first study in Egypt proved a link between the widely used phthalate and ASD.
Conclusions

This study can suggest a link between phthalates and ASD. Exposure to phthalates might be a triggering factor in the development of ASD in genetically susceptible children. In this study, it is observed that children with ASD had higher concentrations of MEHP, monobenzyl, and monobutyl phthalates in urinary samples compared to controls. The study declined any relationship of the studied sources of phthalate exposure to ASD except the exposure to wall painting with plastic. The higher levels of urinary phthalate metabolites in children with ASD could be due to defective metabolism, endocrine defect, or epigenetic changes, all of which need further longitudinal studies to be proved.

Acknowledgments

The authors would like to express their gratitude to all the participants of this study.

Authors’ Contributions

EMS and MMY conceived and designed the study. MAS, IRE, EAA carried out the assessment on the participants. EMS, MAS, and IRE were major contributors in writing the manuscript. ASG and WSN were responsible for the laboratory work, MMA analyzed and interpreted the patients data. All authors read and approved the final manuscript.

References


PMid:23173732

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