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Comparing Urinary Effect Size Related to Behavioral Symptoms between Total Antioxidant Capacity and Hexanoyl-lysine in Individuals with Autism Spectrum Disorders

Kunio Yui[1,*], Hitomi Sasaki[1], Ryoichi Shiroki[1], Yohei Kawasaki[2]

[1]Department of Urology, Fujita health University Graduate School of Medicine, Toyoake, 470-1192, Aichi, Japan
[2]The Chiba University Clinical Research Center, Chiba, Japan
*Corresponding author: yui16@bell.ocn.ne.jp

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Abstract The imbalance between oxidative stress such as increased free radicals and decreased antioxidant capacity has been implicated in the etiology of autism spectrum disorders (ASD). However. which of these has a greater effects on ASD behavioral symptoms is still unclear. We measured urinary levels of the oxidative stress biomarker hexanoyl-lysine (HEL) and the total antioxidant capacity (TAC) and the plasma levels of the oxidative stress biomarker superoxide dismutase (SOD) and of the anti-inflammatory fatty acid eicosapentaenoic acid (EPA). We examined the relationships between these biomarkers and behavioral symptoms in 19 individuals with ASD (mean age 10.9 ± 5.3 years) and 11 healthy controls (mean age 14.3 ± 6.3 years). Ages were not no significant difference between the two groups. Behavioral symptoms were assessed using the Aberrant Behavior Checklist (ABC). In the ASD group, urinary TAC levels were significantly lower and urinary HEL levels were significantly higher than in the control group without plasma SOD and EPA levels. ABC scores were significantly higher in the ASD group than in the control group. Stepwise regression analysis and the standardized regression coefficient revealed that urinary TAC levels provided greater impact for distinguishing the two groups, and that that TAOC levels had a larger effect size than HEL levels in urine. Plasma SOD levels were significantly correlated with the ABC irritability and stereotypy score. Thus, urinary TAP levels may be important factor in the pathophysiology of ASD, and altered plasma SOD levels may contribute to the autistic behaviors.

Keywords: *urinary effect size, behavioral symptoms, total antioxidant power, hexanoyl-lysine, superoxide dismutase, eicosapentaenoic acid, autism spectrum disorder*

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1. Introduction

Accumulating evidence implied critical roles for a various set of environmental factors [1] coupled with genetic vulnerability to the development of autism spectrum dfisorders (ASD) on its gradual and prolonged processes of emergence. [2] Notably, dyshomeostasis between antioxidant capacity and redox activity [3] or defects in detoxification systems [4] are implicated in the pathophysiology of ASD. However, which factor has a greater impact on autistic behavioral symptoms remains unclear.

Previous studies reported on the set of oxidative stress related markers in urine as follows: 1) elevated hexanoyl-lysine (HEL) levels in 24 children [5]; 2) higher the DNA methylation marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in 33 young children with ASD [6]; 3) lowered TAP levels in young subjects with [7] or in 15 young children with ASD. [8] Recently, a set consisting of the oxidative marker HEL, total antioxidant capacity (TAC) and 8-OHdG in urine has been proposed as a useful biomerker set for studying the role of oxidative stress in brain damage. [9,10] However, there are a few studies using this set of oxidative stress-related biomarkers in urine.

Many previous studies have reported that alterations in superoxide dismutase (SOD), a major antioxidant enzyme contribute to ASD pathophysiology. [11,12] In ASD subjects, SOD levels have been shown to decrease in serum [11,13,14] and plasma [15], and to increase in erythrocytes [16,17] and plasma However, the role of the plasma SOD levels in relation to the urinary oxidative stress-related biomarkers remains unclear.

Omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid

(DHA) have been fond as a protective therapeutic regimen for mitigating oxidative stress. [18] Of reference, EPA has shown a stronger capacity to resolve inflammation than DHA. [19] We have already reported papers on relationship between urinary HEL, TAP and 8-OHdG levels, and plasma SOD levels, and autistic social symptoms in individuals with ASD, [20,21] and that urinary TAP levels have an extensive effect size compared to urinary HEL levels in autistic social interaction. [22] The present study calculated the effect sizes of the urinary and plasma variables incliding urinary HEL, TAP levels and 8-OHdG levels, and plasma SOD and EPA levels for distinguishing the ASD group from the control group in autistic behaviors. [23]

2. Subjects and Methods of This Study

2.1. Subjects

Subjects were a total of 30 young, physically healthy children in Hanshin area (Kobe and Osaka prefectures). They were recruited between October 2015 and September 2017. ASD was diagnosed based on the DSM-5 criteria [24] and was confirmed by the Autism Diagnostic Interview-Revised (ADI-R) [25] by two psychiatrists specializing in developmental disorders. Among the 30 participants, 19 were diagnosed as ASD (12 males and 7 females, mean age: 10.9 ± 5.3 years old, age range: 6-22 years old), and the remaining 11 were normal healthy controls (7 males and 4 females, mean age: 14.3 ± 6.3 years old, age range: 5-21 years old). The 11 normal controls were considered to be physically and mentally healthy based on initial physical and mental screening tests. These individuals with ASD had the core symptoms of the DSM-5 diagnostic criteria for ASD without any abnormal neurological symptoms (e.g., epileptic seizure or neuronal diseases). The 19 individuals with ASD and the 11 individuals in the control group were matched on habits of dietary intake, age, gender and full intelligent quotient (IQ) scores (Table 1). There were no abnormalities in physical (resting blood pressure and heart rate) and clinical laboratory examinations (hematology and plasma chemistry, including plasma fatty acids) at the initial health screening tests in the two groups. The other inclusion criteria were as follows: (a) the absence of any other medical or comorbid psychiatric disorders; (b) a baseline verbal or full IQ was greater than 70 as evaluated by the WAIS-III [26] or the adult version of this scale [27] because young individuals with high-functioning pervasive developmental disorders were required to have a total IQ of at least 70; [28] and (c) no treatment with antidepressants, anxiolytic medications or neuroleptics within the three months prior to the study.

The present work complied with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. This study was performed with the approval of the Ethics Committee of the Fujimoto Medical Clinic in Kobe City, Japan. This ethics committee is registered with Pharmaceuticals and Medical Devices Agency of Japan to register the IRB information (http://www.info.pmda.go.jp/). Most of the participants in this study were young people under the legal age of 20 years; thus, we obtained parental permission and applied information on the behalf of these individuals. Written informed consent was obtained from the participants and/or their parents. This study was registered at https://dbcentre3.jmacct.med. or.jp/jmactr (the Clinical Trials Registry, Japan Medical Association, 2018/7/5; renewal; ID: JMA-IIA00162).

Table 1. Subject characteristics; urinary HEL, 8-OHdg and TAP levels; and the ABC scores in the 19 individuals with ASD and the 11 normal controls

Variables	ASD	Controls	U	*P* value
		(n= 19)		(n= 11)
Age (years)				
Mean ± S.D.	10.9 ±5.3	14.3 ±6.37	3.00	0.19
Sex (male/female)	6/13	4/7	$\chi^2 = 0.00$	1.00
Full IQ	100.90 ± 31.4	112.6 ± 17.4	27.5	0.33
Scores of Autism Diagnostic Interview-Revised (ADI-R)				
Domain A (social)	13.3 ± 6.3	—		
Domain B (communication)	7.4 ± 5.3	—		
Domain C (stereotyped)	11.2 ± 6.0	—		
Urinary levels				
HEL (pmol/mg Cre)	75.55 ± 31.00	51.18 ± 26.09	55.00	0.033*
8-OHdG (ng/mL Cre)	11.27 ± 5.64	9.62 ± 3.359	2.50	0.61
TAP (μMUric Acid equivalents)	2969.87 ± 820.14	4152.85 ± 13.1.60	56.00	0.037*
Plasma SOD levels (U/ml)	3.87 ± 3.26	3.58 ± 2.85	87.50	0.47
Plasma EPA levels (μg/ml)	31.73 ± 23.66	21.79 ± 11.36	79.00	0.29
Subscale scores of the ABC				
Irritability	13.37 ± 8.00	0.73 ± 1.10	1.00	0.00***
Social withdrawal	19.32 ± 10.13	0.36 ± 0.92	2.00	0.00***
Stereotypy	4.53 ± 4.42	0.36 ± 0.67	23.00	0.00***
Hyperactivity	20.37 ± 11.70	0.91 ± 2.12	3.00	0.00***
Inappropriate speech	4.68 ± 3.38	0.27 ± 0.65	17.50	0.00***
Total	62.79 ± 29.93	2.45 ± 4.55	0.50	0.00***

Note: HEL, hexanolyl-lysine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; TPA, Total Antioxidant Power; SOD, superoxide dismutase; ABC, Aberrant Behavior Checklist. Values are mean ± SD. * $p< 0.05$ and*** $p< 0.001$compared to the normal controls

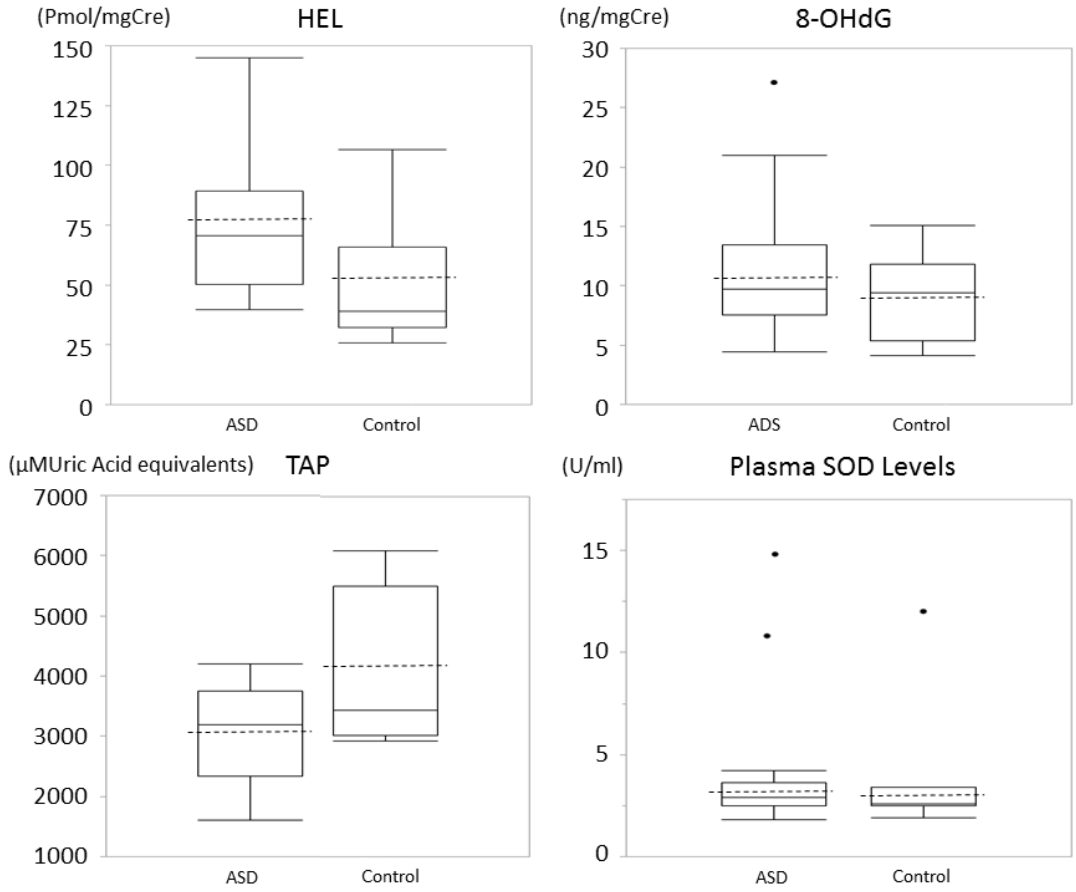

Figure 1. Urinary levels of HEL, 8-OHdG and TAP, and plasma levels of SOD

2.2. Urinary Levels Of Hexanoyl-lysine (HEL), the Total Antioxidant Capacity (TAC) and 8-hydroxy-2′-deoxyguanosine (8-OHdG)

Urines were collected as a spot sample and immediately stored at-80C until analysis. After the dissolving process, the urines were centrifuged to remove all insoluble materials. The specialists at the Department of Pediatrics, Tokyo Metropolitan Fuchu Medical Center for the Disabled (Tokyo, Japan) measured the urinary levels of HEL, 8-OHdG and TAC.

2.2.1. Urinary Levels of HEL

The urinary levels of HEL were measured in duplicate using a competitive ELISA kit (Japan International Cooperation Agency-JICA, Shizuoka, Japan). [29]

2.2.2. Urinary Levels of 8-OHdG

Urine samples were centrifuged and the supernatant after proper dilution was used in duplicate for assessment with a competitive enzyme-linked immunosorbent assay kit (8-OHdG check ELISA kit, JalCA, Japan Institute for the Control of Aging, Shizuoka, Japan). These results were then corrected to the urinary concentration of creatinine, and the urinary 8-OHdG/creatinine levels were used in subsequent analyses. [30]

2.2.3. Urinary Total Antioxidant Capacity

As described in the manufacturer's instruction manuals (Oxford Biomedical Research), the urinary antioxidant capacitywas determined by competitive enzyme-linked Immunosorbent assay (ELISA). [30] This assay provides accurate determination of the total antioxidant activity in a sample that results from the combined activities of the constituents (Oxford Biomedical Research).

2.3. Plasma Levels of Superoxide Dismutase

Plasma SOD levels were estimated from the rate of decrease in nitrite produced by hydroxylamine and the superoxide anions based on the nitrite method, using a Versa max instrument (Molecular Devices Co, Tokyo, Japan). Human plasma was assayed using an SOD Assay Kit (Takara Bio, Tokyo) according to the cytochrome c method, and the plasma SOD levels are expressed as units per milliliter. The assay sensitivity was 0.3 U/ml. The intra-assay and inter-assay coefficients were 2.11 and 2.10 U/ml, respectively.

2.4. Assessment of Behavioral Symptoms

The Aberrant Behavior Checklist (ABC) was used to assess the behavioral symptoms of the 19 individuals with ASD and the 16 normal controls. The ABC is primarily intended to evaluate treatment responses in psychopharmacological and behavioral intervention trials for children and adolescents with mental retardation [31] and normal IQ levels.[32] The subscales are as follows: (1) irritability (15 items); (2) social withdrawal (16 items); (3) stereotypic behavior (seven items); (4) hyperactivity (16 items); and (5) inappropriate speech (four items). The ABC is a broad assessment that captures a wide variety of behavioral problems. [33]

2.5. Statistical Analyses

To identify the significant variables associated with the two groups, the five ABC subscale and total scores, we used a linear regression analysis with forward stepwise methods, in which the relationship of the oxidative stress-related biomarkers (urinary HEL, TAP and 8-OHdG levels and plasma SOD and EPA levels) as independent variables and the two subject groups and the scores on the five subscales and total scores of the ABC as dependent variables. To assess the effect sizes of oxidative stress-related variables (HEL, TAC and 8-OHdG levels in urine and plasma SOD and EPA levels), we used the standardized regression coefficient. [23] Spearman's correlation coefficients were used to evaluate the correlation between the urinary and plasma variables and the ABC scores. We conducted the statistical analyses using SPSS version 18.0 (IBM Tokyo, 2009).

3. Results

3.1. Study Population

Clinical symptoms in the19 individuals with ASD were characterized by repetitive patterns of interest (n = 8), social withdrawal (n = 19) and hyperactivity (n = 4). The mean ABC total score for 19 individuals with ASD was 62.79 ± 29.9 (Table 1). A previous study reported average ABC scores of 60.14 in 29 children and adolescents with moderate or severe ASD. [34] Thus, our patients exhibited moderate or severe behavioral symptoms.

3.2. Oxidative Stress Marker Levels in Urine

There was a significant increase in urinary HEL levels and a significant decrease in urinary TAC levels in the 19 individuals with ASD compared to the 11 normal controls. Urinary 8-OHdG levels and plasma SOD and EPA levels were not significantly different between the groups (Table 1).

3.3. Magnitudes of Variables

As shown in Table 2, stepwise regression analysis revealed that urinary TAP levels were significantly associated with two groups (R^2 = 0.283, p = 0.002). Using Group used as a dependent variable showed a significance of contribution to the urinary TAP levels (unstandardized coefficients, B =1282.98 ± 385.900, β = 0.532, and p = 0.002). The ABC irritability subscale being used as the dependent variables, the statistical significance of contribution of this subscale scores to the urinary HEL levels (unstandardized coefficients, B =1.661 ± 0.587, β = 0.460, and p = 0.009) was shown. Thus, urinary HEL levels were significantly associated with the ABC irritability scores. Additionally, The ABC stereoypy subscale being used as the dependent variables, the statistical significance of contribution of this subscale scores to the plasma SOD levels (unstandardized coefficients, B =0.707 ± 0.145, β = 0.935, and p = 0.000) was shown (Table 2).

The standardized regression coefficients revealed that effect size of each oxidative stress related variables in order of large effect size as TAP (-0.8578), HEL (0.2702), SOD (- 0.1354), and EPA (- 0.1065). Thus, the urinary TAC levels have larger effect that is more powerful compared to urinary HEL.

Taken together, urinary TAC can be used in a first-order regression model to distinguish the ASD group from the control group (Table 2).

3.4. Association among the Oxidative Stress Related Markers

There were significant correlations between urinary TAC levels and plasma SOD levels (r = 0.23, p = 0.04) in the ASD group. Plasma SOD levels significantly correlated with SRS subscale scores of social withdrawal (r = 0.58, p = 0.009), stereotypy (r = 0.55, p = 0.03), hyperactivity (r = 0.56, p = 0.01) and inappropriate speech (r = 0.54, p = 0.02), as well as with total scores (r = 0.60, p = 0.006).

Table 2. Results from the stepwise regression analysis

Model	Coefficients				
	R^2	P-value	B	Beta	*P* value
TAP	0.283	0.002*			
Group (1 = ASD, 2 = control)			1282.98 ± 385.900	0.532	0.002*
Standardized regression Coefficient	-0.8578	0.0108*			
HEL	0.222	0.009*			
ABC irritability scores			1.661 ± 0.587	0.460	0.009*
Standardized regression Coefficient	0.2702	0.4347			
SOD	0.474	0.000**			
ABC stereotypy scores			0.707 ± 0.145	0.935	0.000*
ABC irritability scores			-0.185 ± 0.067	- 0.553	0.010*
Standardized regression coefficient	-0.1354	0.6289			
EPA	0.433	0.017*			
ABC irritability scores			0.997 ± 0.392	0.443	0.017*
Standardized regression coefficient	-0.1065	0.7631			

Note: TAP = total antioxidant power; R^2 = R-squared values; B = unstandardized coefficients; HEL, hexanolyl-lysine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; TPA, Total Antioxidant Power; SOD, superoxide dismutase; ASD = autism spectrum disorder; SRS =Social Responsiveness Scal; *p< 0.05 and **p<0.001, significant contribution.

4. Discussion

We for the first time reported the measurements of the set of HEL, TAC and 8-OHdG levels in urine. Urinary TAC levels were significantly decreased, whereas urinary HEL levels were significantly increased, in the ASD group compared with the control group. The two groups showed no significant differences in the urinary 8-OHdG levels or in the plasma levels of SOD, DHA and EPA. Stepwise multiple regression analysis identified significant correlations between urinary TAC and the two groups. Plasma SOD and EPA levels contributed to the ABC irritability subscale scores. Importantly, the standardized regression coefficients revealed that urinary TAC levels had a larger effect size than urinary HEL levels and plasma levels of SOD and EPA (Table 2). Thus, urinary TAC levels were a more powerful explanatory variable than the urinary HEL levels, and they fit a first-order regression model for distinguishing the ASD group from the control group.

A previous study reported that 29 ASD children aged 6–12 years exhibited significantly lower urinary TAC levels without increases in catalase activity or total thiol molecules, which are urinary indicators of antioxidant capacity, [35], compared to 24 age-matched healthy controls. [7] With respect to urinary HEL levels, 24 children with ASD aged 5 - 12 years had significantly increased HEL levels, without corresponding increases in urinary 8-OHdG levels or erythrocyte SOD levels, compared to 20 gender- and age-matched healthy controls, [5] perhaps due to deficits in membrane fluidity. [5] Further, a previous clinical study revealed that 33 autistic children aged 4-17 years showed a trend of an increased urinary 8-OHdG level compared to 29 healthy controls aged 5-16 years. [36] These previous studies reported urinary TAP levels alone, [7] urinary 8-OHdG levels, [36], or (3) urinary 8-OHdG concomitant with HEL levels. [5]. A set of oxidative stress-related biomarkers, such as urinary levels of HEL, TAC and 8-OHdG, may provide useful information on oxidative stress-induced neuronal damage. [9] This study presented the first report of this set of three oxidative stress-related biomarkers in ASD.

The significant increase in urinary HEL levels and the reduction in urinary TAC levels in the 19 individuals with ASD indicated an imbalance between oxidative stress and antioxidant capacity. Moreover, stepwise regression analysis revealed that the urinary level of TAC was a reliable index for distinguishing the two groups. Moreover, such imbalance between oxidative stress-related ROS production and TAC may correlate with ASD. [37,38] These previous studies indicated a more significant role of oxidants; [37,38] however, the important question of whether oxidant or antioxidant factors have a stronger impact remained unclear. In this study, urinary TAC may be used in a first-order regression model for distinguishing the ASD group from the control group. Thus, the present findings for the first time revealed that urinary TAP levels had a greater impact than the urinary levels of HEL and plasma levels of SOD and EPA on the imbalance between TAP (antioxidant) and HEL (oxidant) in urine.

Interestingly, our stepwise multiple regression analysis and Spearman's correlation coefficients indicated that plasma SOD levels contributed significantly to ABC irritability and stereotypy scores. A previous clinical study reported that raw milk including SOD significantly improved the behavioral features of ASD. [39] In addition, reduction of the mitochondrial SOD accelerated the onset of behavioral changes in transgenic mouse model of Alzheimer's disease-like pathology. [40] Further studies will be needed to elucidate the effect of plasma SOD levels on ABC subscale scores.

Stepwise regression analysis indicated significant correlation of plasma omega-3 fatty acid EPA levels and ABC irritability scores. Several clinical studies reported that low dose omega-3 fatty acids including EPA decreased irritability in 19 subjects with bipolar depression, indicating therapeutic effects on irritability of psychiatric condition, [41] and that closely relationship between low plasma levels of omega-3 fatty acids and vulnerability for irritability. [42] These previous studies may support our findings on significant correlations between plasma EPA and ABC irritability scores.

Oxidative stress contributes to many pathological conditions. Aerobic organisms have integrated antioxidant systems that include enzymatic and nonenzymatic antioxidants that are usually effective in blocking the harmful effects of ROS. [43] Such systems include the endogenous antioxidant defense system. [44] These antioxidant defense systems may be a part of the endogenous antioxidant systems. Indeed, recent research on antioxidant networks has demonstrated that antioxidant enzymes such as SOD, glutathione peroxidase, and glutathione act as an antioxidant network within extracellular components of the antioxidant system. [45] Further, a recent report has suggested that the autophagy-lysosomal activities of these antioxidant enzymes may serve an essential function in preventing neurodegenerative diseases by removing damage as part of an essential cellular antioxidant pathway. [46] Taking these considerations together, the endogenous intrinsic antioxidant system may be impaired in the 19 individuals with ASD.

This study has some limitations. First, previous studies examined urinary oxidative stress biomarkers such as F2-isoprostanes and their association with the activity of plasma enzymatic antioxidants such as glutathione peroxidase. [47] In this study, although the urinary levels of F2-isoprostanes and the plasma levels of glutathione were not measured, a useful and informative set of oxidative stress-related biomarkers were studied, and this work revealed novel, important information on impaired antioxidant capacity that was not reported in previous studies. [5,7,15] Second, ASD is most prevalent in males, with a male to female ratio of 4 to 1; [48] however, in this study, the ASD and control groups were matched for age and gender. Finally, the small sample size affects our ability to generalize our results to the entire population of individuals with ASD.

In conclusion, the present study reported that reduced levels of TAC and increased levels of HEL in urine may contribute to the behavioral sequence in individuals with ASD, without significant alterations in urinary 8-OHdG levels. As stepwise regression analysis and standard regression coefficients revealed that urinary TAC levels has lager effect size than the other variables, and thus may be preferentially used for distinguishing the ASD group from the control group. These finding suggested that endogenous antioxidant defense systems may be impaired in young individuals with ASD. Plasma SOD levels may

be associated with urinary TAC levels, suggesting an effect of the impaired antioxidant capacity.

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Conflicts of Interest

The authors declare no conflict of interest.

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