Original article / *Araştırma*

A comparative study of the oxidative stress indices of children with autism and healthy children

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ABSTRACT

Objective: *This study was aimed to analyze the serum total antioxidant/total oxidant (TAS/TOS) levels and thiol/disulphide balance of children with autism spectrum disorders (ASD) and draws a comparison with those of healthy children.* ***Methods:*** *The study involved 46 children diagnosed with ASD, along with 45 healthy children. The developmental stage, degree of intelligence and autism symptom severity of the ASD group were assessed using the Autism Behavior Checklist (ABC), Problematic Behavior Control List (PBCL), Childhood Autism Checklist Scale (CARS) and developmentally appropriate screening or IQ tests. Clinical, sociological and demographic data were obtained for both groups. The TAS/TOS levels and thiol/disulphide balance of both groups were analyzed and their oxidative stress index was obtained from the formula TAS/TOSx100. SPSS 17.0 was used for statistical analyses. p<0.05 was accepted to be statistically significant.* ***Results:*** *While lower TAS, native thiol and total thiol levels were found in the ASD group, other indices such as TOS, OSI and serum thiol-disulphide were similar for both groups.* ***Discussion:*** *Although no measurable effect upon the severity of autism was noted, the total antioxidant and thiol levels may be a significant cause of oxidative imbalance in ASD and our findings may suggest a predictive usefulness for TAS/TOS and thiol/disulphide balance, suggesting that treatments for ASD that take advantage of the potential advantages of antioxidants should be developed.* ***(Anatolian Journal of Psychiatry 2018; 19:xx-xx)***

Keywords: *autism, oxidative stress, antioxidants, thiol-disulphide, TAS, TOS, OSI, child*

Oksidatif stres göstergelerinin otizmli ve sağlıklı çocuklarda karşılaştırmalı bir çalışması

ÖZ

Amaç: *Bu çalışmada otizm yelpazesi bozukluğu (OYB) olan çocuklarda ve sağlıklı çocuklarda serum total antioksidan/oksidan (TAS/TOS) düzeyleri ile tiyol-disülfid dengesi çalışılarak karşılaştırılması amaçlandı.* ***Yöntem:*** *OYB'li 46 çocuk ile 45 sağlıklı çocuk çalışmaya alındı. OYB'li çocukların gelişim evreleri, zeka düzeyleri ve otizm belirti şiddeti düzeyleriOtizm Davranış Kontrol Listesi (ODKL), Sorun Davranış Kontrol Listesi (SDKL), Çocukluk Otizmi Değerlendirme Ölçeği (ÇODÖ) ve yaş gruplarına uygun gelişim tarama ölçeği ve/veya zekâ testleri ile değerlendirildi. İki grubun klinik, sosyodemografik değişkenlere ait verileri toplandı. TAS/TOS düzeyleri, tiyol-disülfid dengesi iki grupta da analiz edildi. Oksidatif stres indeksleri (OSI) TAS/TOSx100 formülü kullanılarak hesaplandı. İstatistiksel analizlerde SPSS 17.0 kullanıldı. p<0.05 anlamlılık düzeyi olarak kabul edildi.* ***Sonuç:*** *TAS, native tiyol*

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ve total tiyol düzeylerinin OYB'li grupta daha düşük olduğu, TOS, OSI ve serum tiyol-disülfid dengesinin iki grupta birbirine benzer oldukları saptandı. ***Tartışma:*** *Otizmin şiddeti ile arasında bir ilişki bulunamamasına karşın, total antioksidanlar ve tiyol düzeyleri OYB' de oksidatif dengesizliğe yol açabilir. Bulgularımız TAS/TOS ve tiyol-disülfid dengesinin öngördürücü olabilirliğini desteklemekte olup antioksidanların potansiyel tedavi seçenekleri arasında olduğunu düşündürmektedir.* ***(Anadolu Psikiyatri Derg 2018; 19:xx-xx)***

Anahtar sözcükler: *Otizm, oksidatif stres, antioksidanlar, tiyol-disülfit, TAS, TOS, OSI, çocuk*

INTRODUCTION

Oxidative stress arises primarily due to mismatch between reactive oxygen species (ROS) and the antioxidant defensive response, and exerts a degenerative effect upon inflammation, cell signaling, gene expression, and mitochondrial metabolism. This leads to alterations in morphological and functional architecture of the brain and to cognitive dysfunction and retardation.[1] Thus, achieving balance between antioxidants and ROS is central to the management of oxidative stress.

The management of oxidative stress involves maintaining a balance between oxidants and antioxidants and thiol-disulphide balance, which play a central role in the regulation of antioxidants, removal of metabolic waste products, cell signaling, apoptosis, enzyme functions, management of transcription factors, and information transfer.[2] Therefore, the total antioxidant system (TAS) and total oxidant system (TOS), along with thiol-disulphide regulation, is of key significance. Recent studies have indicated that abnormal thiol-disulphide balance is involved in the origin of AIDS, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), cancer, cardiovascular disease, chronic kidney disease, diabetes, Friedreich's ataxia, multiple sclerosis, Parkinson's disease, and rheumatoid arthritis.[3] Studies employing neuropsychiatric scatters in patients with attention deficit hyperactivity disorder (ADHD), obsessive compulsive disorder (OCD), borderline personality disorder (BPD) and schizophrenia, have indicated an imbalance between antioxidants and cancer prevention formulations.[4]

Research has also suggested that oxidative stress and abnormal DNA methylation play a part in the etiopathogenesis of autism by inducing cell damage and unanticipated gene expression changes.[5,6] Although studies have indicated a central contribution of oxidative stress and ROS in ASD etiopathogenesis,[7,8] (these reviews include 73 and 43 studies, respectively, which are different from each other) the relevant roles of thiol-disulphide balance and serum TAS/TOS levels have not yet been examined. Therefore, in the present study, serum TAS/TOS and thiol-disulphide levels of healthy children and children with ASD were analyzed to elucidate the role of oxidative stress in ASD etiology.

METHODS

The present study included forty-six children diagnosed with autism spectrum disorders (ASD) at our clinic, with an age- and sex-matched control group consisting of forty-five healthy children chosen from three different schools using stratified sampling. Required permissions from local authorities and parental consent were obtained.

Forty-five healthy controls and 46 ASD cases were assessed by psychiatric interviews based on DSM-V in terms of diagnosis and co-morbidity and evaluated by child and adolescent psychiatry specialists.

Potential subjects were excluded from the control group if they received past psychiatric treatment or had a family member or ancestor diagnosed with ASD. Potential subjects were excluded from either group if they had a history of smoking or alcohol consumption, suffered a communicable illness within the past week, or been administered any antioxidant factors such as vitamin C or E.

The costs incurred in biochemical marker analysis were funded by the Ataturk Training and Research Hospital Research Grants Unit, Ankara, Turkey. Ethical approval (reference 2015/042) was provided by the Pediatric Hematology Oncology Training and Research Hospital, Ankara, Turkey. The Autism Behavior Checklist (ABC)[9] and the Problematic Behavior Checklist (PBCL)[10] were filled-in by parents of children diagnosed with ASD, and the childhood autism checklist scale (CARS) was applied to all autistic children by interviewers.[11] Sociodemographic forms were completed by clinicians. Children with ASD were diagnosed during clinical consultations with two child psychiatrists using DSM-5 criteria.[12] The ASD group was also subjected to developmental psychometric assessment. Se-

rum samples from all study subjects were analyzed for TAS/TOS and thiol-disulpide levels.

Assessment measures

The ABC has 57 items and 'sensory', 'relating', 'body and object use', 'language' and 'social and self-help' subscales scores that are summed to obtain the total ABC score. AbBC has 58 items and generates five subscale scores: irritability, social withdrawal, stereotypic behaviour, hyperactivity, inappropriate speech. CARS comprises 15 items that are used to generate a total score defining the severity of autism. An expert psychologist with experience treating children with ASDs administered age-appropriate developmental tests or intelligence tests (Ankara Developmental Screening Inventory, Stanford-Binet).

Sample preparation

The study subjects underwent a 12h (overnight) fast, followed by collection of10 mL venous blood. After 30 min of incubation. Samples were centrifuged for 15 min at 5000xg for TAS/TOS analysis, and for 10 min at 1500xg for thiol-disulphide analysis. The samples were stored at -20°C until analysis.

Biochemical analyses

i. Thiol/disulfide balance: A new technique introduced by Erel and Neselioglu[5] was employed to determine serum thiol-disulphide balance using a Shimadzu UV-1800 spectrophotometer and a Roche cobas c501 autoanalyser. Oxidative stress levels of the participants were measured using the oxidative stress index (OSI). The Shimadzu UV-1800 spectrophotometer and Roche cobas c501 autoanalyzer were also used in TAS/TOS analysis.

ii. Measurement of TAS levels: A Rel Assay Kit was used to assess serum TAS levels. Oxidative reaction of hydroxyl radicals with colorless o-dianisidine generates the bright yellow-brown dianisyl radical. Reaction initiation by hydroxyl radicals in the reaction mix is inhibited by serum antioxidants, preventing immediate color change upon serum sample addition. Reaction progress was monitored using the spectrophotometer and auto analyzer to obtain the TAS level. Results were represented as mmol Trolox Eqv./L.[13]

iii. Measurement of TOS levels: A Rel Assay Kit was used to assess serum TOS levels. Oxidative reaction of a ferrous ion-chelate complex with ferric ions causes a color change monitored by the spectrophotometer. Color intensity provides a measure of total oxidant molecules.[14]

iv. Determination of OSI: The oxidative stress index (OSI) was determined using the formula OSI (arbitrary unit)=[TOS (μmol H_2O_2Equiv/L)/ TAS (mmol Trolox Equiv/L)]x100.

v. Statistical treatment: Statistical analysis was done using SPSS 17.0. The Kolmogorov-Smirnov (K-S) test was used to assess goodness of fit (normal distribution). Quantitative variables were analysed using the student's t-test or the Mann-Whitney U test. The chi-squared test or Fisher's exact test were applied to assess categorical variables. Where relevant, the Pearson correlation coefficient was also calculated. The sample size needed for confident detection of a significant effect was evaluated using power analysis. Receiver Operating Characteristics (ROC) curve analysis was applied to assess the predictive nature of serum TAS values with respect to autism. The sensitivity, specificity, and positive and negative predictive values were evaluated upon acquisition of a significant cut-off point, and a 5% type-I error level was applied to define statistically significant predictive behavior of test variables when examining the Area Under Curve (AUC).

RESULTS

The age and sex distributions were identical in both groups (p=0.682 and p=0.600, respectively) and results indicated that children with ASD displayed higher levels of TAS, native thiol, and total thiol (p<0.001) than the control group. The two groups displayed identical levels of TOS (p=0.149) and disulphide (p=0.761). The ASD group displayed higher disulphide/native-sulphydril (SS/native-SH) (p=0.061) and disulphide /total-sulphydril (SS/total-SH) (p=0.065) ratios than the control group, but statistical significance was insufficient (Table 1). Figure 1 also indicates marginal statistical significance for OSI distribution in ASD and control groups.

The ASD group was divided into two subgroups based on autism grade; mild to intermediate (CARS score 30-36), or severe (CARS score37 and over). Results in Table 2 indicate that ASD symptom severity had no impact on experimental outcome.

As shown in Figure 2, ROC analyses of TAS levels indicate a confidence interval between 0.729 and 0.909 (p<0.001) when examining the AUC. Hence, TAS levels <1.20 were predictive of autism with a specificity of 82.6%, a sensitivity of 71.1%, a positive predictive value of 80%, and a negative predictive value of 74.5%.

Table 1. Demographics and laboratory findings of children with ASD and their healthy counterparts

	ASD (n=46) Mean±SD	Control (n=45) Mean±SD	t or χ^2	p
Age (months)	43.5±9.0	44.4±11.6	-0.41	0.682
Gender				
Boys (n=63) (n, %)	33 52.4	30 47.6	0.28	0.600
Girls (n=28) (n, %)	13 46.4	15 53.6		
Laboratory			t or z	
TAS	1.13±0.11	1.26±0.10	-5.54	<0.001
TOS	8.22±5.83	6.70±3.96	1.45	0.149
OSI*	19.80 (5.01-47.82)	23.09 (7.82-295.65)	-1.83	0.067**
Native thiol-SH	416.66±55.66	456.89±44.89	-3.79	<0.001
Total thiol-SH	459.67±60.88	499.40±48.65	-3.43	0.001
Disulphide-SS	21.63±6.09	21.26±5.23	0.31	0.761
SS/native-SH (%)	5.18±1.41	4.66±1.17	1.90	0.061
SS/total-SH (%)	4.67±1.16	4.24±0.97	1.86	0.065

TAS: Total Antioxidants Levels, TOS: Total Oxidants Levels, OSI: Oxidative Stress Index, SH: Sulphydril
**: Median (minimum-maximum) **: Mann Whitney-U test*

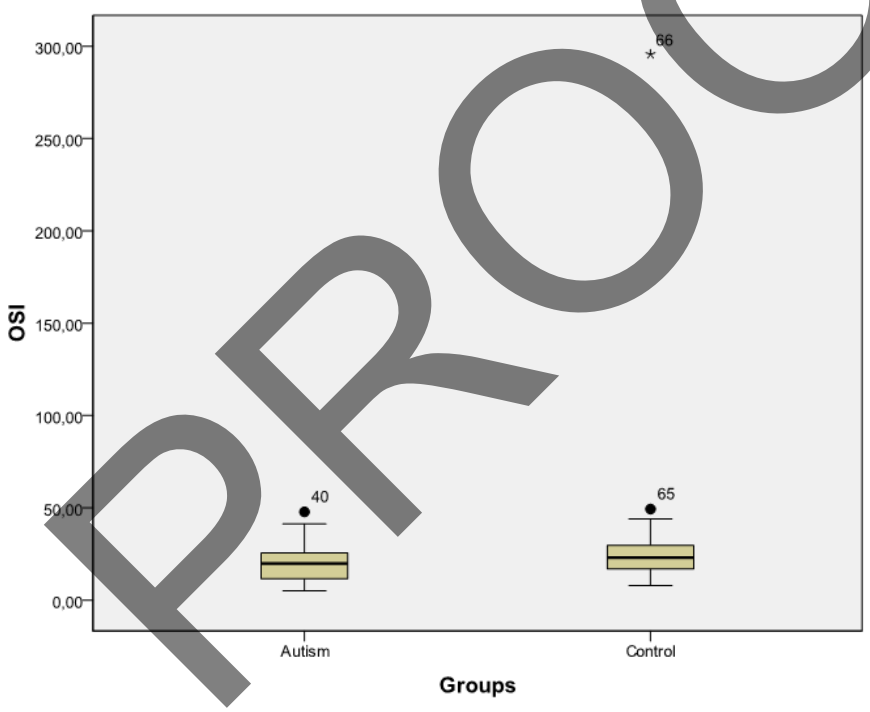

Figure 1. OSI levels in ASD and control groups

Boys with ASD had significantly higher SS/native-SH and SS/total-SH ratios than boys in the control group. Hence, there was no gender-based difference in outcome. The results indicated no correlation between age and oxidative stress indices, but a statistically significant positive link between TAS and native-SS (r=0.404, p<0.001), between TAS and total-SH (r=0.395, p<0.001), between native-SH and total-SH (r=0.983, p<0.001), and between native-SH and disulphide levels (r=0.272, p=0.009). Results in Table 3 indicate a statistically significant (r=0.446, p<0.001) positive link between total-SH and SS levels.

DISCUSSION

To the best of our knowledge, the present work

Table 2. Laboratory findings in accordance with ASD severity

	Mild-moderate autism (n=30) Mean±SD	Severe autism (n=15) Mean±SD	t or z	p
TAS	1.13±0.10	1.11±0.13	0.457	0.650
TOS	8.28±6.35)	7.18±3.20	0.626	0.535
OSI*	21.05 (5.01-47.82)	15.24 (8.71-34.10)	-0.072	0.942**
Native thiol-SH	411.28±54.05	424.71±60.47	-0.755	0.454
Total thiol-SH	453.97±61.50	467.27±60.93	-0.686	0.496
Disulphide-SS	21.46±6.59	21.40±4.93	0.035	0.973
SS/native-SH (%)	5.17±1.39	5.11±1.50	0.128	0.898
SS/total-SH (%)	4.66±1.16	4.61±1.21	0.135	0.893

TAS: Total Antioxidants Levels, TOS: Total Oxidants Levels, OSI: Oxidative Stress Index, SH: Sulphydril
**: Median (minimum-maximum); **: Mann Whitney-U test*

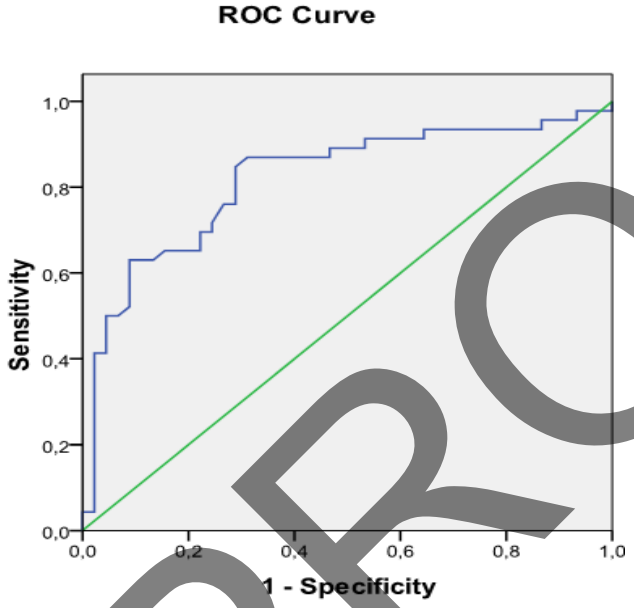

Figure 2. Receiver operating characteristic curve showing the performance of total antioxidants system in autism spectrum disorders

Table 3. The relation of the biochemical markers with age and within each other

	TAS	TOS	Native-SH	Total-SH	SS
Age	0.041 0.699	0.168 0.111	-0.164 0.120	-0.148 0.160	0.022 0.837
TAS		0.183 0.083	0.404* 0.001	0.395* 0.001	0.090 0.399
TOS			-0.077 0.468	-0.043 0.689	0.154 0.145
Native-SH				0.983* 0.001	0.272* 0.009
Total-SH					0.446* 0.001

**: p<0.001*

constitutes the first study of the relationship between TAS, TOS, OSI, thiol/disulphide indices, and ASD in children. Our results indicate that children with ASD have lower levels of TAS and thiol, and higher OSI values, compared to healthy children.

As indicated in the literature, TAS/TOS and thiol-disulphide imbalance are underlying causes of several physiological disorders. ASD onset and progression are highly likely to be influenced by oxidative stress and aberrant DNA methylation, due to resulting cellular damage and inappropriate gene expression alterations.[15]

The antioxidant system, including enzymatic and non-enzymatic factors, has been presumed to be impaired in ASD. A published study showed that ASD patients have diminished glutathione peroxidase activity in erythrocytes and plasma.[16] The study also described lower erythrocyte superoxide dismutase activity in children with autism. Studies have demonstrated reduced glutathione (GSH) and increased glutathione disulphide (GSSG) in plasma of children with ASD, resulting from lower NADPH, a glutathione reductase cofactor.[9,17] Studies have also demonstrated lower vitamin E in children with ASD.[7]

The findings regarding TAS and thiol levels in this study are consistent with a previous study[18] which demonstrated lower TAS in autistic patients relative to controls and patients suffering their first psychotic episode. Post-mortem investigations support the idea that lower TAS and thiol aggravate neuronal damage due to typical or increased oxidant levels, undermining defenses in susceptible patients. An investigation revealed a lower GSH/GSSG ratio, indicative of lower antioxidant capacity, in patients with autism.[19] The study also demonstrated an inverse correlation between GSH/GSSG ratio and 8-oxo-deoxyguanosine (8-oxo-dG) levels, indicative of oxidative DNA damage. The present results indicate a plausible link between reduced antioxidant capacity and ASD.

While the present study found a difference in TAS and thiol levels between children with ASD and healthy individuals, no measurable difference was found in TOS and disulphide levels. This is in contrast to previous results. It should be noted that previous studies evaluated oxidant parameters separately in a complex, challenging, and expensive procedure. Measured separately, TOS and TAS levels may not reveal measurable negative correlations in individuals. The present study is the first to evaluate collective oxidant effects and homeostasis of TAS, TOS, OSI, and thiol/disulphide indices, providing potentially useful data regarding oxidative stress in patients.

Oxidants may arise from normal physiological or abnormal (disease) processes. The majority of studies indicate higher oxidant factors in children with autism.[20,21] Increased nitric oxide (NO) has been consistently demonstrated.[20] Increased thiobarbituric acid reactive substances (TBARS), the degradation products of fats, and increased xanthine oxidase activity in red blood cells have also been reported in autistic patients,[21] as have higher levels of the lipid peroxidation biomarker 8-isoprostane (8-iso-PGF2α).[19]Higher levels of these compounds in autistic individuals are clear indicators of elevated oxidants which may lead to cell degradation and death.[22] Investigations into causes of elevated oxidants have pointed to mitochondrial dysfunction in autistic patients.[7,23] The role of mitochondria in the electron transport system (ETS) makes them the major source of oxidants and primary targets of free radicals (FRs).[24]

Free radicals are highly toxic to neurons and they are generated significantly in the human brain, which is extremely sensitive to oxidative stress. The susceptibility of the brain to FRs can be aggravated by significant quantities of readily oxidized polyunsaturated fatty acids. Neuronal cell damage can be further aggravated by a comparatively weak antioxidant response.[25] Heightened oxidative stress may be instrumental in development of neuropsychiatric disorders such as ASD that arise from brain damage. This attests to the importance of the present study, which is the first to assess and demonstrate high OSI values in ASD patients.

Previous studies suggest that oxidative damage results from loss of mitochondrial membrane potential and ETS function caused by increased lipid peroxidation.[26,27]Lipid peroxidation in autism may also be linked to increased brain volume and loss of Purkinje cells.[28] Increased quantities of lipofuscin containing cells, indicative of oxidative stress in the brain, have been reported in post-mortem investigations on autistic patients,[29] as have raised levels of 3-nitrotyrosine, diagnostic of cerebellar protein oxidation.[30]

The literature also points to DNA and RNA damage in autistic patients due to oxidative stress. A link between DNA damage and increased oxidative stress in individuals with borderline personality disorder (BPD) has been reported.[31] Notable increases in the major DNA

oxidation product 8-Oxo-2'-deoxyguanosine (8-oxo-dG) were also noted in a post-mortem study of autistic patients.[19] Another study suggested a possible link between reduced methylation capacity and increased oxidative stress leading to hindered transfer of methionine from S-adenosylmethionine to DNA, RNA, proteins, phospholipids, and neurotransmitters in autistic children.[32] Published research has also demonstrated impaired neuronal cell migration due to free-radical favored mutation of genes central to brain development.[33-34]

Previous studies have shown that oxidative stress results in mast cell activation, promoting production of numerous proinflammatory and neuro-sensitizing substances such as NO, IL-6, histamine, and bradykinin with a concurrent risk of damage to the blood-gut and blood-brain barriers.[35] Some studies have postulated that the resultant ingress of enterotoxic substances into the brains of autistic patients might provoke inflammation of nervous tissue.[36] Thus, oxidative stress could result in neuronal damage and the development and progression of autism by promoting lipid and protein peroxidation, neuroinflammation, and damage to DNA and RNA. The present study, highlighting increased OSI in autistic patients, supports the above hypothesis.

While autism is currently diagnosed based on behavioral criteria, research efforts have been directed towards improved and prompt diagnosis using specific biological indices for disease risk and onset, evaluated using simple biochemical tests.[37]Numerous investigations have addressed the potential use of biological indices related to oxidative stress as autism indicators.[7,32] The present study employed ROC analysis to show that autism may be diagnosed with 71.1% sensitivity and 82.6% specificity, at a serum TAS level <1.20. Larger sample sizes are needed in future studies.

Another significant outcome of the present investigation is the agreement between TAS, TOS, OSI, and thiol-disulphide values, and the severity according to CARS scores. No previous study has compared OSI and autism symptom severity. The present study reports the novel implication that oxidative imbalance in autistic children is independent of disease severity.

CONCLUSIONS

This study found a decrease in antioxidant capacity and compromised oxidative balance in autistic children, implicating oxidative stress as a key factor in ASD onset and progression. Structural and functional neuronal damage due to oxidative stress via several mechanisms may affect susceptible individuals. The effect of oxidative stress appears to be independent of symptom severity. Hence, antioxidant administration may benefit autism treatment. Understanding detailed effects of oxidative stress on ASD onset and progression requires additional investigations with larger study groups.

This investigation had limitations of cross-sectional design and was not population based. Therefore, cause-and-effect relationships could not be elucidated. Future studies could study birth patterns or oxidative stress markers in cord blood, ideally using targeted monitoring of prospective subjects at regular intervals to confirm oxidative stress levels. By examining subjects at an earlier age, or pre-natally, it may be possible to pinpoint changes in healthy individuals at critical stages. Recognition and improved understanding of such variables could result in enhanced treatment for underlying ASD symptoms.

In summary, results of this study indicate that children with ASD have lower levels of TAS and thiol, and higher OSI values, compared to healthy children. No measurable difference was found in TOS and disulphide levels, in contrast to previous investigations. ROC analysis showed that autism may be diagnosed with 71.1% sensitivity and 82.6% specificity, at a serum TAS level <1.20.

This study is consistent with previous studies suggesting that oxidative stress may cause neuronal damage and the development and progression of autism, by promoting lipid and protein peroxidation, neuroinflammation, and DNA and RNA damage.

Authors' contributions: *Ç.U.: designed and coordinated the study, collected and analyzed the data, contributed to the writing of the manuscript; H.T.: collected and analyzed the data, contributed to the writing of the manuscript; E.S.: contributed to the collection of data; M.A.: supervised the study, making and interpreting biochemical analyzes; O.E.: making and interpreting biochemical analyzes; O.U.: supervised the study, collected and analyzed the data; E.Y.S.: contributed to the collection of data, wrote and revised the content of the manuscript.*

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