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Abnormal transsulfuration metabolism and reduced antioxidant capacity in Chinese children with autism spectrum disorders

Yu Han, Qian-qian Xi, Wei Dai, Shu-han Yang, Lei Gao, Yuan-yuan Su, Xin Zhang*

School of Public Health, Tianjin Medical University, Tianjin, China

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ABSTRACT

Autism spectrum disorder (ASD) is a neurological disorder that presents a spectrum of qualitative impairments in social interaction, communication, as well as restricted and stereotyped behavioral patterns, interests, and activities. Several studies have suggested that the etiology of ASD can be partly explained by oxidative stress. However, the implications of abnormal transsulfuration metabolism and oxidative stress, and their relation with ASD are still unclear. The purpose of this study was to evaluate several transsulfuration pathway metabolites in Chinese participants diagnosed with ASD, to better understand their role in the etiology of this disorder. Fifty children (39 male, 11 female) diagnosed with ASD and 50 age- and gender-matched non-ASD children (i.e., control group) were included in this study. This prospective blinded study was undertaken to assess transsulfuration and oxidative metabolites, including levels of homocysteine (Hcy), cysteine (Cys), total glutathione (tGSH), reduced glutathione (GSH), oxidized glutathione (GSSG), and glutathione ratio (GSH/GSSG). The clinical severity of ASD was evaluated with the Childhood Autism Rating Scale (CARS), and the autistic children's present behavior was measured by the Autism Behavior Checklist (ABC). The results indicated that Hcy and GSSG levels were significantly higher in children diagnosed with ASD, Cys, tGSH and GSH levels as well as the GSH/GSSG ratio showed remarkably lower values in ASD children compared to control subjects. Hcy levels correlated significantly with increasing CARS scores and GSSG levels in children with ASD. Our results suggest that an abnormal transsulfuration metabolism and reduced antioxidant capacity (i.e., hyperhomocysteinemia and increased oxidative stress), and Hcy level appears to have a potentially negative impact on clinical severity of autistic disorder.

1. Introduction

Autism spectrum disorder (ASD) is a neurological disorder with a spectrum of qualitative impairments in social interaction, communication, as well as restricted and stereotyped behavioral patterns, interests, and activities (American Psychiatric Association, 2013). ASD affects males much more than females (i.e., 4–5 males per 1 female), and usually manifests in children before the age of three (Fombonne, 2005). Over the past two decades, the number of individuals diagnosed with ASD has increased dramatically (Boyle et al., 2011). A recent meta-analysis showed that between 2010 and 2011, the Chinese population presented the highest pooled prevalence (16.4 per 10,000) of ASD (Sun et al., 2013). However, the etiology of ASD remains unknown despite numerous efforts to elucidate the causes of this disorder.

It is becoming clear that the etiology of ASD involves complex interactions between genetic predisposition and environmental exposures or triggers (Gentile et al., 2013). A recent study of dizygotic twins estimated that the environment contributes a greater percentage of the risk of developing autistic disorder compared with genetic factors (Hallmayer et al., 2011). Preconception, gestational and early childhood exposure to environmental toxicants has been suggested to increase the risk of autism (Rossignol et al., 2014).

Recent evidence suggests that a broad range of children with ASD have impairments in glutathione (GSH) redox metabolism, and suffer from chronic oxidative stress (James et al., 2006; Rose et al.,

Abbreviations: ASD, autism spectrum disorders; Hcy, homocysteine; Cys, cysteine; tGSH, total glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH/GSSG, glutathione ratio; CARS, Childhood Autism Rating Scale; ABC, Autism Behavior Checklist; CBS, cystathionine β-synthase; SD, standard deviation; IQR, interquartile range; MTHFR, methylenetetrahydrofolate reductase; RFC, reduced folate carrier; BHMT, betaine-homocysteine methyltransferase; NAC, *N*-acetylcysteine.

* Corresponding author at: School of Public Health, Tianjin Medical University, 22# Qixiangtai Road, Tianjin 300070, China.
E-mail address: zhangxin@tijmu.edu.cn (X. Zhang).

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2012). It is suggested that oxidative stress may play a central role in the pathogenesis of ASD as a result of the cumulative influence of toxic environmental insults, which can promote neuronal damage in genetically predisposed individuals (Frustaci et al., 2012). Abormal transsulfuration metabolism will indirectly impact GSH synthesis, leading to oxidative stress.

The transsulfuration pathway begins with homocysteine (Hcy), which can be either be remethylated to methionine, or irreversibly removed from the methionine cycle by the cystathionine β-synthase (CBS). This one-step reaction permanently removes Hcy from the methionine cycle, and initiates the transsulfuration pathway for the synthesis of cysteine (Cys), glutathione, sulfate, and taurine (Finkelstein, 1998). Hcy is an excitatory amino acid that markedly enhances the vulnerability of neuronal cells to excitotoxicity and oxidative injury (Kruman et al., 2000), because protein-related Hcy metabolism produces Hcy-thiolactone, *N*-Hcy-protein, and *N* epsilon-Hcy-Lys, which can cause protein damage and structural changes. These structural changes generate proteins that are toxic and induce an autoimmune response (Jakubowski and Glowacki, 2011). Cys is the limiting amino acid for glutathione synthesis because it has a low concentration compared to glycine and glutamate (Reed et al., 2008). Glutathione is the principal non-protein thiol involved in antioxidant cellular defense, and plays a key role in neutralizing reactive oxygen species (or free radicals) and removing toxic substances from the body (Margis et al., 2008). Therefore, abnormal transsulfuration metabolism will directly affect the redox status in vivo.

Several recent studies have focused on the transsulfuration pathway in ASD, reporting that children diagnosed with ASD had abnormal levels of Hcy, Cys, and glutathione (Ghanizadeh, 2013; James et al., 2004; Pasca et al., 2006). But the results from these studies proved inconsistent. To our knowledge, there has been no study investigating the relation between transsulfuration metabolism parameters and autistic behaviors and clinical severity. In this study, we wanted to assess if abnormal levels of transsulfuration pathway and oxidative stress metabolites were related to ASD in Chinese children, and associations between these metabolites and clinical severity of autism were also examined, with the purpose of identifying potential biomarkers that could be used for the early diagnosis and intervention in ASD.

2. Material and methods

2.1. Participants

From April 2012 to December 2013, we recruited 50 children diagnosed with ASD (mean age ± SD: 7.64y ± 4.22y, 39 male and 11 female), and 50 non-ASD control children (mean age ± SD: 8.38y ± 3.45y, 39 male and 11 female). The recruited children came from four special education schools, and a preschool for children diagnosed with ASD. All cases were diagnosed in Tianjin Children's Hospital, China. Every case had a confirmed diagnosis of ASD, which was provided by the major hospitals in Tianjin and Beijing. In addition, after the children with ASD entered the study, they were evaluated by pediatric psychologists using the Childhood Autism Rating Scales (CARS) (Schopler et al., 1980). The childhood autism rating scale (CARS) is a widely used rating scale for the detection and diagnosis of autism, the cutoff scores of 30 was used to diagnose autistic disorder (Chlebowski et al., 2010) and the score of 25.5 as the CARS cutoff was recommended for an ASD diagnosis (Chlebowski et al., 2010; Tachimori et al., 2003). 24 ASD children with CARS scores ≥30 were considered to have autistic disorder and used to analyze their clinical severity and behavior problems. The Autism Behavior Checklist (ABC) was applied for them, around the same period, with the parents and teachers, during an interview with the focus on the children's present behavior. The ABC is completed by teachers and consists of a series of 57 questions, selected from various sources, which are grouped in five areas: sensory, relating, body/object use, language, and social and self-help (Volkmar et al., 1988). The 50 controls were frequency matched to cases on gender and age. The controls were typically-developing children aged 3–18 years old who attended a regular preschool, a regular elementary school, a middle school or a high school, all public schools, in Tianjin between October and December, 2013. These schools were randomly selected from the schools in District Hedong, whose socioeconomic level is average and representative of that in Tianjin.

All control cases were also clinically examined by the pediatricians to exclude the possibility that the controls could have any sub-clinical autistic features. Exclusion criteria for both groups included history of malnutrition, presence of active infections, or known genetic diseases.

2.2. Ethics approval and consent

This research was carried out in accordance with the Declaration of Helsinki, and was approved by the Institutional Review Board at Tianjin Medical University. Written consent was obtained from the parents of each child, according to the guidelines of the ethics committee.

2.3. Sample collection

Fasting blood samples were collected from both ASD children and controls. 5 ml of venous blood were drawn from the antecubital vein in serum separator tubes. Following centrifugation, the serum was transferred to an Eppendorf tube, and stored at −80 °C prior to measurements.

2.4. Biochemical assays

2.4.1. Measurement of glutathione status

Levels of reduced glutathione (GSH), total glutathione (tGSH), and oxidized glutathione (GSSG) were measured using a commercially available assay kit (Nanjing Jiancheng Bioengineering Institute, China).

2.4.2. Determination of Hcy and Cys levels

Hcy and Cys levels were measured blinded for the diagnoses of the study participants using a HPLC Spectrofluorimetric technique: In an Eppendorf tube, 120 μl of serum was mixed with 50 μl of 1.43 M sodium borohydride and 8 μl *n*-octyl alcohol. The mixture was allowed to stand for 30 min at 4 °C. Then, 50 μl of 0.6 M perchloric acid was added, and after mixing, the mixture was allowed to stand for 10 min at 25 °C.The tube was centrifuged for 20 min at 14.000 rpm; then, 20 μl of supernatant was recovered and mixed with10 μL of 1.55 M NaOH, 125 μl of 0.125 M boric acid, and 50 μl of 1 g/L solution of 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium (SBD-F), and incubated at 60 °C in a shaker for 60 min, cooled on ice, and 200 μl was injected in the HPLC system.

For the separation of serum Hcy and Cys, we used an HPLC system with a Waters 700HPLC pump and a reversed-phase C18 column (5 μm bead size; 4.6 × 250 mm; Waters, Milford, MA). The mobile phase consisted of 0.08 M acetate buffer and 5% (v/v) methanol adjusted to pH 4.0 by adding concentrated acetic acid, and then filtered through a 0.45 μm membrane filter. The isocratic elution was performed at a flow rate of 1.0 ml/min at 30 °C, and a pressure of 100–110 kgf/cm^2 (1500–1,800 psi). A fluorescence detector with excitation wavelength set at 390 nm, and emission set at 470 nm was used to detect Hcy and Cys. Serum Hcy and