Original Paper

DOI: 10.5455/bcp.20160323105909

Oxidative Imbalance in Children and Adolescents with Autism Spectrum Disorder

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ABSTRACT:

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Objective: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interactions and behavioral problems. Various genetic and environmental factors, including oxidative stress, are claimed to play a role in the etiopathogenesis of ASD. In this study, we aimed to examine the status of oxidative metabolism in ASD and the association between oxidative parameters and ASD symptom severity and subtype of ASD.

Method: Thirty-three children and adolescents diagnosed with ASD (16 children diagnosed with autistic disorder, 13 children with pervasive developmental disorder not otherwise specified, and 4 children with Asperger syndrome) according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) and 28 healthy controls, aged 2-17 years, were recruited in this study. Total oxidant status (TOS) and total antioxidant status (TAS) were evaluated using Rel Assay Kit in children and adolescents. The oxidative stress index (OSI) was calculated by dividing the TOS values by the TAS values. Autistic symptoms for these patients were scored on the Childhood Autism Rating Scale (CARS).

Results: In patients with ASD, TAS was statistically significantly lower and OSI statistically significantly higher than in healthy controls. There were no statistically significant differences in TOS between the ASD and control groups. There were no statistically significant differences between the subtypes of ASD in terms of oxidative stress parameters. In addition, TAS, TOS, and OSI values did not differ statistically significantly between the patients' CARS groups, and were not associated with the CARS scores of the patients.

Conclusion: Our findings suggest that oxidative imbalance is present in ASD and that oxidative stress may play a role in the etiopathogenesis of ASD. Therefore, it is suggested that antioxidants may have beneficial effects on ASD and may be a new therapeutic target in treating ASD.

Keywords: autism spectrum disorder, total oxidant status, total antioxidant status, oxidative stress index, oxidative stress

Klinik Psikofarmakoloji Bulteni - Bulletin of Clinical Psychopharmacology 2016;26(3):257-64

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Date of submission:
January 27, 2016

Date of acceptance:
March 23, 2016

Declaration of interest:
O.O., O.B., B.K.B., H.A., A.B., B.K., Y.E., M.D., O.F.T., A.C.K.: The authors reported no conflict of interest related to this article.

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders characterized by problems related to social interaction and behavioral area[1]. The causes and pathophysiology of ASD are not understood completely. It is likely that multiple genetic and environmental factors contribute to its etiology[2]. In recent years, the number of biological studies of ASD have increased in an attempt to determine the role of environmental factors in the etiology and pathophysiology of ASD[3]. Studies have suggested a pathophysiological role for oxidative stress, which

may be a crucial environmental factor in various psychiatric disorders, including anxiety disorders[4], obsessive compulsive disorder (OCD)[5], panic disorder[6], attention deficit hyperactivity disorder (ADHD)[7], bipolar disorder (BPD)[8], and schizophrenia[9]. Oxidative damage may play a central role in the pathogenesis of ASD, just as it does in these other neuropsychiatric disorders[10]. Previous studies have reported increased oxidant stress, including nitric oxide, in ASD patients[10-12]. Normally, antioxidant defense mechanisms balance the activity of free radicals (FRs), creating homeostasis between oxidants and antioxidative systems. Oxidative stress is characterized by the production of excessive amounts of FRs, decreased levels of antioxidants, or both. In certain neuropsychiatric disorders this homeostasis is disrupted, and enhanced concentrations of FRs which can lead to neuronal damage in genetically predisposed persons[13]. The number of various antioxidants and FRs makes it difficult to measure them separately. Understanding the levels of total oxidative and antioxidative metabolism in patients with ASD may help clarify the biochemical mechanisms underlying these disorders. The process for measuring total antioxidative status (TAS) and total oxidative status (TOS) uses novel methods developed by Erel[14,15]. TAS test reflects a patient's total antioxidant status, TOS test reflects total oxidant status, and the oxidative stress index (OSI) reveals current oxidative balance.

The present study aimed to examine oxidative metabolism in patients with ASD by measuring TOS, TAS and OSI values to determine changes in oxidative stress parameters that may contribute to the etiopathogenesis of ASD. In addition, we examined the association between these oxidative parameters and symptom severity and subtype of ASD.

METHODS

Participants and procedures

The study recruited 33 children and adolescents from 2-17 during routine visits between January and July 2015, diagnosed with ASD at the Pamukkale University School of Medicine, Department of Child and Adolescent Psychiatry. Patients were examined using a two-stage evaluation. In the first stage, they were diagnosed with ASD including autistic disorder (AD), Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS), and Asperger syndrome (AS) according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria by the senior child psychiatrist. In the second stage, autistic symptoms of these patients were evaluated with the Childhood Autism Rating Scale (CARS)[16,17]. Finally, the study enrolled 16 children diagnosed with AD, 13 children with PDD-NOS, and 4 children with AS. Based on hospital staff and those attending the pediatric outpatient clinic for routine check-ups and various other reasons, the study recruited 28 healthy controls from 2-17, matched for age and gender. The study excluded children with a history of psychiatric treatment in the control group. In addition, healthy controls had no family history of ASD. The study also excluded patients and healthy controls with an active infectious disease the previous week, who received any antioxidant agents, such as vitamin C or vitamin E, or who had a history of smoking or alcohol use.

All participants were informed about the nature of the study and signed an informed consent form in accordance with the Declaration of Helsinki. The local Ethics Committee of Pamukkale University School of Medicine approved the study.

Blood Samples and Measurement Tools

Venous blood samples were obtained from an antecubital vein after a 12-h overnight fasting period; 10 mL of venous blood from each participant was collected in biochemistry tubes. Samples were centrifuged at 5000 rpm for three min and serum obtained from the blood samples were frozen and stored at -20°C prior to analysis. After all samples were collected, biochemical analyses were made in the Biochemistry Laboratory of the Pamukkale University School of Medicine.

Measuring TOS

The samples were first brought to room temperature (18-26 °C). Serum TOS was measured using a Rel Assay Kit. In this method, oxidants present in the sample oxidize the ferrous ion–chelator complex to ferric ion. The presence of ferric ion leads to a change in color that can be measured spectrophotometrically, whereby the color intensity reflects the total amount of oxidant molecules present in the sample[14].

Measuring TAS

Before analyses, the serum was brought to room temperature (18-26°C) and the serum TAS was measured using a Rel Assay Kit. In this method, the hydroxyl radical reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown. Upon addition of a serum sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the serum, preventing color change. This reaction is measured spectrophotometrically in automatic analysers to obtain the TAS value. Results are expressed as mmol Trolox Eqv./L[15].

Calculating OSI

The OSI was obtained by dividing the TOS by the TAS. The TAS mmol unit value was first translated to micromol units (i.e., the units used in the TOS test), then the OSI was calculated according to the following formula: OSI (arbitrary unit)=[TOS (μmol H_2O_2 Equiv./L)/TAS (mmol Trolox Equiv./L)] x 100.

Statistical Analysis

SPSS for Windows (version 17.0) was used for statistical analysis. Categorical variables were analyzed using the Chi-square test. The distributions of the variables were assessed by the Shapiro–Wilk W test, and non-parametric tests were used for variables with non-normal distributions. The Mann-Whitney U test was used for continuous variables with two categories, and the Kruskal-Wallis tests were used for continuous variables with three categories. Spearman's correlation analysis was performed to evaluate correlations between age, CARS scores, and TAS, TOS, and OSI levels. Statistical significance was defined as $p<0.05$.

RESULTS

The study included 33 patients with ASD (25 males and 8 females) and 28 healthy controls (15 males and 13 females). The mean age of the ASD group was 8.72±3.73 years, and the mean age of the control group was 10.71±4.35 years. There were no statistically significant differences in mean age ($p=0.60$) or gender ($p=0.69$) distribution between the groups. Sociodemographic and clinical characteristics of the participants are shown in Table 1.

The mean TAS values were statistically significantly lower and OSI values statistically significantly higher in the ASD group than in the control group ($p<0.001$). There were no statistically significant differences in TOS between the groups ($p=0.297$; Table 2).

We found no statistically significant correlations

Table 1: Demographic characteristic of patients with ASD and HC

	ASD (n=33)	HC (n=28)	p
Age (mean years±SD)	8.72±3.73	10.71±4.35	0.060*
Sex (male/female)	25/8	15/13	0.069†
ASD subtypes (n,%)			
Autism	16 (48.5)		
PDD-NOS	13 (39.4)		
Asperger disorder	4 (12.1)		

*Independent-Samples T-Test, †Chi-squared test. ASD: Autism Spectrum Disorders, PDD-NOS Pervasive developmental disorder not otherwise specified, HC: healthy control, SD: standard deviation

Table 2: Comparison of the oxidative stress parameters among groups

	ASD (n=33)	HC (n=28)	p
TAS	0.42 (0.28-0.51)	0.76 (0.47-0.87)	<0.001*
TOS	44.93 (43.56-48.56)	47.53 (43.69-49.52)	0.297
OSI	10.74 (8.64-16.80)	6.20 (5.19-9.80)	<0.001*

Variables were expressed as median (25^{th}–75^{th} percentile). *Mann Whitney-U test, statistically significant p<0.05, ASD: Autism Spectrum Disorders, HC: healthy control, TAS: total antioxidant status, TOS: total oxidant status, OSI: oxidative stress index

Table 3: Comparison of the oxidative stress parameters among ASD subgroups and CARS groups

	ASD subgroups			
	Autism (n=16)	**PDD-NOS (n=13)**	**AS (n=4)**	**p***
TAS	0.31 (0.18-0.45)	0.49 (0.35-0.61)	0.47 (0.40-0.53)	0.09
TOS	44.11 (43.15-45-58)	45.89 (44.11-49.31)	44.31 (42.46-48-42)	0.22
OSI	15.03 (9.74-25.06)	10.07 (7.25-14.11)	10.08 (8.23-10.86)	0.14
	CARS groups			
	Non-autistic group (n=5)	**Mild to moderate autism group (n=14)**	**Severe autism group (n=14)**	**P***
TAS	0.52(0.41-0.70)	0.36 (0.27-0.49)	0.41 (0.24-0.53)	0.79
TOS	44.93(42.87-48.21)	44.93 (43.15-49.17)	44.79 (43.62-46.36)	0.91
OSI	9.52(6.61-10.79)	12.68 (9.41-17.33)	11.98 (8.25-20.03)	0.35

Variables were expressed as median (25^{th}–75^{th} percentile). *Kruskal-Wallis test, statistically significant p<0.05, ASD: Autism Spectrum Disorders, PDD-NOS: Pervasive developmental disorder not otherwise specified, AS: Asperger syndrome, TAS: total antioxidant status, TOS: total oxidant status, OSI: oxidative stress index

between CARS scores and TAS (r=-0.072, p=0.69), TOS (r=-0.020, p=0.91), and OSI (r=0.097, p=0.59) values for the patients. Patients were divided into three groups according to the CARS scores as follows: non-autistic group (patients with CARS scores between 15-29.5 points), mild to moderate autism group (patients with CARS scores between 30-36.5 points) and severe autism group (patients with CARS scores greater than 36.5 points). The oxidative parameters including TAS, TOS and OSİ values were not statistically significantly different between the patients' CARS groups. In addition, there were no statistically significant differences between the subtypes of ASD in terms of oxidative stress parameters (Table 3).

DISCUSSION

To our knowledge, this is the first study to examine TAS, TOS and OSI parameters in children with ASD. We demonstrated the presence of lower TAS and higher OSI levels in children with ASD.

The antioxidant system consists of enzymatic and non-enzymatic components[10]. In general, it has been suggested that ASD patients have a weakened antioxidant system. Previous studies have reported lower glutathione peroxidase activity in ASD patients than in controls when measured in erythrocytes[10] and in erythrocytes and plasma[18]. Similarly, lower erythrocyte superoxide dismutase activities were reported in autistic children than in healthy controls[18]. In addition, due to decreased nicotinamide adenine dinucleotide phosphate (i.e., the cofactor for glutathione reductase), lower plasma concentrations of glutathione (GSH) and higher plasma concentrations of glutathione disulfide (GSSG) were reported in ASD children[10,19]. Vitamin E levels were reported to be consistently lower in ASD children than in heathy controls[10]. Parellada et al. (2012)[20] demonstrated that the TAS of patients with AS was lower than those of the healthy controls and patients with psychosis at first episode. Our finding of decreased TAS in patients

with ASD supported these previous results. Decreased TAS may increase neuronal damage caused by normal or enhanced oxidants impairing the defense mechanisms of vulnerable individuals. Post-mortem studies seem to support this view. A post-mortem study reported that the GSH/GSSG ratio, an indicator of antioxidant capacity, was lower in autism patients than in healthy controls, and the ratio was inversely correlated with 8-oxo-deoxyguanosine (8-oxo-dG), which is a biomarker of oxidative deoxyribonucleic acid (DNA) damage[21]. Therefore, the results of the present study suggest that decreased antioxidant capacity in ASD children may play a crucial role in ASD pathogenesis.

Oxidants may be produced by physiological or pathological processes[22]. Most studies have demonstrated increases in various oxidant markers in ASD children. In particular, elevated nitric oxide (NO) levels were consistently reported among ASD patients in various studies,[11,23,24] as were elevated thiobarbituric acid reactive substances and xanthine oxidase activity in red blood cells[25] and increased levels of 8-isoprostane (8-iso-PGF2a), a lipid peroxidation biomarker[26] in autism. Increases in these substances indicate excess oxidants in autistic patients compared to controls. An elevated concentration of oxidants in cells may lead to cell damage and death[22,27]. Studies investigating the reason for the elevated oxidants found evidence for mitochondrial dysfunction in patients with ASD[28,29]. Because of the electron transport system (ETS), mitochondria is the predominant source of oxidants and the major target of FRs[30,31]. Mitochondrial dysfunction may cause increased reactive oxygen species and oxidative damage[32].

Interestingly, in contrast with TAS, the present study found no significant differences in TOS values between ASD children and the control group. This result seems to contradict those of previous studies. However, in previous studies, oxidant parameters were evaluated separately, which is a fairly difficult and expensive method. The present study was the first to measure the cumulative oxidant effects of these agents. In addition, TOS and TAS may not always show strong negative correlations in individuals. Therefore, measuring the OSI parameters provides better information about patients' oxidative stress.

The present study demonstrated an increase in OSI values in patients with ASD. An oxidative imbalance between oxidants and antioxidant systems has been reported in neuropsychiatric disorders, including ADHD[33], OCD[34], BPD[8], and schizophrenia[9]. However, this study was the first time that OSI parameters were evaluated in ASD patients.

The human brain is very sensitive to oxidative stress[35]. The brain utilizes 20% of the oxygen taken up by the body, even though it constitutes approximately 2% of body weight[35,36]. The expected result is that large amounts FRs, which are toxic to neurons, are produced in the brain. Large amounts of polyunsaturated fatty acids, which are oxidized easily, can increase the vulnerability of brain to FRs. In addition, a relatively weak antioxidant system in the brain contributes to damage to neuronal cells[37]. Therefore, increased oxidative stress may contribute to the pathogenesis of neuropsychiatric disorders like ASD that are caused by brain damage.

Oxidative damage is caused by an increase in lipid peroxidation[38], which leads to a loss of mitochondrial membrane potential and destruction of ETS activity[39]. Kern and Jones (2009) hypothesized that increased brain volume and loss of Purkinje cells are associated with lipid peroxidation in autism[40]. Post-mortem studies, conducted in patients with autism, have reported increased lipofuscin containing cells, which is a marker of oxidative stress in the brain[41], and increased levels of 3-nitrotyrosine, which is an indicator of protein oxidation in the cerebellum[42].

It is thought that oxidative stress can cause DNA and RNA damage in patients with autism. A previous study showed an association between elevated oxidative stress and DNA damage in patients with BPD[43]. A post-mortem study reported significant increases in 8-oxo-dG in patients with autism[21]. James et al. (2004) reported that decreased methylation capacity may be associated with increased oxidative stress and impaired

transfer of methionine from S-adenosylmethionine to DNA, RNA, proteins, phospholipids, and neurotransmitters in children with autism[44]. In addition, studies investigating the effects of FRs on the brain have reported that they impair neuronal cell migration by promoting mutations in genes important to brain development[45,46].

In previous studies mitochondrial dysfunction, which causes oxidative imbalance[32], was reported in ASD[28,29]. The oxidative imbalance may initiate mitochondrial and non-mitochondrial dependent cascades, which results in apoptosis[37]. Oxidative stress can lead to mast cell activation that promotes the secretion of various proinflammatory and neuro-sensitizing molecules, including bradykinin, histamine, IL-6, and NO[47]. These molecules may damage the gut-blood-brain barrier. Theoharides et al. (2009) speculated that permitting entero-toxic molecules to enter the brain may trigger neuro-inflammation in patients with autism[48].

Taken together, these findings suggest that oxidative stress may lead to neuronal damage and may contribute the pathogenesis of ASD by causing lipid and protein peroxidation, DNA and RNA damage, and neuroinflammation. Therefore, our findings of increased OSI in patients with ASD were concomitant with those of previous studies. The other major finding of the present study is that there was no difference between the TAS, TOS, and OSI values and ASD subtypes and disease severity (using CARS scores). These results cannot be compared with those of previous studies, because no other study has compared oxidative stress parameters in ASD subtypes. This finding may indicate that oxidative imbalance in ASD children is independent of subtypes and symptom severity.

The most prominent limitation of the present study is its relatively small sample size. The other limitation is the cross-sectional design of our study. Although there is no statistically significant difference in the gender distribution between the patient and control groups, another limitation is disproportion in the distribution of girls and boys in the ASD and control groups, which is in turn related to the small sample size. Finally, we evaluated a wide range of ages. Therefore, these results may be affected by possible variations in the effects of age on oxidative parameters. To our knowledge, this is the first study to examine TOS, TAS, OSI, and overall oxidative balance in children and adolescents with ASD.

CONCLUSIONS

This study found decreased antioxidant capacity and impaired oxidative balance in children diagnosed with ASD. Given these findings, oxidative stress may play a crucial role in the pathogenesis of ASD, causing structural and functional neuronal damage in more than one pathway in vulnerable individuals. However, the effect seems to be independent of subtype and severity of ASD. Antioxidants may have beneficial effects on ASD and may be a new therapeutic target for treating ASD. Prospective studies with larger samples are needed to understand the exact effect of oxidative stress on ASD pathogenesis.

Conflict of interest: *The authors have no financial relationships or conflicts of interest to disclose.*

Acknowledgments: *The present study was supported by Scientific Research Projects Coordination Unit of Pamukkale University. The authors would like to thank Raziye Unlubas for her contributions to the study.*

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