Research Article

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The urinary biopterin in autism spectrum disorder

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Abstract

Objective – The aim of the study was to determine whether biopterin is present in significantly lower quantities in urine samples of patients with autism spectrum disorder (ASD) compared to healthy individuals.

Methods – The concentration of biopterin in urine samples was measured by ELISA using commercially available kit. The study involved 53 children aged 3–16 years with ASD and 60 healthy children aged 2–14 years.

Results – Significantly lower biopterin concentration was observed in autistic patients compared to the control group. However, no significant difference was observed between mild, moderate, and severe ASD.

Conclusion – One of the potential causes of decrease in urinary biopterin levels may be tetrahydrobiopterin (BH₄) deficiency, which has extensive and serious health consequences for the nervous system. The results of measuring biopterin as a fully oxidized form of BH₄ may suggest that biosynthesis or regeneration of BH₄ may be decreased in children with ASD. On the other hand, decreased urinary biopterin levels in children with ASD may be due to BH₄ overuse, a good regeneration process, and decreased urinary excretion; and abnormalities in BH₄ metabolism appear to be related to the aetiology of ASD or may be due to ASD.

Keywords: autism, biopterin, tetrahydrobiopterin, pterins, urine

1 Introduction

Neurodevelopmental disorders, including the most frequent autism spectrum disorder (ASD), affect approximately 1–2% of the world's population of children [1]. According to the Centers for Disease Control and Prevention, almost 1 in 60 children in the USA is being identified with ASD [1]. Thus, ASD is not a rare phenomenon. However, after 75 years since Leo Kanner (1943) described ASD as a set of developmental disabilities in communication and social interactions with repetitive behaviours, the aetiology of it still remains unknown [2]. Current research suggests that autism is a multicausal disorder, i.e. it means there might be no single reason for its appearance. Recently, autism is considered as a complex and heterogeneous disorder with genetic basis with the possibility of heritability, which might be supported by environmental factors influencing phenotypic expression [3]. It is widely believed that environmental factors (e.g. neurotoxins, infections and maternal infections) in the presence of host genetic susceptibility and immunogenetic background affect the initiation of some current CNS abnormalities and the activation of inflammatory processes in the nervous system. In addition, there is some evidence that ASD also includes physiological system anomalies that go beyond specific organ dysfunction. Abnormalities in the immune system, methylation and transsulfuration cycle disturbances, mitochondrial dysfunction and increased risk of oxidative stress have been implicated in the pathophysiology of autism [4–6].

In the biological system, pterins mostly occur as unstable reduced forms, where around 80–90% of that is tetrahydrobiopterin (BH₄) with the highest biological activity, which is confirmed by its appearance in many metabolic pathways. Under conditions of homeostasis, BH₄ may act in its physiological role as a cofactor for nitric oxide synthase (NOS enzyme). Nitric oxide as a product of this reaction is involved in immunological mechanisms, neurotransmission, or regulation of blood vessel tone. Unfortunately, BH₄ is very unstable and

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oxidizes easily, especially under oxidative stress. Oxidized form of BH₄ (biopterin and dihydrobiopterin) cannot act as a cofactor in this reaction and stabilize the coupling of molecular oxygen with arginine. Oxidation of BH₄ by free radicals leads to an excess of unconjugated NOS, which generates peroxide anion and peroxynitrite, contributing to increased oxidative stress. Thus, the reduction in bioavailability of BH₄ may be due to overactivation of the immune system and inflammatory processes during excessive production of nitric oxide [7–10]. BH₄ in the human body also plays an essential role as a cofactor for the production of monoamine neurotransmitters such as dopamine or serotonin, and transformation of phenylalanine into tyrosine [7]. BH₄ takes part as a cofactor in many processes requiring enzymatic activity and occurs probably in every cell, tissue, or fluid in the human body [9,11], but its predominant sites of synthesis are liver and dopaminergic and serotonergic synaptosomes of the central nervous system [8]. The most critical metabolic pathways of BH₄ include the production of monoamine neurotransmitters such as dopamine, norepinephrine, or serotonin; conversion of phenylalanine to tyrosine; and the production of nitric oxide from L-arginine [7,11–13]. Therefore, any imbalance in BH₄ metabolism can lead to the disruption in the secretion of neurotransmitters and afterwards to serious neurological impairments. This assumption can be supported by the depletion of BH₄ that had been found in some neurodegenerative disorders such as Parkinson's [14,15] and Alzheimer's diseases [15,16]. Low content of biopterin had also been reported in other neurodevelopment disorder like schizophrenia [17]. Moreover, BH₄ deficit had been reported even in autism when samples of cerebrospinal fluid were examined [18,19].

According to the literature, there are not many reports on the determination of biopterin in the urine of children diagnosed with autism. On the other hand, papers that are related to this topic do not give a definite answer about the correlation with the occurrence of autism in children.

Eto et al. [8] reported that the concentrations of biopterin did not differ statistically between the ASD and control groups. However, biopterin concentrations were lower in the tested group than in the control group. The biopterin concentration was determined in urine and plasma samples for 16 subjects from ASD group (aged 6–18; 11 male and 5 female) and 11 or 12 patients from the control group (aged 7–14; 4 male and 8 female). From this experiment, the results indicate a lack of dependence between urinary and plasma biopterin concentration and children's incidence of autism [8]. Harrison and Pheasant [20] obtained a similar result by measuring

total biopterin concentration in urine. Thus, no significant difference was observed in biopterin concentration between both the groups; but in this case, the tendency was opposite; biopterin concentrations were higher in the tested group (aged 3–21 years; 2 female and 15 male) than in the control group (age range 3.5–14.5 years; 4 female and 13 male). The authors obtained significant difference in biopterin concentration by determining the native and oxidizable forms of biopterin. The native biopterin was higher in autistic children ($p < 0.05$). However, the oxidizable form was much lower in autistic children ($p < 0.005$). They pointed out this shift in redox form of biopterin could occur *in vivo* and that would have been caused by the disruption in BH₄ metabolism or it could be a result of sample collecting and delivering conditions [20]. Messahel et al. [9] measured native biopterin concentration in urine within three groups of children: with diagnosed ASD (aged 3–5 years; 12 male and 2 female), their healthy siblings (aged 1–19 years; 10 male and 11 female), and other healthy children (aged 3–5 years; 8 male and 8 female). Their results show a significant difference between all of these groups, where the highest concentration of biopterin was determined in the ASD group and the lowest in the control group. This is explained by the increase in biosynthesis of BH₄ under the influence of interferon γ , resulting in an increased loss of biopterin with urine. They claimed that increased loss of fully oxidated biopterin is a result of lower activity of the dihydropteridine reductase because of the lack of differences between concentration of fully oxidated biopterin and total biopterin. Thus, according to the authors, it suggests a lower concentration of reduced biopterins (dihydrobiopterin and BH₄) in children with infantile autism, which might explain neurological deficit [9].

Given these facts, the aim of our study was to evaluate the concentrations of fully oxidized metabolite biopterin in the urine of children with ASD and to compare it with healthy children.

2 Material and method

In order to include children from the Silesian agglomeration in Poland to the research, they were diagnosed by a psychiatrist from the Psychiatry Department of the Medical University of Silesia in Katowice. Eventually, 53 selected children with ASD according to ICD-10 (10th revision of the International Statistical Classification of Diseases and Related Health Problems) recommended by the WHO were enrolled to obtain a homogeneous group with the infantile autism (F.84.0 – 90.5%); 3.8%

of subjects was with the Asperger's syndrome (F.84.5) and 5.7% with the atypical autism (F.84.1). The subjects were also evaluated using the Childhood Autism Rating Scale (CARS); the mean and the median were equal to 32, indicating mild to moderate autism. The subjects were divided into three groups according to the severity of autism: mild (28%), moderate (53%), and severe (19%). The median age in the study group (48 males and 5 females) was 8 years (range: 3–15 years). Children live in Silesia in the south of Poland, which is a highly industrialized area with a high degree of environmental pollution, especially air pollution. As many as 81.5% of children with ASD lives in large cities with more than 100,000 inhabitants, and the rest – 18.5% – lives in towns and villages.

This group included individuals with comorbidities such as asthma (13%), hyperactivity (11.1%), inhalation and food allergies (7.4%), atopic dermatitis (5.6%), mild mental retardation (5.6%), and hypothyroidism (3.7%). The most frequently taken drug is risperidone (13%); some of the individuals also took haloperidol (5.6%), depakene (3.7%), concerta (3.7%), anafranil (1.9%), perazine (1.9%), pulmicort (5.6%), flixotide (3.7%) and euthyrox (3.7%).

The control group consisted of 60 healthy children (32 male and 28 female) without any history of neuro-developmental disease (the median age 6, range: 2–14 years).

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration and has been approved by the Local Bioethical Committee of the Silesian Medical University.

Informed consent: Every caregiver of patient was informed about the purpose of the study and agreed in writing to participate in this research.

3 Urine collection

Before the biological material was taken, an original questionnaire was performed with parents of autistic children. Based on this, socio-demographic data about children with ASD and their parents were obtained. Morning urine samples (approximately 100 mL) were collected and stored at -80°C until analysis. All samples were protected against UV light.

3.1 Assay of bipterin

The concentration of bipterin in urine samples was measured by ELISA using commercially available kit (Bipterin ELISA kit; Aviva Systems Biology, Beijing, China) according to the manufacturer's detailed manual. The mean precision for intra-assay and inter-assay was below 7.6 and 10.5%, respectively. Sensitivity of the kit is 1.23 nmol/L.

3.2 Measurement of urinary creatinine

The concentration of creatinine in urine samples was measured by Jaffe's kinetic method using a commercially available set (Stamar, Poland), according to the manufacturer's detailed manual. The mean precision for intra-assay and inter-assay was below 5.7 and 2.5%, respectively. Sensitivity of the kit is below 0.168 mg/dL.

3.3 Statistical analysis

The obtained results were presented using the basic parameters of descriptive statistics. Normal distribution of data was measured using Shapiro–Wilk's test. Independent data between two groups of patients with ASD and the controls were compared using non-parametric Mann–Whitney U test. The Spearman's rank test was used to measure correlations. Independent data between three groups of patients with mild, moderate, and severe ASD were compared using non-parametric ANOVA and Kruskal–Wallis's test. Regression analysis was also applied. The $p < 0.05$ was considered statistically significant. Calculations were performed with STATISTICA for Windows 10.0 software (StatSoft, Cracow, Poland).

4 Results

Significantly lower bipterin concentration in autistic patients as compared with the control group was observed (mean $\pm$ SD: 1.60 ± 0.88 vs 2.33 ± 0.95 $\mu\text{mol/mol}$ creatinine; $p < 0.001$) (Figure 1). However, no significant difference ($p > 0.05$) was observed between mild (mean $\pm$ SD: 1.50 ± 0.90 $\mu\text{mol/mol}$ creatinine), moderate (mean $\pm$ SD: 1.58 ± 0.88 $\mu\text{mol/mol}$ creatinine), and severe ASD (mean $\pm$ SD: 1.81 ± 0.89 $\mu\text{mol/mol}$ creatinine). Moreover, bipterin

Figure 1: Urinary bipterin concentration in patients with diagnosed ASD and healthy children. The concentration was significantly lower in children with ASD compared with the control group (median: 1.32 vs 2.13 $\mu\text{mol/mol}$ creatinine; $p < 0.001$).

concentration did not significantly correlate with CARS ($R = 0.17$; $p = 0.24$).

Bipterin concentration significantly correlated with age (Figure 2), weight, and height in both groups (Table 1). But in the control group no significant difference was observed between bipterin concentration depending on gender (mean $\pm$ SD: $2.28 \pm 0.9 \mu\text{mol/mol}$ creatinine for male vs $2.39 \pm 0.98 \mu\text{mol/mol}$ creatinine for female; $p > 0.1$). If only male subjects were taken into account, still no correlation was observed in bipterin concentration between ASD and control group (mean $\pm$ SD: 1.63 ± 0.88 vs $2.28 \pm 0.90 \mu\text{mol/mol}$ creatinine; $p < 0.001$).

Bipterin concentration in urine was positively dependent on strong disability in communication connected with social impairments ($\beta = 0.29$, $p = 0.036$). But no relationship was observed between bipterin concentration and the presence of emotional problems (aggression and hyperactivity) or speech disorders.

5 Discussion

Although we were able to distinguish children with ASD from healthy ones by taking into account the concentration of bipterin in urine as a parameter, particular group of ASD divided according to the severity of the disorder (mild, moderate, and severe ASD) showed lack of variety. However, it can also be observed that the higher mean concentration of bipterin in urine of children with ASD, the more severe ASD. Interestingly though, such tendency can be a result of more complex phenomenon which occur in BH_4 cycle rather than easy dependence between secretion of BH_4 and bipterin excretion in urine. Our results may suggest general reduction of BH_4 synthesis but might also implicate the presence of other factors which disrupts in regeneration and supports poor recycling of BH_4 , ultimately leading to increased loss of BH_4 as a fully oxidated bipterin with urine in severe ASD. This theory is supported by the complexity of BH_4 metabolism cycle in which a lot of enzymes takes part; lower activity of dihydropteridine reductase was suggested by Messahel *et al.* to be one of the reason for increased loss of bipterin with urine [9]. It could mean

Figure 2: Moderate negative correlation between bipterin concentration in urine and age of children from ASD and control group ($R = -0.63$ and $R = -0.44$, respectively; $p < 0.001$).

Table 1: Correlation between biopterin concentration in urine and age, weight, and height in both groups

Group	Age		Weight		Height	
	<i>R</i> Spearman	<i>p</i>	<i>R</i> Spearman	<i>p</i>	<i>R</i> Spearman	<i>p</i>
ASD	−0.6278	0.000000	−0.6641	0.000002	−0.6308	0.000008
Control	−0.4382	0.000462	−0.4665	0.000172	−0.4905	0.000069

that children with mild and moderate ASD are characterized by better functioning regeneration system of BH₄; and they reuse higher percentage of BH₄ than in the severe ASD despite the general smaller synthesis of BH₄.

We have also shown that sex has no statistical significance in this research. Despite the fact that mainly boys suffer from ASD, the control sample may contain more girls, without affecting the biopterin concentration in the entire study group, which facilitates the stage for collecting samples. Such a phenomenon may result from the lack or slight effect of the gonadal endocrine system in such young people on BH₄ metabolism cycle. Instead, there is a clear negative correlation between the biopterin concentration and age, weight, and height of children. These three parameters unanimously confirm that the highest concentration of biopterin is in the early years of life when the size of the body is not large. This means that BH₄ synthesis decreases over the years or its regeneration is more efficient.

In addition, we also showed that the concentration of biopterin in urine correlated with typical manifestations characteristic of autism and the problems of communication and social impairments. These results seem to be consistent with BH₄-deficit hypothesis and its function, which is necessary for the synthesis of several neurotransmitters. Disturbances in their syntheses are responsible for neurocognitive problems, developmental, and neuropsychiatric symptoms [21].

Wide range of action of catecholamines and phenylalanine in the CNS, which coincide with typical symptoms of autism, suggests that this may be the result of a cofactor deficiency (BH₄) needed for the action of three enzymes from the group of hydroxylases (phenylalanine-4-hydroxylase, tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase) [7]. Disturbance in BH₄ production is responsible for the impaired conversion of phenylalanine to tyrosine and, at the same time, the reduced availability of tyrosine affects the transport of amino acid to the brain and its further conversion to its metabolites like L-DOPA, dopamine, epinephrine, and noradrenaline. Hence, the elevated phenylalanine to tyrosine concentration ratio in blood can be a good indicator for impaired BH₄ availability [22]. The combination of reduced BH₄ with neurotransmitter

dysfunction and typical symptoms of autism seems to be a complement theory of dependence existing in the whole spectrum of autistic disorders or affecting its genesis.

6 Conclusion

In summary, we reported a significant difference in urinary biopterin concentrations between the tested ASD and control groups. It is thought that this may be due to a deficiency of BH₄, which has extensive and serious health consequences for the nervous system. However, the relationship between urinary biopterin concentration and the severity of ASD was inversely expected, which may suggest more complicated disorders throughout the entire metabolism cycle of BH₄ changes. This issue undoubtedly requires closer examination to be able to clearly determine why biopterin levels are lower in these children and whether this affects ASD development.

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Data availability statement: The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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