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Study on oxidative stress and inflammatory/antioxidant substance levels in autism spectrum disorder

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Abstract

Background: The etiology of autism spectrum disorder (ASD) includes oxidative stress and brain inflammation. We investigated the relationship among oxidative stress markers, in vivo inflammatory substances, and antioxidants that can be easily measured in the clinic and compared them between children with ASD and those with typical development (TD).

Methods: Sixty-one children with TD and 199 with untreated ASD were investigated. They were Japanese children aged 2–15 years and were divided into those aged <7 and ≥7 years. Serum levels of reactive oxygen metabolites (ROMs), high-sensitivity C-reactive protein (hsCRP), prolactin (PRL), albumin (Alb), total bilirubin (T-Bil), and uric acid (UA) were measured. These measurements were compared between TD and ASD, and the relationship between oxidative stress and relevant laboratory parameters was analyzed.

Results: The hsCRP and PRL levels were significantly higher in patients with ASD than in those with TD. Among those aged <7 years, hsCRP and PRL were significantly higher in those with ASD than in those with TD. Among those aged ≥7 years, ROMs, hsCRP, and PRL were significantly higher in those with ASD than in those with TD. In ASD, ROMs were significantly correlated with hsCRP, Alb, T-Bil, and PRL. In contrast, no significant correlations were found in the TD group except for the relationship between ROMs and hsCRP in those aged <7 years.

Conclusion: The results suggest that serum levels of in vivo inflammatory substances, stress-related substances, and antioxidants are altered in ASD under oxidative stress.

Keywords: Autism spectrum disorder; Hydroperoxide; Inflammation; Oxidative stress

1. INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by the core symptoms of "persistent impairment in reciprocal social communication and interpersonal interaction" and "restricted, repetitive patterns of behavior, interests, or activities."[1] The etiology, pathogenesis, and treatment evaluation of ASD are not well established.

Genetic and environmental factors have been implicated in the etiopathogenesis of ASD.[2] Additionally, inflammation and biochemical changes in the body may be risk factors for ASD. Oxidative stress, which is involved in the pathogenesis of various diseases, has also been suggested to be associated with ASD.[3]

Oxidative stress is caused by an increase in the levels of reactive oxygen species (ROS) or free radicals and a decrease in the levels of antioxidant enzymes/substances. Inflammation and antioxidants are involved in the elimination of cytosolic and plasma ROS. They also help prevent the lipid peroxidation of the plasma membrane,[4] thereby playing crucial roles in maintaining the redox equilibrium in vivo.

We compared typical development (TD) and ASD using reactive oxygen metabolites (ROMs), a marker of oxidative stress, and found that high oxidative stress is involved in the development of ASD.[5]

However, no studies have examined the interrelationship between oxidative stress and high-sensitivity CRP (hsCRP) or prolactin (PRL) in ASD. Therefore, we focused on blood investigations related to ASD, which are easy to measure clinically, minimally invasive, and quick to determine, and results can be quantified and are easy to understand.

This study measured oxidative stress, inflammatory substances, and endogenous antioxidants, which can be measured

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in daily practice. They were then compared and examined in TD and ASD.

2. METHODS

2.1. Subjects

This study included Japanese children aged 2–15 years. We enrolled those seen for the first time between April 2018 and March 2021, and their consent was obtained. There were 61 children with TD (30 males and 31 females) and 199 children with untreated ASD (149 males and 50 females). It has been reported that ROM values are high in TD in infancy and decrease with growth and development. Adult levels are achieved after 7 years of age.[6] Subjects were divided into preschool (<7 years of age: TD, n = 20 and ASD, n = 74) and school (≥7 years of age: TD, n = 41 and ASD, n = 125) children.

Children were included in the TD group if a pediatrician assessed them as mentally and physically healthy. The exclusion criteria were: (1) the presence of underlying disease, (2) presence of perinatal abnormalities, (3) history of drug or supplement use for treating or preventing ASD, (4) history of any disease diagnosed within the month before enrollment in the present study, and (5) presence of a suspected developmental disorder.

Eligibility criteria for enrollment in the ASD group were: (1) diagnosis of ASD by a pediatric neurologist according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, parent interview ASD Rating Scales–Text Revision scores, and clinical symptoms, and (2) absent history of ASD treatment. The exclusion criteria were: (1) presence of underlying disease other than developmental disorders and (2) history of drug or supplement use for treating or preventing ASD.

Attention deficit hyperactivity disorder and mental retardation were comorbidities in 78.4% and 26.2% of ASD subjects, respectively.

2.2. Items measured

The serum ROM level was measured as an indicator of oxidative stress. In the ROM test, ROS and free radicals in vivo were not directly measured, but the blood hydroperoxides generated by ROS and free radicals were measured based on the color reaction to evaluate the oxidative stress level in vivo. Hydroperoxides are stable chemical substances generated by the oxidation of various organic molecules (eg, proteins, amino acids, peptides, glucosides, lipids, and nucleotides). Hydroperoxides are indicators of oxidative stress because they produce free radicals in the presence of metal ions.

ROS and free radicals cannot be easily measured *in vivo* because of their short lifespan and high reactivity. Therefore, the ROM test was developed to reproduce the "in vivo" phenomena "in vitro." The ROM test is characterized by its simplicity, rapidness, and high reproducibility.[7] Additionally, it has been confirmed that there is no diurnal variation, sex difference, or correlation with electron spin resonance.[7]

Tests were performed to measure the levels of inflammatory/antioxidant substances in the serum. We assessed the levels of serum hsCRP, which is an indicator of inflammation, and those of albumin (Alb), total bilirubin (T-Bil), and uric acid (UA), which are antioxidants in vivo. PRL levels, influenced by the degree of stress, were also measured.

2.3. Measurement methods

Blood samples were collected from the subjects between January 2017 and August 2019. Serum samples were collected simultaneously at a single point during the day, centrifuged at 1469*g* for 10 minutes, and measured after frozen storage in a deep freezer where the serum samples were obtained. Blood samples were taken in normal physical condition, avoiding when the patient was sick or after exercise. No restrictions on diet or sleep were applied.

2.4. ROM test

ROMs were measured using the ROM [FOR FREE] reagent (R1, R2) (Wismerll, Tokyo, Japan) and the free radical analyzer "FREE CARRIO DUO" (Diacron International, Grosscto, Italy), according to the manufacturer's instruction. Twenty microliters of plasma were placed in a cuvette containing acetic buffer (pH 4.8) and mixed ($Fe2^+$ and $Fe3^+$ were released from blood proteins). In this process, hydroperoxides in the blood are degraded to alkoxyl radicals and peroxy radicals, catalyzed by $Fe2^+$ and $Fe3^+$. Next, by adding 20 μL of a coloring chromogen (N, N-diethyl paraphenylenediamine), free radicals oxidize chromogen substrates to generate red-colored radical cations. The solution is mixed, and the cuvette is placed in a photometer for optical measurement at 505 nm. The measurement time was 5 minutes. The results are expressed in U. CARR of 1 U corresponded to 0.08 mg/dL of H_2O_2.

2.5. General blood tests

Serum hsCRP was measured by latex nephelometry using the "fully automated immunochemistry analyzer BN-II" (Siemens Healthcare Diagnostics, Tokyo, Japan). Serum Alb and T-Bil levels were measured using the bromocresol purple improvement method and the Japan Society of Clinical Chemistry standardization method, respectively, using "LABOSPECT 006" (Hitachi High-Tech, Tokyo, Japan). Moreover, serum UA was measured by the uricase/peroxidase method using "LABOSPECT 006" (Hitachi High-Tech). Serum PRL was measured by chemiluminescence immunoassay using the "Cobas 8000 e801 module" (Roche Diagnostics, Tokyo, Japan).

2.6. Statistical analysis

Two-sided tests were performed, with a significance level of $p < 0.05$. A nonparametric test (Mann–Whitney U test), for comparing independent groups, was used to compare the ASD and TD groups. In the ASD group, the F test showed that the serum ROM levels were nonparametrically correlated with other laboratory parameters examined, and Spearman's rank correlation coefficient was used to calculate the correlation coefficient ρ and the *p* value. EZR was used for receiver operating characteristic (ROC) analysis, and IBM SPSS Statistics version 21 was used for other analyses.

2.7. Ethical considerations

This study was conducted with the approval of the Ethics Committee of the Japanese Red Cross Tokushima Hinomine Rehabilitation Center for People with Disabilities. A written explanation was given to the subjects and their parents, and their informed consent was obtained in writing. Care was taken to protect the subjects' personal information and ensure that no disadvantages accrued for the subjects in accordance with the principles of the Declaration of Helsinki.

3. RESULTS

Fig. 1 shows the serum hsCRP levels of children with TD and ASD of all ages. Serum hsCRP levels were significantly higher in the ASD group than in the TD group. The area under the curve (AUC) by ROC analysis was 0.788, with a 95% CI of 0.719-0.837. The cutoff value was 0.015 mg/dL (sensitivity 0.780, specificity 0.714).

Fig. 2 shows the serum PRL levels of the children with TD and ASD of all ages. PRL levels were significantly higher in the ASD

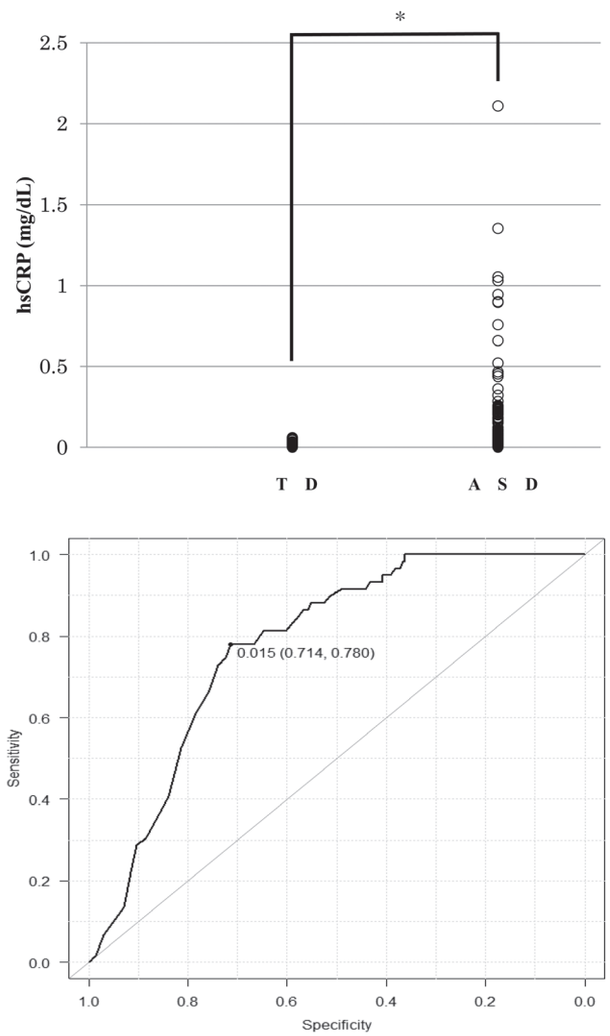

Fig. 1 Comparison of hsCRP in TD and ASD. (All subjects) n = 260 (TD = 61, ASD = 199) TD: 0.014±0.013, ASD: 0.117±0.244, $p < 0.001$. Mann–Whitney's U test was used for statistical analysis. AUC was 0.788, with a 95% CI of 0.719-0.837. ROC analysis was used for statistical analysis. 95% CI = 95% confidence interval; ASD = autism spectrum disorder; AUC = Area under the curve; hsCRP = high-sensitivity C-reactive protein; TD = typical development; *$p < 0.05$.

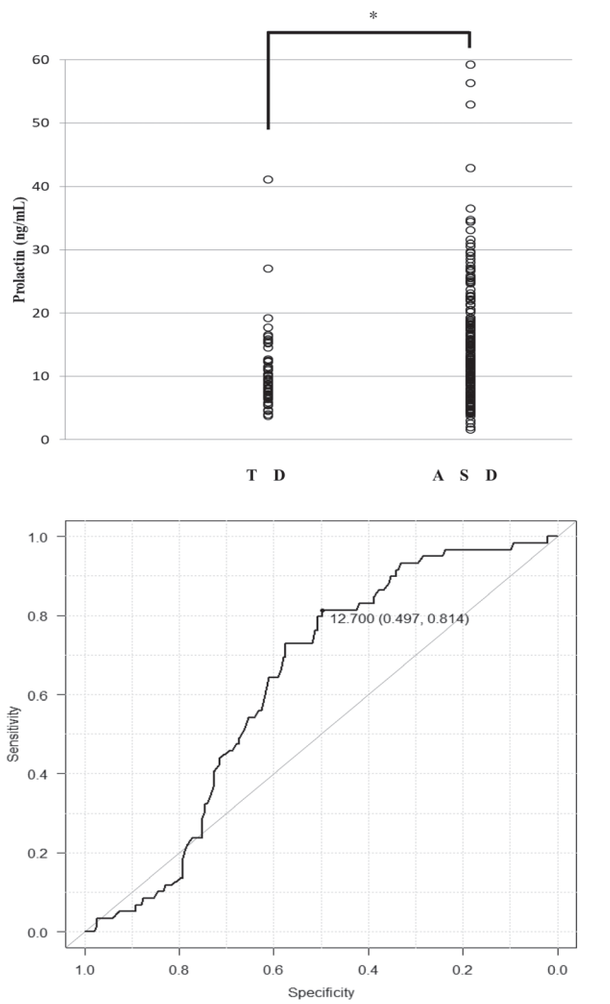

Fig. 2 Comparison of PRL in TD and ASD. (All subjects) n = 260 (TD = 61, ASD = 199) TD: 10.5±5.72, ASD: 14.8±9.51, $p = 0.002$. Mann–Whitney's U test was used for statistical analysis. AUC was 0.63, with a 95% CI of 0.558-0.702. ROC analysis was used for statistical analysis. 95% CI = 95% confidence interval; ASD = autism spectrum disorder; AUC = Area under the curve; TD = typical development; PRL = prolactin; *$p < 0.05$.

group than in the TD group. The AUC was 0.63 (95% CI 0.558-0.702), with a cutoff value of 12.7 ng/mL (sensitivity 0.814, specificity 0.497). The hsCRP and PRL cutoffs were exceeded in 152 (76.4%) and 136 (68.3%) children with ASD, respectively.

Table 1 shows the serum levels of oxidative stress and inflammation markers, PRL, and antioxidants in children with TD and ASD at all ages. The serum ROM, hsCRP, and PRL levels were significantly higher in the ASD group than in the TD group. However, the serum Alb and T-Bil levels were significantly higher in the TD group than in the ASD group. There was no significant difference in serum UA levels between the ASD and TD groups.

In those aged <7 years, serum ROM levels were not significantly different between the two groups but tended to be higher in the ASD group. The serum hsCRP and PRL levels were significantly higher in the ASD group than in the TD group. There was no significant difference in the serum levels of any antioxidant marker between the two groups.

In those aged ≥7 years, serum ROM, hsCRP, and PRL levels were significantly higher in the ASD group than in the TD group. However, the serum Alb and T-Bil levels were significantly higher in the TD group than in the ASD group, and there was no significant difference in the serum UA levels between the two groups.

Table 2 shows the relationship between serum ROM, hsCRP, PRL, and antioxidant levels in children with TD and ASD aged <7 and ≥7 years.

In the TD group, no significant correlations were found except for hsCRP in those aged <7 years. In the ASD group, serum ROM levels were significantly correlated with serum hsCRP, PRL, Alb, and T-Bil in both age groups. A significant correlation between serum ROM and UA levels was observed only in those aged ≥7 years.

4. DISCUSSION

This study showed that oxidative stress levels are higher in children with ASD than in children with TD, with significant differences in the blood levels of inflammatory/antioxidant substances and PRL between the two groups. Additionally, the results revealed that the serum levels of oxidative stress markers were correlated with the serum levels of inflammatory/antioxidant

Table 1

Comparison of oxidative stress, inflammation, and antioxidants in TD and ASD

		All subjects (TD = 61, ASD = 199)			<7 y (TD = 20, ASD = 74)			≥7 y (TD = 41, ASD = 125)		
		TD	ASD		TD	ASD		TD	ASD	
	Unit	Mean ± SD		*p*	Mean ± SD		*p*	Mean ± SD		*p*
ROM	U.CARR	328.2 ± 61.1	400.8 ± 60.9	<0.001	399.2 ± 58.1	430.2 ± 63.8	0.073	292.1 ± 24.6	383.8 ± 52.3	<0.001
hsCRP	mg/dL	0.014 ± 0.013	0.117 ± 0.244	<0.001	0.025 ± 0.016	0.148 ± 0.220	<0.001	0.009 ± 0.007	0.099 ± 0.257	<0.001
PRL	ng/mL	10.5 ± 5.72	14.8 ± 9.51	0.002	9.83 ± 3.75	17.2 ± 8.29	<0.001	10.0 ± 4.15	14.1 ± 7.79	0.042
Alb	g/dL	4.58 ± 0.27	4.41 ± 0.27	<0.001	4.45 ± 0.274	4.37 ± 0.299	0.111	4.64 ± 0.247	4.43 ± 0.250	<0.001
T-Bil	mg/dL	0.36 ± 0.20	0.29 ± 0.11	0.016	0.28 ± 0.11	0.29 ± 0.11	0.625	0.40 ± 0.23	0.30 ± 0.11	0.01
UA	mg/dL	4.23 ± 1.41	4.32 ± 1.25	0.755	4.00 ± 0.716	3.99 ± 1.34	0.437	4.49 ± 1.63	4.52 ± 1.16	0.39

Alb = albumin; ASD = autism spectrum disorder; hsCRP = high-sensitivity C-reactive protein; 1U.CARR = Hydrogen peroxide water at 0.08 mg/dL; ROMs = reactive oxygen metabolites; PRL = prolactin; T-Bil = total bilirubin; TD = typical development; UA = uric acid.

Table 2

Relationship between ROM and in vivo substances in TD and untreated ASD (Comparison between <7-year group and ≥7-year group)

	ROM value							
	T D				ASD			
	<7 y		≥7 y		<7 y		≥7 y	
	ρ	*p*	ρ	*p*	ρ	*p*	ρ	*p*
hsCRP	0.672	<0.001[a]	0.295	0.065	0.355	0.003[a]	0.564	<0.001
PRL	0.048	0.831	–0.244	0.126	0.374	0.002[a]	0.198	0.045[a]
Alb	–0.408	0.074	0.254	0.113	–0.325	0.006[a]	–0.261	0.004[a]
T-Bil	–0.201	0.381	–0.165	0.302	–0.237	0.046[a]	–0.221	0.016[a]
UA	–0.291	0.204	–0.131	0.411	–0.054	0.649	–0.201	0.027[a]

TD: < 7 years, n = 20; ≥7 years, n = 41; ASD: < 7 years, n = 74; ≥7 years, n = 125.

Alb = albumin; ASD = autism spectrum disorder; hsCRP = high-sensitivity C-reactive protein; ROMs = reactive oxygen metabolites; PRL = prolactin; T-Bil = total bilirubin; TD = typical development; UA = uric acid.

[a]Statistically significant differences $p < 0.05$.

substances in children with ASD and that these values and their correlations were age-dependent. Moreover, the ROM level in the TD was significantly correlated with hsCRP alone in those aged <7 years but not with the other measured substances or in those aged ≥7 years. This indicates a different result between TD and ASD.

Regarding the relationship between inflammation and oxidative stress, reports indicate that serum ROM levels are elevated in inflammatory diseases such as Crohn's disease and rheumatoid arthritis and correlated with serum CRP levels.[8,9] These results suggest that inflammation associated with oxidative stress exists in vivo in ASD children. The study showed that the serum ROM level increased in children with ASD.

Inflammatory and immune system abnormalities are reportedly involved in the etiopathogenesis of ASD.[10] In particular, the activation of brain microglia, which plays a vital role in brain inflammation and immunity, has attracted attention. Gandal et al reported that the number of microglia and astrocytes in the brain increases in ASD and that gene expression abnormalities in the microglia are characteristic of ASD.[11] Furthermore, according to the hypothesis by Kato et al on changes in microglial activity during the growth/development process, microglial activity is high in infancy and decreases with growth.[12] This agrees with the results of our study in which age-related changes in serum ROM levels were investigated. Microglial activity that decreases with age is reactivated by psychological stress, and the elevated activity levels persist.[12] This finding is consistent with the reported relationship between age and high serum ROM levels in children with ASD.

Reports indicate that nerve inflammation is associated with damage, increased permeability, and decreased function of the blood–brain barrier (BBB).[13] It has also been noted that BBB dysfunction can cause infiltration of peripheral substances, such as immune cells, which can affect inflammation and oxidative stress.[14] Although it is unclear whether all these results apply to ASD, it seems possible that oxidative stress and inflammation in the brain can also cross the BBB in ASD and be confirmed in peripheral blood.

In addition, changes in the intestinal microbiota have been reported to be associated with ASD. The frequency of increased intestinal permeability caused by disturbances in the intestinal microbiota is about 7.5 times higher in ASD than in controls.[15] This suggests that increased intestinal permeability may allow pathogens to enter the body through the intestinal tract and increase blood levels of inflammatory cytokines, which may pass through the BBB and cause neuroinflammation. The relationship between ASD and gut microbiota may be one of the mechanisms for the elevated ROM and hsCRP levels observed in peripheral blood in this study.

Therefore, measuring serum hsCRP and ROM levels in patients with ASD might be a simple and valuable method for clinical diagnosis and treatment. However, it will be necessary to measure and evaluate CRP levels in cerebrospinal fluid in the future to eliminate the effects of oxidative stress and inflammation in peripheral blood.

Regarding PRL, exposure to stress in humans causes the medullary noradrenaline neurons to activate prolactin-releasing peptide (PrRP). The activated PrRP promotes prolactin secretion and stimulates adrenocorticotropic hormone release to promote cortisol secretion.[16] Specifically, exposure to stress increases serum PRL levels. This study showed significantly elevated PRL levels in children with ASD compared to those with TD. Moreover, the serum ROM levels were significantly correlated

with serum PRL levels. This suggests that the stress and anxiety felt by children with ASD affect serum PRL levels.

Serum Alb and T-Bil, which are antioxidants in vivo, showed significantly higher levels in children with TD than in children with ASD. Moreover, the serum Alb and T-Bil levels showed a significant negative correlation with serum ROM levels in ASD children. There was a significant negative correlation between serum UA and ROM levels in ASD children aged ≥7 years. Alb is oxidized by ROS, thereby serving as an antioxidant for other substances in vivo.[17] Furthermore, T-Bil and UA, which are carried by Alb, function as scavengers of radicals generated in vivo; therefore, Alb also plays a role in suppressing the radical chain reaction.[17] T-Bil has been reported to act as an antioxidant in the metabolic conversion of heme to biopyrin and is reported to have a stronger antioxidant effect than α-tocopherol.[18] UA has been reported to form a complex with Fe^{3+}, have an antioxidant effect,[19] suppress the degradation of superoxide dismutase, and exert an antioxidant effect (equivalent to ascorbic acid) against ROS.[20] The present study suggests that, in vivo, these three antioxidants tend to be consumed when the oxidative stress level increases. If the oxidative effects of free radicals and ROS are equivalent to the effects of antioxidants in vivo, serum ROM levels will be balanced with the serum levels of antioxidant substances, and abnormally elevation is unnoted. However, it is suggested that the oxidative stress level is often too high to be suppressed by antioxidants alone in vivo in ASD.

A previous study involving only TD children reported that serum ROM levels were significantly higher in children aged <7 years than in those aged ≥7 years and that the younger the age, the higher the serum ROM levels.[6] Furthermore, similar results have been reported with other oxidative stress markers (eg, 8-hydroxy-2′-deoxyguanosine, nitrite/nitrate, and pentosidine).[21] Regarding the relationship between the examined parameters and age, there were no significant differences in the serum levels of antioxidants in vivo, such as the serum levels of Alb and T-Bil, between children with TD and ASD aged <7 years. However, the serum levels of the antioxidants were significantly higher in children with TD aged ≥7 years compared to children with ASD aged ≥7 years. These results seem influenced by the physiological profiles of children, as oxidative stress levels are high in infancy, even in children with TD, and decrease with growth/development. We speculate that antioxidants in vivo, such as Alb, T-Bil, and UA, are consumed by the influence of oxidative stress in infancy, resulting in the absence of any significant difference between children with TD and those with ASD.

This study has some limitations. The TD group had a smaller sample than the ASD group, which may have affected the results. Furthermore, oxidative stress is involved in depression and anxiety disorders, which are secondary disorders of ASD, and these secondary disorders were not examined in this study. Therefore, the presence of symptoms other than the core symptoms of ASD should be examined in the future. Regarding the antioxidant effects, the relationship between ASD and substances in vivo cannot be fully explained only by the effects of the endogenous antioxidants examined in this study. Future studies should include additional studies using comprehensive data, including the measurement of antioxidant enzymes and environmental changes for each subject.

ASD is diagnosed clinically, and the changes in oxidative stress and antioxidant markers obtained from the results of this study may improve the accuracy of diagnosis as objective biological data. It is also speculated that it could be applied to monitoring changes in the severity of illness and treatment. Based on the results of this study, oxidative stress assessment by ROMs, inflammatory changes by hsCRP, decreased in vivo antioxidants (such as Alb and T-Bil), and increased PRL levels may aid the objective assessment of ASD for easy use in clinical practice.

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