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The Large Effect Size of Urinary Total Antioxidant Capacity in Behavioral Symptoms of Young Autistic Individuals: Comparion with Omega-3 Fatty Acid and Superoxide Dismutase in Plasma

Kunio Yui[1*], Hitomi Sasaki[1], Nasoyuki Tanuma[2] and Yohei Kawasaki[3]

[1]Department of Urology, School of Medicine, Fujita Health University, Toyoake, Japan

[2]Department of Pediatrics, Tokyo Metropolitan Fuchu Medical Center for the Disabled, Tokyo, Japan

[3]Chiba University Clinical Research Center, Chiba, Japan

*Corresponding author: Kunio Yui, Department of Urology, School of Medicine, Fujita Health University, Toyoake, Japan, Tel: +81 78 791 8981; E-mail: yui16@bell.ocn.ne.jp

Abstract

Objective: The imbalance between increased oxidative stress and reduced antioxidant defense has been implicated in the pathophysiology of autism spectrum disorders (ASD). Which of these has a greater impact on ASD behavioral symptoms is still unclear. We measured urinary levels of the oxidative stress biomarker hexanoyl-lysine (HEL), the total antioxidant capacity (TAC) and the DNA methylation biomarker 8-hydroxy-2′-deoxyguanosine (8-OHdG) and their relation to the plasma levels of the oxidative stress biomarker superoxide dismutase (SOD) and of the anti-inflammatory fatty acid eicosapentaenoic acid (EPA).

Methods: We studied 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). Behavioral symptoms were evaluated using the Aberrant Behavior Checklist (ABC).

Results: Ages were not significant difference between two groups. The ASD group showed significantly reduced levels of urinary TAC and significantly increased levels of urinary HEL compared to the control group. Urinary 8-OHdG levels or plasma SOD and EPA levels were not significantly different between the two groups. The ABC subscale and total scores were significantly higher in the ASD group had significantly higher ABC subscale and total scores than he control group. Stepwise regression analysis and the standardized regression coefficient indicated that urinary TAC levels provided greater impact for distinguishing the two groups. There was significant correlation between the urinary TAC levels and plasma EPA levels and the ABC irritability scores.

Conclusion: Urinary TAP levels may be important in the imbalance between the urinary levels of HEL and TAC, and altered plasma SOD levels may contribute to this imbalance.

Keywords: Autistic disorders; Antioxidans; Oxidative stress; Superoxide dismutase; Eicosapentaenoic acid; Hexanoyl-lysine; 8-Hydroxy-2′-deoxyguanosine

Introduction

Accumulating evidence indicated that dyshomeostasis between antioxidant capacity and redox activity [1] and defects in detoxification systems [2] are implicated in the etiology of autism spectrum disorders (ASD). However, which factor has a greater impact on ASD behavioral symptoms remains unclear.

Previous studies reported findings on the set of oxidative stress markers in urine as follows: 1) elevated hexanoyl-lysine (HEL) levels in 24 children with ASD aged 5 to 12 years [3]; 2) higher 8-OHdG levels in 33 children with ASD aged 3–10 years [4]; 3) lowered urinary TAP levels in subjects with ASD in 29 subjects with ASD aged 6 to 12 years [5] or in 15 children with ASD aged 4 to 12 years [6]. Recently, a set consisting of the oxidative marker hexanoyl-lysine (HEL), total antioxidant capacity (TAC) and the DNA methylation marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) in urine has been studied to examine the role of oxidative stress in brain damage [7-9]. There were few previous studies using a set of oxidative stress-related biomarkers such as HEL, TAC and 8-OHdG levels in urine.

A lot of former studies have reported that alterations in superoxide dismutase (SOD), an important main antioxidant enzyme [10], contribute to pathophysiology of ASD [11]. Erythrocyte SOD levels in ASD have been reported to decrease in serum [12] and plasma [13], and to increase in erythrocytes [10,11] and plasma [14]. Nevertheless, the relationship between plasma SOD levels and the urinary oxidative stress-related biomarkers is still unclear. In addition, ω-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) has anti-inflammatory capacity to resolve inflammation than DHA [15].

Taking these considerations together, we examined the links among HEL, TAC and 8-OHdG levels in urine, and their association to plasma levels of SOD and EPA in behavioral symptoms of juvenile individuals with ASD, and then estimated the effect sizes of these oxidative stress-related variables using standardized regression coefficients [16].

Urinary TAC levels are influenced by dietary intake [17]; therefore, dietary TAC was assessed in this study.

Methods

Subjects

Subjects were a total of 30 young, physically healthy individuals in Kobe and Osaka prefectures. The diagnosis of ASD was based on the DSM-5 criteria [18] and was confirmed by the Autism Diagnostic Interview-Revised (ADI-R) [19] by two physicians. Among the 30 participants, 19 were diagnosed with ASD (12 males and 7 females, mean age: 10.9 ± 5.3 years old, age range: 6-22 years old), and the rest of 11 were healthy normal controls (7 males and 4 females, mean age: 14.3 ± 6.4 years old, age range: 5-21 years old). These individuals with ASD showed the main symptoms of the DSM-5 diagnostic criteria for ASD, and had not any abnormal neurological symptoms (e.g., epileptic seizure or neuronal diseases). The 19 individuals with ASD and the 11 normal controls were matched on habits of dietary intake, age, gender and full intelligent quotient (IQ) scores (Table 1). These 30 subjects have no any extraordinary data in physical and laboratory examinations. The other criteria for inclusion were as follows: (a) no history of any other medical illnesses or comorbid psychiatric disorders; (b) a full IQ greater than 70 as estimated by the Wechsler Intelligence Scale Revised (WISC-R) [20] or the respective scale for adults [21] because high-functioning ASD were defined as subjects with an IQ above 70 required to have a total IQ of at least 70 [22]; and (c) no treatment with any other neuroleptics within the three months prior to the study.

This study was conducted according to the Ethics Committee approval of the Fujimoto Medical Clinic in Kobe City, Japan. As the most of the 30 subjects in the current study were youth under the legal age of 20 years; we gained parental permission and applied information on the behalf of these individuals. The participants and/or their parents provided written informed consent.

Sample size calculation

In this paper, Primary outcome was the Total Antioxidant Power (TAP). However, we provided estimated power of some parameters as below. Sample size calculation were calculated based on O'Brien-Castelloe approximation method for the Wilcoxon-Mann-Whitney test [23] using SAS 9.4 for Windows (SAS Institute Inc; Cary, NC).

TAP: Based on some previous study and studies using TAP, we estimated the mean difference of TAPs between the two groups as 1,000 points, with 850 (ASD group) and 150 (Control group) points of standard deviation (SD). Total sample size of urinary TAP levels was estimated as 30 for two groups, with a power level of 0.95 to detect a difference of TAPs and a 2-sided alpha level of 0.05.

HEL and 8-OHdG: We estimated the mean difference of HELs between the two groups as 25 points, with 30 (ASD group) and 25 (Control group) points of standard deviation (SD). Total sample size of urinsry HEL was estimated as 30 for two groups, with a power level of 0.68 to detect a difference of HELs and a 2-sided alpha level of 0.05. Total sample size of urinary 8-OhdG levels was estimated as 30 for two groups, with a power level of 0.27 to detect a difference of 8-OHdG s and a 2-sided alpha level of 0.05.

SOD: We estimated the mean difference of SODs between the two groups as 1 points, with 3 (ASD group) and 3 (Control group) points of standard deviation (SD). Total sample size of plasma SOD levels was estimated as 30 for two groups, with a power level of 0.15 to detect a difference of SODs and a 2-sided alpha level of 0.05.

Urinary assay of HEL, 8-OHdG and TAC levels

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 Pediatrics, in Tokyo Metropolitan Fuchu Medical Center for the Disabled (Tokyo, Japan) assayed the urinary levels of HEL, 8-OHdG and TAC.

Urinary levels of HEL: The urinary HEL levels were measured in duplicate using a competitive ELISA kit (Japan International Cooperation Agency-JICA, Shizuoka, Japan) [24].

Urinary levels of 8-OHdG: Urine speciemens 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). The urinary 8-OHdG/creatinine levels were used for analyses [25].

Urinary total antioxidant capacity: The urinary antioxidant capacity was determined by competitive enzyme-linked Immunosorbent assay (ELISA) [25].

Plasma levels of SOD: Plasma SOD levels were assayed using the reduce rate in nitrite induced by hydroxylamine and the superoxide anions based on the nitrite method (Molecular Devices Co, Tokyo, Japan).

Estimation of nutritional TAC: Urinary levels of TAC have been reported to be affected by dietary food intake [26]. Thus, a nutritional TAC value was assigned to each food item in the DHQ15.

Assessment of behavioral symptoms

The Aberrant Behavior Checklist (ABC) (Japanese version, Jiho, Ltd, Tokyo) was employed to evaluate the behavioral symptoms in the 19 individuals with ASD and the 11 normal controls. The ABC is a standardized rating scale for problematic behaviors for children and adolescents with normal IQ levels [27]. The ABC is a broad measure to assess a broad range of problematic behavior [28]. The internal consistency of the Japanese version based on Cronbach's alpha showed reliability ranged from .85 to .95 across five subscales [29] or from 0.75 to 0.98 [30], indicating high reliability of this version [28,29]. The Japanese version of the ABC showed good correlation with the Repetitive Behavior Scale-Revised (RBS-R) [31].

Moreover, irritability subscale scores of the Japanese version showed improvement similar to Clinical Global Impression in 47 ASD children who received aripiprazol compared to 45 children who received placebo [32]. With respect to validity of the ABC, a previous review article reported that a few of the seven behavioral showed test-retest and inter-rater reliability and structural validity scales for assessing behavioral symptoms in ASD [33], however, one recent study described the cross-cultural stability and validation for assessment of overall behavior symptoms in the ABC [34]. Additionally, the Brazikian Portuguese version of the ABC showed cress-cultural stability of the ABC [35].

Statistical analyses

As the data were not normally distributed, the non-parametric Mann-Whitney U test for multiple comparisons was employed to elucidate significant differences between the groups. Multiple regression analysis was conducted to determine the relationship between the urinary (HEL, 8-OHdG and TAC) and plasma oxidative stress-related biomarkers (SOD and EPA), and the other variables (two groups, and the ABC scores) (Table 2).

Importantly, to estimate the effects size of the urinary and plasma oxidative stress-related biomarkers, standardized regression coefficients were employed. The standardized coefficients were useful to determine the most important predictor variables [16]. We conducted statistics using SPSS version 18.0 (IBM Tokyo, 2009).

Results

Study population

Behavioral symptoms of the 19 individuals with ASD were characterized by repetitive patterns of interest and activities (n=8), social withdrawal (n=8) and irritability (n=3). The mean ABC score was 62.7 ± 30.0 (Table 1).

According to a previous study, total ABC scores were 60.1 in children and adolescents with moderate or severe ASD [36], and 83.4 ± 31.8 in 18 children with ASD who had severe maladaptive behaviors such as self-injurious behavior [37]. Thus, the patients in this study suffered moderate or severe behavioral symptoms. Ages were not significantly different between the two groups.

Oxidative stress marker levels in urine

The Mann-Whitney U-test revealed that a significant increase in urinary HEL levels and a significant decrease in urinary TAC levels in the 19 individuals with ASD as compared to the 11 normal controls. There were no significantly differences in urinary 8-OHdG levels and plasma SOD and EPA levels between the groups (Table 1).

Variable	ASD (n=19)	Control (n=11)	U	P-value
Age (years)	10.9 ± 5.3	14.3 ± 6.4	3	0.19
Sex (male/female)	13-Jun	4-Jul	χ2=0.00	1
Full IQ	100.9 ± 31.4	112.6 ± 1 7.4	27.5	0.33
Scores of ADI-R				
Domain A (social)	13.3 ± 6.3			
Domain B (communication)	7.4 ± 5.3			
Domain C (stereotyped behavior)	11.2 ± 6.0			
Urinary levels				
HEL	75.55 ± 31.00	51.18 ± 26.09	55	0.033*
8-OHdG	11.27 ± 5.64	9.62 ± 3.35	2.5	0.61
TPA	2969.87 ± 820.14	4152.85 ±131.60	56	0.037*
Plasma SOD	3.87 ± 3.26	3.58 ± 2.85	87.5	0.47
Subscores of ABC				
Irritability	13.37 ± 8.00	0.73 ±1.10	1	0.000**
Social withdrawal	19.32 ± 10.13	0.36 ± 0.92	2	0.000**
Stereotypy	4.53 ± 4.42	0.36 ± 0.67	23	0.000**
Hyperactivity	20.37 ± 11.70	0.91 ± 2.12	3	0.000**
Inappropriate speech	4.68 ± 3.38	0.27 ± 0.65	17.5	0.000**
Total	62.79 ± 29.93	2.45 ± 4.55	0.5	0.000**
*p<0.05 and **p<0.001, significant differences				

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. ADI-R, Autism Diagnostic Interview-Revised; ABC, Aberrant Behavior Checklist; HEL, hexanolyl-lysine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; TPA, total antioxidant power; and SOD; superoxide dismutase.

Model	R^2	P value	Coefficients 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	0.011*			
HEL	0.222	0.009*			
ABC irritability score			1.661 ± 0.587	0.46	0.009*
Standardized regression Coefficient	0.2702	0.4347			
SOD	0.4741	0.000**			
ABC irritability score			0.185 ± 0.067	-0.553	0.010*
Standardized regression Coefficient	-0.1354	0.6289	-		
EPA	0.433	0.017*			
ABC irritability score			0.997 ± 0.392	0.443	0.017*
Standardized Regression Coefficient	-0.1065	0.7631			
*p<0.05 and **p<0.001, significant contribution					

Table 2: Results from the stepwise regression analysis. TAP=total antioxidant power; R^2=R-squared values; B=unstandardized coefficients; ASD=autism spectrum disorder; ABC=Aberrant Behavior Checklist and EPA=Eicosa-pentaenoic acid.

Magnitudes of variables

As shown in Table 2, stepwise regression analysis indicated significant association of urinary TAP with two groups. Meanwhile, urinary HEL levels and plasma SOD and EPA levels were significantly associated with the ABC irritability scores. The group, as used a dependent variable, made a significant contribution to the urinary TAP levels. The ABC irritability scores as used dependent variables were significantly associated with urinary HEL levels, plasma SOD and EPA levels. Importantly, urinary TAP levels contributed to discriminating the ASD group from the control group.

The standardized regression coefficients indicated that effect size of each oxidative stress related variables in order of large effect size as TAP, HEL, SOD, EPA, and 8-OHdG. Thus, the urinary TAC levels have larger effect size that is more powerful compared to urinary HEL (Table 2).

Dietary total antioxidant power

The ASD group absorbed significantly more dietary TAC in the form of chocolate (p=0.02), cookies and biscuits (p=0.04), and jam and marmalade (p=0.007) than in the control group. While, there were no significant relationships between dietary TAC levels and TAP urinary levels (r=0.02–0.57, p=0.95–0.053) in the ASD group.

Discussion

Urinary TAC levels were significantly reduced, whereas urinary HEL levels were significantly increased, in the ASD group compared with the control group. Importantly, the standardized regression coefficients indicated that urinary TAC levels showed a larger effect size compared with urinary HEL levels or plasma SOD and EPA levels. Thus, urinary TAC levels were a more powerful explanatory variable than the urinary HEL levels, indicating first order multiple linear regression models for distinguish the two groups.

Previous studies reported findings on the set of oxidative stress markers in urine as follows: 1) elevated urinary hexanoyl-lysine (HEL) levels in 24 children with ASD aged 5 to 12 years, while other oxidative stress marker such as urinary8-hydroxy-2'-deoxyguanosine (8-OHdG) levels or erythrocytes SOD levels were unchanged [3] suggesting oxidative stress and erythrocyte membrane alterations as a role of the pathogenesis of ASD [3]; 2) significantly higher levels of urinary 8-OHdG levels in 40 children with ASD aged 3–10 years as compared with 40 age-matched normal controls [4]; 3) lowered activity of urinary total antioxidant capacity (TAP) and total thiol molecules which has antioxidant capacity [38] concomitant with higher urinary catalase activity, which is regulated by oxidative stress [39], in 29 subjects with ASD aged 6 to 12 years compared with 24 normal controls [5]. This study suggested that increased vulnerability to oxidative stress may contribute to the development and clinical symptoms of ASD [5]. Additionally, urinary TAC levels were significantly lower in 45 ASD children aged 4-12 years as compared with age matched controls [6]. Collectively, it is unclear which of these markers has a greater impact on ASD symptoms. The present study firstly revealed that urinary TAC levels were a more powerful explanatory variable than the urinary HEL levels.

The significant increase in urinary HEL levels and the significant 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. A former review article indicated that the imbalance between reactive oxygen species (ROS) production in relation to oxidative stress and TAC may correlate with pathophysiology of ASD [1,40]. However, the important question of whether oxidant or antioxidant factors have a stronger impact remained unclear. The present findings firstly reveal 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.

Our stepwise multiple regression analysis indicated that plasma SOD levels contributed significantly to ABC irritability. However, 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 above findings (Figure 1).

Figure 1: The findings and limitations in this study.
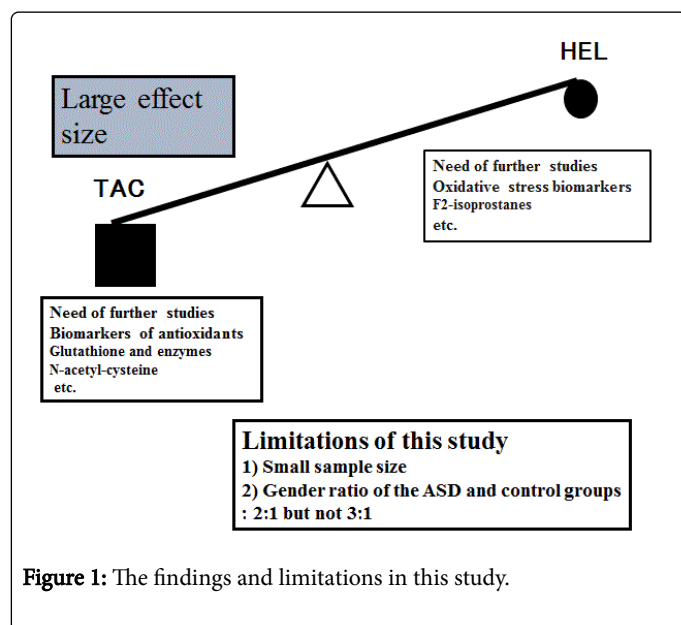
Oxidative stress was concerned with various pathophysiological conditions, and the human body prevent gainst the harmful effects of oxidative stress damage with the the antioxidant defense system which comprises both enzymatic and nonenzymatic mechanisms [43,44]. Such systems include the endogenous antioxidant system (43,44). Most previous studies on antioxidant capacity in individuals with ASD have suggested deficient antioxidant defense mechasnism especially at young children [45] or a chronically low detoxifying capacity [46]. 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 specific intracellular or extracellular components of the antioxidant system [47]. 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 [48]. Taking these considerations together, the endogenous antioxidant system may be deficit in individuals with ASD. While a former clinical study on TAC in plasma indicated that in a group of adolescents with Asperger syndrome, impaired detoxifying capacity was not fond in the first years of illness despite of chronic low detoxifying capacity [46]. Impaired detoxifying capacity may therefore c oxidative stress damage. Further studies will be need to confirm which specific factor may be impaired and whether deficit TAC is intrinsic impairment or secondary deficient resulting from oxidative stress in ASD.

The ASD group revealed significantly higher dietary TAC for chocolate, biscuits and cookies and jam and marmalade as compared with the control group. Cocoa products including chocolate [49], cookies containing chocolate chips [50], and jam and marmalade [51] increase antioxidant capacity. Reduced urinary TAC levels in the ASD group suggest that antioxidant defense systems i.e., endogenous antioxidant defense system [44] or intrinsic antioxidant defenses [47] may be deficit.

Excess oxidative stress against the endogenous antioxidant defense sometimes induced disruption of the blood-brain barrer (BBB), and then brain-specific proteins circulating inside the brain are observed in the peripheral blood as an index for the increase in BBB permeability and brain damage [52]. Overproduction of ROS and the imbalance between ROS and antioxidant capacity induces toxic effects on brain neurons. Thus, imbalance between increased HEL levels and decreased TAC levels in urine may interrupt the BBB, resullting behavioral abnormalities in the ASD group.

This study has some limitations. Firstly, previous studies examined urinary oxidative stress biomarkers such as F2-isoprostanes and their association with the activity of plasma enzymatic antioxidants such as SOD and glutathione peroxidase [53]. In current study, an informative set of oxidative stress-related biomarkers were examined, and this work revealed novel, important information on impaired antioxidant capacity that was not reported in previous studies [3,5,13]. Secondly, ASD is most prevalent in males, with a male to female ratio of 4 to 1 [53]; however, in the current study, the ASD and control groups were matched for age and gender, suggesting influence of on the male to female ratio. Finally, the small sample size limit the ability to generalize findings our findings to all patients with ASD.

Conclusion

This study firstly report that reduced levels of TAC and increased levels of HEL in urine may be conductive to the behavioral sequence in individuals with ASD, without significant changes in urinary 8-OHdG levels. Importantly, reduced urinary TAC levels could be preferentially used for distinguishing the ASD group from the control group. The endogenous antioxidant systems may be impaired in young subjects with ASD.

Acknowledgements

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