

# Imbalance between Urinary Hexanoyl-lysine and Total Antioxidant Capacity Levels and Plasma Superoxide Dismutase Levels in Individuals with Autism Spectrum Disorder

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**Abstract Background and aim:** The imbalance between oxidants and antioxidants contributes to the pathophysiology of ASD. **Methods:** We measured the urinary levels of an oxidative stress biomarker, hexanoyl-lysine (HEL), the total antioxidant capacity (TAC) and the DNA methylation biomarker 8-hydroxy-2'-deoxyguanosine (8-OHdG), and the plasma levels of a major antioxidant enzyme, superoxide dismutase (SOD). We examined the relationship between these four biomarkers and social responsiveness in 20 individuals with ASD and in 11 healthy controls. **Results:** The sex and age distributions ( $11.4 \pm 5.1$  years vs.  $13.9 \pm 6.6$  years) were not significantly different between the groups. Social responsiveness was assessed using the Social Responsiveness Scale (SRS). Dietary TAC from chocolate, biscuits and cookies, jam and marmalade was significantly higher in the ASD group than in the control group, although the nutrient intake was not significantly different between the two groups. Urinary TAC levels were significantly lower, but the urinary HEL levels were significantly higher in the ASD group compared to the control group. Urinary 8-OHdG levels and plasma SOD levels were not significantly difference between the groups. SRS scores were significantly higher in the ASD group than in the control group. Plasma SOD levels significantly correlated with the urinary TAC levels. Stepwise regression analysis revealed that urinary TAC and HEL levels may predict differences in the biomarkers and SRS scores between the groups. **Discussion and Conclusion:** A critical imbalance between the urinary HEL and TAC levels may contribute to impaired social responsiveness in individuals with ASD without the DNA methylation. Plasma SOD levels may affect the impaired antioxidant capacity.

**Keywords:** urinary levels of total antioxidant power, urinary levels of hel and 8-ohdg levels, social responsiveness scale, autism spectrum disorder, endogenous antioxidant capacity

**Cite This Article:** Kunio Yui, Nasoyuki Tanuma, George Imataka, and Yohei Kawasaki, "Imbalance between Urinary Hexanoyl-lysine and Total Antioxidant Capacity Levels and Plasma Superoxide Dismutase Levels in Individuals with Autism Spectrum Disorder." *Journal of Translational Medicine and Developmental Disorders*, vol. 3, no. 1 (2017): 1-10. doi: 10.12691/jtmdd-3-1-1.

## 1. Introduction

The prevalence of ASD has markedly increased in recent years [1]. ASD symptoms usually appear during early development and generally lead to serious lifelong limitations in function; thus, both genetic and environmental factors may contribute to its development [2,3]. The shift in the balance between oxidants and antioxidants in favor of oxidants is termed "oxidative stress" [4]. Regulation of reduction/oxidation (redox) state is important for cell viability, proliferation and organ function [4], however, imbalance of oxidant/antioxidant balance such as overproduction of ROS became toxic to

neurons, inducing DNA methylation [5] and various tissues damage [6]. Because the brain is highly vulnerable to oxidative stress during early development [6], oxidative stress-induced neuronal damage may occur in genetically predisposed individuals [7]. Thus, deficits in antioxidant capacity may important in the etiology of ASD. The total antioxidant capacity (TAC) in urine and plasma is a parameter used to characterize the antioxidant status of the body [8,9].

Recent studies in ASD have shown significantly reduced urinary levels of TAC, indicating increased vulnerability to oxidative damage [6], and significantly higher urinary levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [10]. further, the urinary levels of oxidative stress markers such as hexanoyl-lysine (HEL) were found

to be elevated in children with ASD, and higher HEL values correlated with the hyperactivity component of the Childhood Autism Rating Scale [11]. Thus, urinary oxidative stress-related biomarkers, including HEL, which is reportedly a more sensitive biomarker of oxidative stress [12], TAC and biomarkers of DNA methylation may provide useful information about oxidative stress-related brain damage. However, few studies have measured urinary levels of HEL, TAP and 8-OHdG.

Increased ROS with a concomitant decrease in antioxidant capacity may induce more serious damage in diseases such as leukemia [13,14]. However, few reliable studies have shown increased HEL to be concomitant with decreased antioxidant capacity in ASD.

Interestingly, a recent study revealed that decreased serum levels of SODs, which are major antioxidant enzymes [15] and contribute to human neurodegenerative disorders [16], may also be implicated in the pathophysiology of ASD [17]. Previous studies have also reported that altered plasma levels of enzymatic antioxidants such as SOD, ceruloplasmin (Cp), and transferrin (Tf) play an important role in ASD [18-25]. In spite of the important pathophysiological contribution of SOD in ASD, the role of the plasma SOD levels in relation to the urinary levels of oxidative stress-related biomarkers remains unclear.

These considerations taken together, we pursued three avenues of research in this work. First, we evaluated the link between the urinary levels of HEL, TAC and 8-OHdG and social responsiveness in individuals with ASD. Next, we assessed the role of plasma SOD levels in relation to these urinary biomarkers. Finally, we determined the relationship between HEL and TAC in urine of individuals with ASD. Because previous clinical studies have found that urinary levels of TAC are affected by diet [26,27], we also assessed dietary nutrient intake and estimated dietary TAP.

## 2. Subjects and Methods

### 2.1. Subjects

This study included a total of 31 young, physically healthy individuals in Hanshin area (Kobe and Osaka prefectures) participated in this study. They were recruited from the medical care facilities of the Research Institute of Pervasive Developmental Disorders of Ashiya University based on their presenting for a medical consultation between October 2013 and September 2015. ASD was diagnosed based on the DSM-5 criteria [28] and was confirmed by the Autism Diagnostic Interview-Revised (ADI-R) [29]. Two psychiatrists specializing in ASD and developmental disorders diagnosed all 20 individuals with ASD. Among the 31 participants, 20 had an independent clinical diagnosis of ASD (13 males and 7 females, mean age:  $11.1 \pm 5.2$  years old, age range: 6-22 years old), and the remaining 11 were normal healthy controls (7 males and 4 females, mean age:  $13.9 \pm 6.6$  years old, age range: 5-21 years old). These individuals with ASD had the core symptoms of the DSM-5 diagnostic criteria for ASD without any abnormal neurological symptoms (e.g., seizure or movement disorders). The sex and age distributions were not significantly different between

the two groups. The 20 individuals with ASD and the 11 individuals in the control group were matched on home environment, feeding habits and full intelligent quotient (IQ) scores (Table 1). The 11 normal controls were considered to be physically and mentally healthy based on initial physical and mental screening tests. At the initial screening, physical (resting blood pressure and heart rate) and clinical laboratory examinations (hematology and plasma chemistry, including plasma fatty acids, cholesterol and triglycerides) were performed on all 31 participants. These 31 participants did not have any abnormalities in their physical examinations and laboratory findings. The IQs of the individuals were estimated using the Wechsler Intelligence Scale for children and adolescents aged 6-16 years old [30] or the respective scale for adults (WAIS-R) (Table 1). Comorbid psychiatric disorders were evaluated based on the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID). None of the ASD or control individuals had any history of neurological conditions, including seizure, movement disorders, head injury, Attention-Deficit Hyperactivity Disorder or learning disorders. The additional inclusion criteria were as follows: (a) the absence of any other medical or comorbid psychiatric disorders; (b) a baseline verbal or full IQ greater than 70 as calculated by the [30] or the respective scale for adults [31] (WAIS-R) because subjects with high-functioning pervasive developmental disorders were required to have a total IQ of at least 70 [32]; and (c) no treatment with antidepressants, anxiolytic medications or neuroleptics within the three months prior to the study (the treatment of ADHD symptoms with stimulants was allowed during this study, provided that the patient's dosage was stable for at least 3 months before and during the study).

All procedures performed in this study were accordance with the ethical standards of the Fujimoto Medical Clinic in Kobe City, Japan with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. 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 (e.g., recognizing whether each participant's urinary TAP and plasma SOD levels were within the standard values according to SRL, Inc, Tokyo, Japan). The standard values of TAP levels were within standard ranges established by the Department of Pediatrics, Tokyo Metropolitan Fuchu Medical Center for the Disabled, Tokyo, Japan ( $3700 - 4000 \mu\text{M}$  uric acid equivalents in 8 normal healthy subjects aged 6-37 years) [33], and those of plasma SOD levels were within standard ranges established by SRL, Inc., Tokyo, Japan ( $1.8-3.2 \text{ U/ml}$  in 164 healthy subjects) [34]. The studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was obtained from the participants and/or their parents. This study was registered at <https://dbcentre3.jmacct.med.or.jp/jmacctr> (the Clinical Trials Registry, Japan Medical

Association, 2010/6/14; renewal, 2015/12/31; ID: JMA-IIA 00162).

## 2.2. Assessment of Dietary Nutrient Intake

Urinary levels of TAC may be affected by dietary food intake may [26,27]. Berries, fruits, nuts, chocolate, vegetables [35], and cafe and soybean oil had the highest antioxidant values [36]. Thus, participants who habitually ingested foods and snacks containing high antioxidant values (berries, fruits, nuts, chocolate, vegetables, coffee and soybean oil) were excluded from the study.

To assess the daily food intake, a semi-constructive questionnaire for the Japanese (DHQ) was performed using the junior high school version (DHQ15) (DHQ Support Center, <http://www.ebnjapan.org/>). The DHQ15 consisted of 72 questions on the frequency of intake of 150 various food and beverage items and cooking methods. DHQ15 was administered one month before the study on randomly selected subsamples of 10 individuals with ASD and 10 normal controls by the order of their submission to medical consultation during January 2013 and June 2015. The food and beverage items and portion sizes in the questionnaire were derived primarily from the data in the Overview of Dietary Reference Intake for Japanese [37]. If there was any missing or unclear information recorded on the DHQ 15 by the parents or subjects, the researcher (KY) questioned them by phone or e-mail, and information was corrected in the DHQ 15. The estimated intake of nutrients was calculated using a dedicated program for the DHQ system (DHQ Support Center, Tokyo, Japan) [38]. The validity of the DHQ15 has been already verified [39].

## 2.3. Estimation of Dietary TAC

Urinary levels of TAC have been reported to be affected by dietary food intake. Grape juice (200 ml) [38] and cocoa powder (40 g) [39] increased urinary TAC. Thus, a dietary TAC value was assigned to each food item in the DHQ15. The 31 participants in this study were children or young adolescents with age range: 6-22 years old, their nutrients were less varied and were of smaller amounts than those of adults. We therefore calculated dietary TAC value of the remaining 34 foods in the DHQ15 according to a previous study [35,42]. TAC values were expressed as  $\text{mmol Fe}^{2+}/100$  g of food for ferric reducing ability of plasma (FRAP) [42] or  $\text{mmol}/100\text{g}$  oxygen radical absorbance capacity (ORAC) [35,42]. Finally, The TAC values of 18 foods were calculated as FRAP or those of 4 foods were calculated as ORAC.

## 2.4. Urinary Assay of HEL, 8-OHdG and TAC Levels

Urinés were collected as a spot sample and immediately stored at -80°C 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.

The urinary levels of HEL were measured in duplicate using a competitive ELISA kit (Japan International Cooperation Agency-JICA, Shizuoka, Japan) [43].

For measurement of the 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 [44].

For measurement of the urinary levels of urinary total antioxidant capacity, the urinary antioxidant capacity was determined by competitive enzyme-linked immunosorbent assay (ELISA) [44]. 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).

## 2.5. Plasma Levels of SOD

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.

## 2.6. Assessment of Social Impairment

The social responsiveness scale (SRS) can be used to distinguish clinically significant ASD from other psychiatric disorders based on varying levels of social impairment [45]. The SRS is a 65-item questionnaire completed by subjects' parents for a quantitative assessment of autistic traits across a wide spectrum of children aged 4-18 or 19 and more years (adult version). The total SRS raw scores range from 0 to 195, with the highest scores corresponding to significant social impairment, as observed in individuals with severe ASD (Di Martino et al, 2009). The SRS is able to reliably distinguish ASD from other psychiatric conditions [46], and assess the severity of autistic trait [47].

## 2.7. Statistical Analyses

Because the data were not normally distributed, the non-parametric Mann-Whitney U test for multiple comparisons was used to determine the significant differences in the urine levels of HEL, 8-OHdG and TAC and plasma SOD levels as well as the five subscale and total scores on the SRS, and the intake of nutrients between the random subsamples of 10 individuals with ASD and 10 of the normal controls. Moreover, stepwise regression analysis was used to confirm the relationship between the urinary oxidative stress-related biomarkers (levels of HEL, 8-OHdG and TAC) and plasma SOD levels, and the other main variables, adjusting for the two

subject groups, and the SRS five subscale and total scores (Table 2). We conducted statistical analyses using SPSS version 18.0 (IBM Tokyo, 2009).

### 3. Results

#### 3.1. Study Population

Clinical features of in the 20 individuals with ASD were characterized by failure of normal back-and-forth conversation ( $n = 8$ ), deficits in socio-emotional reciprocity ( $n = 9$ ) and deficits in understanding relationships ( $n = 3$ ). The mean total SRS score for our patients was  $111.0 \pm 33.1$  (Table 1). Previous studies reported total SRS scores of 120.21 for children and adolescents with moderate-to-severe ASD [48]. Considering that an SRS total score between 60 and 80 is associated with a mild form of ASD [49], our patients suffered moderate or severe social impairment from ASD. Ages did not differ significantly between the ASD and control groups ( $p = 0.30$ ).

#### 3.2. Urinary Levels of Oxidative Stress Markers

There was a significant increase in urinary HEL levels ( $p = 0.018$ ) and a significant decrease in urinary TAC levels ( $p = 0.049$ ) in the 20 individuals with ASD compared to those observed in the 11 normal controls.

Urinary levels of 8-OHdG and plasma levels of SOD were not significantly different between the groups (Table 1).

#### 3.3. Predictor Variables

Table 2 summarizes the results of our stepwise multiple regression analysis. Urinary levels of TAC contributed significantly to the variables after adjusting for the two subject groups, urinary HEL levels, 8-OHdG levels, plasma SOD levels, and the five subscale and total ABC scores (Step 1,  $R^2 = 0.216$ ,  $P = 0.008$ ; step 2,  $R^2 = 0.411$ ,  $p = 0.001$ ). Using Group used as a dependent variable showed a significance of contribution to the urinary TAC levels (step 1, unstandardized coefficients,  $B = 1152.564 \pm 407.338$ ,  $\beta = 0.5465$ , and  $p = 0.008$ ; step 2, unstandardized coefficients,  $B = 3027.688 \pm 713.591$ ,  $\beta = 1.222$ , and  $p = 0.000$ ) and to the SRS cognition scores (unstandardized coefficients,  $B = 83.864 \pm 27.572$ ,  $\beta = 0.876$ , and  $p = 0.005$ ).

Furthermore, the procedure using urinary HEL levels as the independent variable terminated at step 2, explaining 22.1 % of the variance. The SRS subscale and total scores being used as the dependent variables, the statistical significance of contribution of the SRS subscale of awareness to the urinary HL levels (unstandardized coefficients,  $B = 2.312 \pm 0.807$ ,  $\beta = 0.470$ , and  $p = 0.008$ ). These findings indicate that urinary levels of TAC and HEL were predictable measures for differences in the biomarkers and the SRS scores between the ASD group and the control group (Table 2).

**Table 1. Subject characteristics and urinary levels of HEL, 8-OHdG and TAP, and the SRS scores in the 20 individuals with ASD and 11 normal controls**

| Variables   | ASD (n=20)       | Controls (n= 11) | U             | p value  |
|---|------------------|------------------|---------------|----------|
| Age (Year)  |                  |                  |               |          |
| Mean±S.D.   | 11.1±5.2         | 13.9±6.6         | 84.00         | 0.30     |
| Sex (male/female)                                     | 13/7             | 7/4              | $\chi^2=0.00$ | 1.00     |
| Full IQ   | 100.9±25.91      | 109.38±18.45     | 32.00         | 0.52     |
| Scores of Autism Diagnostic Interview-Revised (ADI-R) |                  |                  |               |          |
| Domain A (social)                                     | 13.6±6.3         | —                |               |          |
| Domain B (communication)                              | 8.0±4.5          | —                |               |          |
| Domain C (stereotyped)                                | 11.2±6.0         | —                |               |          |
| Urinary levels  |                  |                  |               |          |
| HEL (pmol/mg Cre)                                     | 77.10± 30.56     | 51.76 ± 26.16    | 53.00         | 0.018*   |
| 8-OHdG (ng/mL Cre)                                    | 11.31± 5.50      | 19.51± 32.16     | 105.50        | 0.86     |
| TAP (µMUrlic Acid equivalents)                        | 2986.18 ± 999.25 | 4169.71±1285.03  | 62.000.       | 049*     |
| Plasma SOD levels (U/ml)                              | 3.86 ± 3.12      | 3.64 ± 2.82      | 103.00        | 0.79     |
| Subscale scores of the SRS                            |                  |                  |               |          |
| Irritability  | 12.80 ± 4.4      | 12.73 ± 3.70     | 8.50          | 0.000*** |
| Social withdrawal                                     | 25.80 ± 7.13     | 3.10±4.7         | 40.00         | 0.000*** |
| Stereotypy  | 41.25 ± 11.05    | 4.82±7.17        | 1.50          | 0.000*** |
| Hyperactivity   | 21.60±5.28       | 4.73±5.49        | 2.50          | 0.000*** |
| Inappropriate speech                                  | 26.35±27.00      | 2.18±3.97        | 0.50          | 0.000*** |
| Total   | 114.94±31.34     | 17.36± 23.40     | 3.00          | 0.000*** |

HEL, hexanoly-l-lysine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine, TPA, Total Antioxidant Power; SRS, Social Responsiveness Scale

Values are mean ± SD

\*  $p < 0.05$ , \*\*\*  $p < 0.001$  compared to normal controls.

**Table 2. Results of the stepwise regression analysis**

| Model                        | Model          |         | Model Coefficients |       |         |
|------------------------------|----------------|---------|--------------------|-------|---------|
|                              | R <sup>2</sup> | P-value | B                  | Beta  | p value |
| <b>TAP</b>                   |                |         |                    |       |         |
| Step 1                       | 0.219          | 0.008*  |                    |       |         |
| Group (1 = ASD, 2 = control) |                |         | 1183.529 ± 415.231 | 0.468 | 0.008*  |
| Step 2                       | 0.416          | 0.001*  |                    |       |         |
| Group (1 = ASD, 2 = control) |                |         | 109.753±725.554    | 1.229 | 0.000** |
| SRS cognition scores         |                |         | 86.149± 28.034     | 0.881 | 0.005*  |
| <b>HEL</b>                   |                |         |                    |       |         |
| SRS awareness scores         | 0.212          | 0.009*  | 2.244± 0.803       | 0.460 | 0.009*  |

TAP = total antioxidant power; R<sup>2</sup> = R-squared values; B =unstandardized coefficients; ASD = autism spectrum disorder; SRS =Social Responsiveness Scale

\*  $p < 0.05$ , \*\*  $p < 0.001$ , significant contribution.

**Table 3. The intake of nutrients in the random subsamples of 10 individuals with ASD and random subsample of 10 normal controls**

|                         | ASD (n = 10) | Control (n = 10) | U    | p value |
|-------------------------|--------------|------------------|------|---------|
| Age (years)             | 11.4± 3.9    | 10.1±3.9         | 35.0 | 0.45    |
| High (cm)               | 142.1±21.7   | 141.1±20.2       | 42.5 | 0.84    |
| Wight (kg)              | 39.5 ± 21.6  | 39.2± 18.1       | 45.5 | 1.00    |
| Energy (kcal)           | 2166.0±523.6 | 2231.8 ±617.5    | 40.0 | 0.72    |
| Fat (g/day)             | 71.6± 26.2   | 78.8±23.3        | 38.0 | 0.60    |
| Protein (g/day)         | 79.2± 23.3   | 80.8± 27.0       | 43.0 | 0.91    |
| Animal protein (mg/day) | 32.4±8.0     | 33.8±12.0        | 41.0 | 0.78    |
| Cholesterol (mg/day)    | 138.4±179.0  | 127.8 ±134.6     | 37.0 | 0.55    |
| Carbohydrates (g/day)   | 297.8±58.6   | 292.2±86.8       | 45.0 | 1.00    |
| Vitamin B2 (mg/day)     | 1.8± 0.7     | 1.7± 0.8         | 44.0 | 0.97    |
| Vitamin B6 (mg/day)     | 1.4± 0.5     | 1.3± 0.4         | 42.5 | 0.84    |
| Vitamin B12 (mg/day)    | 7.3± 4.0     | 7.0±2.3          | 44.5 | 0.97    |
| Vitamin C (mg/day)      | 1678.0± 79.4 | 119.4± 74.5      | 28.0 | 0.18    |
| Omega-3 (mg/day)        | 216.5± 161.6 | 192.7±90.8       | 36.0 | 0.50    |
| Omega-6 (mg/day)        | 384.5 ±230.7 | 357.7± 121.3     | 35.0 | 0.44    |

Values are mean ± SD.

**Table 4. Dietary TAP in the random subsamples of 10 individuals with ASD and 10 normal controls**

|                              | ASD (n = 10) | Control (n = 10) | U    | p value |
|------------------------------|--------------|------------------|------|---------|
| <b>FRAP</b>                  |              |                  |      |         |
| Non alcohol beverages        |              |                  |      |         |
| Green tea                    | 7.13± 5.7    | 8.72±4.17        | 42.5 | 0.58    |
| Fruit juice                  | 2.79± 4.28   | 1.89±4.19        | 31.5 | 0.17    |
| Vegetables                   |              |                  |      |         |
| Cabbage                      | 0.26 ±0.26   | 0.16±0.13        | 39.5 | 0.44    |
| Tomatoes                     | 0.08± 0.08   | 0.10± 0.09       | 43.0 | 0.63    |
| Lettuce                      | 0.09 ± 0.05  | 0.07± 0.06       | 41.0 | 0.53    |
| Fruits                       |              |                  |      |         |
| Oranges                      | 0.65 ± 0.78  | 0.41± 0.51       | 39.5 | 0.44    |
| Strawberries                 | 1.10± 1.78   | 0.44± 0.48       | 43.0 | 0.63    |
| Sugar confectioneri          |              |                  |      |         |
| Chocolate                    | 0.94± 0.80   | 0.29± 0.33       | 20.0 | 0.02*   |
| Jam and marmalade            | 0.15± 0.13   | 0.00 ± 0.00      | 45.0 | 0.007*  |
| Cookies and biscuits         | 0.06± 0.05   | 0.02± 0.02       | 22.0 | 0.04*   |
| Pancakes                     | 0.03± 0.03   | 0.02± 0.02       | 41.0 | 0.53    |
| ORAC                         |              |                  |      |         |
| Breakfast cereals            | 0.63± 0.46   | 0.42±0.35        | 40.5 | 0.48    |
| Egg                          | 0.03± 0.01   | 0.02±0.01        | 35.0 | 0.28    |
| Meat and meet products       | 0.17 ± 0.09  | 0.20±0.20        | 43.5 | 0.63    |
| Poultry and poultry products | 0.06± 0.05   | 0.07±0.07        | 42.5 | 0.58    |

FRAP = ferric reducing ability of plasma; ORAC = oxygen radical absorbance capacity

Values are mean ± SD

\* $p < 0.05$ .

### 3.4. Association between Urinary HEL, TAC and 8-OHdG Levels, and Plasma SOD Levels and Their Relations to the SRS Scores

There were significant correlations between urinary TAC levels, and plasma SOD levels ( $r = 0.46$ ,  $p = 0.04$ ) and SRS subscale scores of cognition ( $r = 0.47$ ,  $p = 0.04$ ) in the ASD group. Urinary HEL levels were significantly correlated with the SRS subscales of awareness ( $r = 0.60$ ,  $p = 0.005$ ) and mannerisms ( $r = 0.69$ ,  $p = 0.001$ ). Plasma SOD levels significantly correlated with SRS subscale scores of communication ( $r = 0.58$ ,  $p = 0.007$ ) and total scores ( $r = 0.65$ ,  $p = 0.02$ ).

### 3.5. Assessment of Nutrient Intake

There were no significant differences in weight, high, energy intake, or in the intake of protein ( $p = 1.00$ ), cholesterol ( $p = 0.49$ ), vitamin B2 ( $p = 0.97$ ), vitamin B12 ( $p = 0.97$ ), vitamin C ( $p = 0.18$ ), omega-6 ( $p = 0.44$ ), iron ( $p = 0.55$ ), copper ( $p = 0.55$ ), omega-3 ( $p = 0.50$ ) and omega-6 PUFAs ( $p = 0.44$ ) between the ASD and control groups (Table 3).

### 3.6. Dietary Total Antioxidant Power

The ASD group consumed significantly more dietary TAC in the form of chocolate ( $p = 0.02$ ), cookies and biscuits ( $p = 0.04$ ), and jam and marmalade ( $p = 0.007$ ) compared to the control group. However, there was no significant correlation between dietary TAC and urinary levels of TAP ( $r = 0.02$ – $0.57$ ,  $p = 0.95$ – $0.053$ ) in ASD groups (Table 4).

## 4. Discussion

The present study first measured a set of urinary levels of HEL, TAC and 8-OHdG, and revealed significantly decreased levels of TAC and significantly increased levels of HEL in urine of the 20 individuals with ASD compared with the 11 healthy controls. It was also shown that there were no significant differences in urinary 8-OHdG levels between the groups.

Stepwise multiple regression analysis identified significant correlations between the urinary levels of both TAC and HEL and the adjusting variables (the two subject groups, the levels of other urinary biomarkers and the total ABC scores) in the whole population. Thus, the urinary levels of TAC and HEL were appropriate models for distinguishing the ASD group from the control group and significantly predicted the adjusting variables. These findings suggest that the urinary levels of TAC and HEL may contribute to social impairment in individuals with ASD.

A previous study revealed that the urinary levels of TAC were significantly lower without a concomitant increase in urinary catalase activity and total thiol molecules, which are the main indicators of antioxidant capacity [50] in 29 children with ASD aged 6–12 years than in 24 age-matched healthy controls [6]. Further, 34 adolescent individuals with Asperger syndrome (mean age

12.89  $\pm$  2.58 years) showed significantly reduced plasma and erythrocyte levels of TAP compared to 34 age-matched controls, indicating a chronically low detoxifying capacity in these ASD individuals [25]. Regarding urinary HEL levels, a previous study reported that 24 children with ASD aged 5 - 12 years showed elevated levels of urinary HEL without concomitant alterations in the urinary levels of 8-OHdG or the erythrocyte levels of SOD, which may result from a deficit in membrane fluidity [11]. Further, a case-control study reported that 68 children with ASD aged 3–10 years showed significantly increased levels of 8-OHdG in peripheral lymphocyte DNA compared to 54 age-matched healthy controls [10], indicating a deficit in antioxidant and methylation capacity that promote cellular damage and alter epigenetic gene expression. As indicated in this earlier works, pro-oxidant environmental stressors may act in conjunction with a genetic predisposition toward the development of ASD [10]. Previous studies evaluated the following parameters: 1) urinary levels of TAP alone [25]; 2) urinary levels of TAC in concomitance with urinary catalase activity and total thiol molecules [6]; 3) urinary levels of 8-OHdG alone [10,51]; and 4) urinary levels of 8-OHdG in concomitance with HEL [11]. According to recent measurements, a set of oxidative stress-related biomarkers such as urinary levels of HEL, TAC and 8-OHdG may provide useful information on neuronal damage by oxidative stress [52,53]. The present study first examined the urinary levels 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 individuals with ASD demonstrated in this study indicated an imbalance between oxidative stress and antioxidant capacity. Moreover, stepwise regression analysis revealed that the urinary levels of TAC and HEL were reliable measures for distinguishing the ASD group from the control group. A previous review article suggested that declining ROS levels are coupled with increased antioxidant enzyme activities, depending on the formation of antioxidant enzymes relative to the changes in the levels of free radicals [54]. Moreover, growing evidence indicates that the imbalance between ROS production and TAC may correlate with ASD [55,56]. Thus, the present study first revealed detailed and concrete findings on the imbalance between oxidative stress and TAC in the urine of individuals with ASD. These findings may provide additional and useful information on the significance of imbalance between increased oxidative stress and impaired urinary antioxidant systems in the pathophysiology of individuals with ASD. Although elevated levels of 8-OHdG in plasma or urine is a commonly used biomarker for assessing oxidative DNA damage during inflammatory disease and pro-oxidant exposures [10], DNA methylation analysis of ASD reveals multiple dysregulated biological pathways [57]. Thus, the ASD group in this study may be a subset of ASD without DNA methylation damage.

Stepwise regression analysis and Spearman correlation coefficients revealed that urinary TAC levels were significantly correlated to plasma SOD levels and the SRS subscale scores for cognition. Previous studies reported significant decrease in plasma and urine SOD and

TAC levels in 47 sarcoma patients [58] and significant increase in urine SOD and TAC levels after administration of L-carnitine in 12 healthy subjects [59], indicating concomitant changes of plasma and urine SOD and TAC, supporting our findings. A previous study reported a significant positive correlation between plasma TAC levels and cognitive function in 105 subjects with early onset-first episode psychosis [60], and an association between lowered plasma TAP levels and cognitive dysfunction in 138 older adults with mild cognitive dysfunction [61] and in 90 healthy care workers [62]. The Spearman correlation coefficient also revealed a significant association between plasma SOD levels and the SRS subscale of communication. A previous study reported significant correlations between blood levels of SOD and psychosocial functioning such as “relation to friends” and “taking initiative and self-sufficiency” in 51 subjects with bipolar depression [63]. Social interaction-mediated lifespan extension of SOD mutants has been found in human [64]. Thus, SOD may be significantly correlated to social behavior, which supports our present findings.

Stepwise regression analysis and Spearman correlation coefficients further revealed that the urinary HEL levels were significantly correlated to the SRS subscale scores for awareness and mannerisms. Of reference, the plasma levels of 3-nitrotyrosine (3-MT), which is an oxidative stress marker [65], have previously been associated with low scores in SRS subscales of awareness in 18 children with ASD [66]. Sulforaphane, which has therapeutic potential against oxidative stress, was shown to improve SRS scores by 17% [48]. Thus, oxidative stress biomarkers are associated with the SRS scores, which supports our findings.

A previous assessment of daily nutrient intake using DHQ 15 revealed no significant differences in the intake of fat, protein, vitamin B2, vitamin B6, vitamin B12, vitamin C, omega-6, or omega-3 PUFAs between random subsamples of 10 individuals with ASD and those of 10 healthy controls. With respect to the relationship between dietary intake and the urinary levels of TAP, intake of virgin olive oil [67] and tryptophan-enriched cereal [68] have been shown to increase urinary TAC. Conversely, refined potato starch resulted in decreased urinary levels of TAC [69]. Therefore, the effect of dietary intake on urinary TAC may be variable, and differences in dietary intake between the groups may not affect urinary TAC.

The dietary TAC of chocolate, biscuits and cookies, jam and marmalade was significantly higher in the ASD group than in the control group. Cocoa products such as chocolate are good sources of dietary antioxidants [70]. Cookies containing chocolate chips have been shown to possess the highest antioxidant capacity [71]. Biscuits including 5 % cocoa increased antioxidant properties [72]. Jam and marmalade processing has been shown to increase antioxidant capacity [73], and bilberry jams have been revealed as a good source of antioxidant compounds [74,75]. Thus, chocolate, cookies, biscuits, jam and marmalade possess higher antioxidant power. However, urinary TAC levels in the ASD group were significantly lower than those in the control group, indicating that antioxidant capacity in the ASD group may be impaired.

Oxidative stress contributes to many pathological conditions. Aerobic organisms have integrated antioxidant

systems, which include enzymatic and nonenzymatic antioxidants that are usually effective in blocking the harmful effects of ROS [76]. Such systems include the endogenous antioxidant defense system [77] and intrinsic antioxidant defenses [78]. Most previous studies on antioxidant capacity in individuals with ASD have suggested a vulnerability in antioxidant enzymes [24] or a chronically low detoxifying capacity [25]. 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 [79]. Further, a recent work has suggested that the autophagy-lysosomal activities of these antioxidant enzymes may serve an essential function in preventing neurodegenerative diseases by removing damaged as part of an essential cellular antioxidant pathway [80]. These considerations taking together suggested that the endogenous intrinsic antioxidant system may be impaired in individuals with ASD. However, the present study did not examine the specific neurobiological roles of urinary TAP and plasma SOD, and further studies will need to determine which specific components may be affected in ASD.

Compromised intrinsic antioxidant defense contribute to blood-brain barrier (BBB) disruption through oxidative damage to cellular molecules [81]. When BBB disruption occurs, brain-specific proteins circulating inside the brain, are observed in the peripheral blood as an index for estimating the extent of the increase in BBB permeability and brain damage [81]. As described above, overproduction of ROS results in oxidative stress the imbalance between ROS and antioxidant capacity (referred to oxidative stress) became toxic to neurons, damaging DNA, proteins and lipids, inducing neurodegeneration [4] such as ASD [82]. Thus, the imbalance between the generation of ROS and TAC may disrupt BBB, and be related to impaired social responsiveness through biochemical processes such as imbalance between increased urinary levels of HEL as oxidants and urinary levels of TAC as antioxidant in individuals with ASD.

This study has some limitations. 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 [83,84]. In this study, the urinary levels of 2-isoprostanes and plasma levels of glutathione were not measured; however, we examined a useful and informative set of oxidative stress-related biomarkers and discovered novel important information on impaired antioxidant capacity that was not revealed in previous studies [6,11,25,85]. Second, ASD is most prevalent in males, with a male to female ratio of 4 to 1 [86]. However, in this study, the ASD and control groups were age- and gender-matched. 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 firstly found reduced levels of TAC and increased levels of HEL in urine in individuals with ASD with a lack of significant alterations in urinary 8-OHdG levels. Thus, the imbalance between

the generation of ROS and TAC was impaired in individuals with ASD. In addition, as stepwise regression analysis revealed that urinary TAC levels showed preferentially contribute to the adjusting variables compared to urinary HEL levels, urinary TAC levels may be more appropriate model for distinguishing the ASD group from the control groups. Thus, endogenous antioxidant defense systems may be impaired in young individuals with ASD. Plasma SOD levels may be associated with urinary TAC levels and may be related to the impaired antioxidant capacity. These defects in the balance between oxidative stress and antioxidant capacity in urine may be related to the pathophysiology of social impairment in individuals with ASD.

## Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (Grant No 21200017) (2010-2012) and a Grant-in-Aid for Scientific Research (C) (2014-2016) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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