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Oxidative Stress and Thiol-disulphide Hemostasis in Children with Anxiety Disorders

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ABSTRACT

Introduction: Anxiety disorders (AD) constitute a significant part of mental health problems; however, their pathogenesis remains not fully elucidated. The balance between the oxidative and antioxidative systems are disrupted in children with AD. The total oxidant/antioxidant status (TOS/TAS) and thiol/disulphide homeostasis (TDH) show oxidative stress through different mechanisms. To date, research in this context has tended to focus on adults rather than children. Despite this, understanding oxidative stress in pediatric populations is increasingly emphasized. Therefore, this research aims to investigate TOS/TAS and TDH in children with AD.

Methods: The study included 40 treatment-naive children with AD and 40 healthy controls matched by age and sex. Sociodemographic data and The Screen for Child Anxiety-Related Emotional Disorders (SCARED) were used for assessment.

Results: The results showed that TOS and the Oxidative Stress Index (OSI) were elevated, and TAS was reduced in children with AD compared to controls. However, when evaluated in terms of TDH, there was no significant difference. Logistic regression analysis identified TOS as a significant predictor of AD (p=0.027; OR=5.49, 95% CI: 1.21–24.84). Although dynamic-disulphide level improved the model's predictive accuracy, they did not reach statistical significance (p=0.063).

Conclusion: These findings suggest a potential oxidative dysfunction in AD. The study highlights the potential utility of TOS as a robust biomarker for distinguishing pediatric AD from HC. Furthermore, the absence of significant changes in TDH suggests that oxidative stress in pediatric AD may primarily involve alternative pathways. This may involve a complex interplay of DNA damage, lipid peroxidation, and protein oxidation processes contributing to the oxidative stress observed in AD. To explore the potential for using oxidative stress markers as novel targets for treatment and diagnostic tools for AD, prospective, large-scale, randomized trials are required.

Keywords: Anxiety disorders, child, oxidative stress, thiol/disulphide hemostasis

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INTRODUCTION

Anxiety disorders (AD) are amongst the most frequently observed mental health conditions in children and adolescents, with an estimated prevalence of approximately 15% (1). While various biological, genetic, and psychosocial theories have been proposed to explain the etiology of AD, the exact cause remains incompletely understood (2). To date, there has been a rise in the number of biological studies that focus on AD. Catecholamines are the primary neurotransmitters involved in the pathophysiology of AD (3). When the catecholaminergic system becomes overactive, reactive oxygen and nitrogen species (ROS and RNS) are produced, leading to oxidative stress. Recent studies suggest that oxidative stress plays a role in the pathophysiology of AD by affecting neuronal structure (4,5). While the exact cause-and-effect link between anxiety disorders (AD) and oxidative stress is still not fully understood, oxidative stress is believed to impact AD by affecting anxiety-regulating systems. Studies involving both adults and children have demonstrated a link between oxidative stress and AD through different mechanisms. Specifically, oxidative stress can influence the hypothalamus-pituitary-adrenal (HPA) axis, which controls the body's stress response. The increased release of stress hormones, such as

Highlights

- **Elevated TOS and OSI levels effectively differentiate children with AD from HC.**
- **TOS has been identified as a key biomarker for predicting AD.**
- **The alterations in TDH among children with AD were not statistically significant.**

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cortisol, triggered by oxidative stress may intensify anxiety symptoms. Furthermore, oxidative stress may disrupt neurotransmitter balance by damaging the lipid membranes of nerve cells, impairing nerve transmission, and contributing to AD. Moreover, oxidative stress can initiate or intensify inflammatory processes in the brain, promoting microglial cell activation and the release of proinflammatory cytokines. Oxidative stress-induced mitochondrial dysfunction and cellular damage may further compromise neuronal function, potentially contributing to anxiety symptoms (6). Although many significant findings have been revealed by studies on oxidative and antioxidative parameters in AD (7,8), our understanding of oxidative metabolism in children with AD remains incomplete. Limited research has been reported regarding the oxidative/antioxidative imbalance shifting towards oxidation in children with AD (9,10).

The comprehensive assessment of oxidative stress may not be fully captured by isolated measurements of antioxidants and oxidants. Therefore, evaluating total antioxidant status (TAS) and total oxidant status (TOS) is considered reliable, as these markers provide a more accurate reflection of oxidative stress. The oxidative stress index (OSI), calculated as the ratio of TOS to TAS, serves as an indicator of the balance between antioxidants and oxidants, representing the overall oxidative state in the organism (11).

Thiols are well-known antioxidative molecules containing a sulfhydryl (-SH) group. They react with oxidant molecules, forming disulphide bonds. Assessing plasma thiol content is of the utmost importance, as thiols are the primary compounds depleted during oxidation within biological systems, offering insights into the effects of oxidation on these systems (12). Maintaining active thiol/disulphide homeostasis (TDH) is essential for detoxification, apoptosis, and enzymatic reactions (13,14). Hence, unsurprisingly, it is suggested that the dynamic balance of TDH can be affected during disease processes triggered by oxidative stress. Within this mindset, increasing evidence suggests that there is a disruption in the dynamic balance of TDH in various psychiatric disorders such as Autism, Attention-Deficit Hyperactivity Disorder (ADHD), and Schizophrenia (15–17). Several papers consistently found that TDH balance is disturbed in the adult population with AD; however, the value of TDH in children with AD has not yet been documented (18,19).

A review of the relevant literature shows no research directly assessing both total oxidant/antioxidant status and TDH in children with AD. Thus, in this cross-sectional study, we aimed to evaluate TOS/TAS and TDH in children with AD. Additionally, we sought to determine whether these molecules, which are detectable in clinical settings, could serve as distinctive biomarkers. As far as we know, this is the first clinical study to offer a thorough analysis of these oxidative stress markers in children with AD.

METHOD

The cross-sectional study included 40 treatment-naive children, aged 6 to 13, who were diagnosed by child psychiatrists at the Child Psychiatry Clinic between April 2021 and August 2023. These children were compared with 40 healthy controls (HC), matched by age and sex. Diagnoses were confirmed using the K-SADS-PL diagnostic interview. Children and adolescents were excluded from the study if they had any chronic disease, any psychopathology other than AD, a BMI ≥30, or dietary restrictions in the past month. After the researcher administered the Screen for Child Anxiety-Related Emotional Disorders (SCARED) to those meeting the inclusion criteria, the children proceeded to provide blood samples.

Measurements

Scales

Sociodemographic Data Form

This form, created by the researchers for this study, includes characteristics such as age, gender, socioeconomic status of parents, number of siblings, BMI, medical conditions, and dietary restrictions.

Kiddie Schedule for Affective Disorders and Schizophrenia – Current and Lifetime Version (K-SADS-PL)

The K-SADS-PL serves as a diagnostic tool, designed as a semi-structured interview format, first introduced by Kaufman et al. (20), aligned with DSM-IV diagnostic guidelines, and serves the purpose of diagnosing mental disorders in children. It comprises sections such as an initial unstructured interview, followed by a screening interview that evaluates over 40 psychiatric disorders and approximately 200 symptoms from both recent (within the last 2 months) and past histories. The adaptation and translation of this instrument into Turkish were undertaken by Gökler et al (21).

The Screen for Child Anxiety-Related Emotional Disorders (SCARED)

The screen for child anxiety-related emotional disorders (SCARED) is a 41-item scale designed to measure anxiety severity during childhood (22). Participants rate the items on a 3-point scale: 0 for "not true or hardly ever true," 1 for "sometimes true," and 2 for "true or often true." This results in a score ranging from 0 to 82, with higher scores indicating greater anxiety. The total scores of SCARED have demonstrated satisfactory validity and reliability for the Turkish population, with a Cronbach's α value of 0.88 (23).

Biochemical Analysis

To minimise the impact of extraneous variables on the study results, all participants were instructed to refrain from consuming coffee, tea, or food prior to blood sampling. Following a 12-hour overnight fast, venous blood samples were drawn from the antecubital vein. Each patient provided 10 mL of blood, collected in biochemistry tubes for analysis. After an incubation period of 30 minutes, the samples were centrifuged at 3000 g for 10 minutes. After centrifugation, the sera were separated and stored at -80°C until analysis. Biochemical analyses were conducted in the Research Laboratory of the Ondokuz Mayıs University Faculty of Medicine, Department of Medical Biochemistry, using a Shimadzu UV-160 VIS-NR spectrophotometer (Kyoto, Japan) and a Biotek Instruments analyser (Highland, USA).

Total Oxidant/Antioxidant Status (TOS/TAS) Measurement

Plasma total oxidant status (TOS) was measured through an innovative automated colorimetric technique outlined by Erel (11). In this approach, oxidants in the sample induce oxidation of the ferrous ion-o-dianisidine complex into ferric ions. Glycerol molecules, abundant within the reaction medium, promote this oxidation. Under acidic conditions, the ferric ions form a coloured complex with xylenol orange, and the resulting colour intensity –measured spectrophotometrically– reflects the total oxidant concentration in the sample. The assay is standarised with hydrogen peroxide, with results reported as micromolar hydrogen peroxide equivalents per litre (mmol H_2O_2 Eqv./L). The inter-assay coefficient of variation (CV) was 3.9%, and the intra-assay CV was 3.2%.

Total antioxidant status (TAS) was evaluated using a novel automated colorimetric technique, also developed by Erel (11). This method relies on the reduction of the dark blue-green ABTS radical cation (2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) to its colourless form by the antioxidants presents in the sample. The absorbance change at 660

nm correlates with the sample's total antioxidant capacity. The assay is calibrated with a stable antioxidant standard solution, known as the Trolox Equivalent –a vitamin E analogue. The inter-assay coefficient of variation (CV) averaged 2.8%, while the intra-assay CV was 3.3%. The measurable range is 0.1–3.5 mmol Trolox Equiv. /L in undiluted samples.

The oxidative stress index (OSI), defined as the ratio of TOS to TAS, was determined. The OSI value was computed using the formula: OSI (arbitrary unit)=[TOS (μmol H2O2 Equiv./L) / TAS (mmol Trolox Equiv./L)] × 100.

Thiol/Disulphide Homeostasis (TDH) Measurement

Thiol/disulphide homeostasis was assessed using the method developed by Erel et al. (Rel diagnostic assay, Türkiye) (24). Serum total and native thiol concentrations were analysed using commercially available total thiol and native thiol kits (Rel Assay Diagnostics, Gaziantep, Türkiye). This method is based on the reduction of disulfide bonds to yield free functional thiol groups. Any unreacted reducing agent, sodium borohydride, is neutralised and removed using formaldehyde. After reacting with 5.5′-dithiobis-(2-nitrobenzoic) acid (DTNB), both reduced and native thiol groups, encompassing all thiols, are measured. Half of the difference between total and native thiols represents the dynamic disulfide content. Ratios for disulfide to total thiol (oxidized thiol ratio), disulfide to native thiol (oxidation-reduction ratio), and native thiol to total thiol (reduced thiol ratio) were subsequently calculated.

Statistical Analysis

The IBM Statistical Package for Social Sciences (SPSS) program version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Categorical variables were presented as frequencies and percentages, while continuous variables were reported as means with standard deviations (SD) for data following a normal distribution, or as medians with interquartile ranges (IQR) for data that did not follow a normal distribution. The Chi-square test was applied to analyse categorical variables, while the independent samples t-test was used for continuous variables with normal distributions, and the Mann-Whitney U test was used for non-normally distributed continuous data. Statistical significance was set at $p < 0.05$.

A binary logistic regression analysis was performed to examine the relationship between TOS and dynamic-disulphide level (independent variables) and group membership (AD vs. HC), with group membership defined as the dependent variable. Total oxidant status and dynamic-disulphide level were identified as pivotal predictors in the binary logistic regression model, selected for their statistical significance and theoretical relevance. Total oxidant status demonstrated a substantial contribution to the model's predictive capacity, as evidenced by the Wald test and a marked improvement in log-likelihood. Dynamic-disulphide level provided additional value by reflecting critical redox balance dynamics, further enhancing the model's explanatory depth. Multicollinearity diagnostics (VIF <5) validated the independence of these variables, ensuring a robust and reliable framework for distinguishing between AD and HC.

Statement of Ethical Considerations

Ethical approval for the study was obtained from the Ondokuz Mayıs University Ethics Committee (Approval No: 2020/599; Date: October 20, 2020). The study was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki. Written informed consent was obtained from both the parents and the children. In cases where the children were unable to provide written consent due to their age, verbal assent was obtained alongside written consent from their parents.

RESULTS

The descriptive data and statistics are presented in Table 1. Significant differences were observed between the two groups in terms of total oxidant/antioxidant status, specifically in TOS (μmol/L), TAS (mmol/L), and OSI values (%). The effect sizes of the measured variables between the AD and HC were evaluated using Cliff's Delta (25). The analysis revealed a substantial negative effect size for TAS (Δ=-0.855), classified as a large effect, indicating that TAS values in HC were significantly higher than those in AD. In contrast, positive and large effect sizes were observed for TOS (Δ=0.975) and OSI (Δ=0.962), reflecting significantly higher levels of TOS and OSI in AD. These findings highlight a pronounced distinction between the two groups across all parameters, with all effect sizes surpassing the threshold for large effects (25).

In children with AD, the parameters of total thiol (Cohen's d=1.206), native thiol (Cohen's d=0.687), and dynamic-disulphide (Cohen's d=1.067) were found to be higher compared to HC. The effect sizes for total thiol and dynamic-disulphide were considered large. The small, medium, and large effect sizes for the independent samples t-test (Cohen's d) are considered to be 0.02, 0.05, and 0.08, respectively (26). There was no significant difference between the groups in terms of reduced thiol, oxidised thiol, and thiol oxidation-reduction ratios (Table 2).

A binary logistic regression analysis was conducted to investigate the association between TOS and dynamic-disulphide level with group membership, differentiating AD and HC. The overall model was statistically significant ($\chi^2(2, N=80)=97.52$, $p < 0.001$), demonstrating the predictors' ability to effectively distinguish between the two groups (Table 3). The model explained 93.9% of the variance (Nagelkerke $R^2=0.939$) and achieved a high overall classification accuracy of 93.8%.

Table 1. Descriptive findings: comparison of categorical and continuous variables between the two groups

Variables	Anxiety disorder (n=40)	Healthy control (n=40)	p
Sex[a]			1.0
Male	22 (55%)	22 (55%)	
Female	18 (45%)	18 (45%)	
Age[b]	10.20±2.00	10.20±2.00	1.0
Education of mothers[a]			0.165
Elementary school	11(27%.5)	4 (10%)	
Junior high school	7 (17%.5)	13 (32%.5)	
Senior high school	2 (5%)	2 (5%)	
University	20(50%)	21(52%.5)	
Education of fathers[a]			0.795
Elementary school	1 (2%.5)	2 (5%)	
Junior high school	14 (35%)	11 (27%.5)	
Senior high school	5 (12%.5)	7 (17%.5)	
University	20(50%)	20(50%)	
Employment of mothers[a]			0.256
Employment	21(52%.5)	26 (65%)	
Unemployment	19 (47%.5)	14 (35%)	
Employment of fathers[a]			1.00
Employment	38 (95%)	38 (95%)	
Unemployment	2 (5%)	2 (5%)	
Number of siblings[c]	1.07±0.61	1.00±0.64	0.595
SCARED[c]	43.12±18.58	23.80±16.55	<0.001

SCARED: the screen for child anxiety-related emotional disorders; [a] Chi-square test; [b] independent sample t-test; [c] Mann-Whitney U test.

Table 2. Biochemical findings: comparison of biochemical variables between the two groups

Variables	Anxiety disorder	Healthy control	Statistics	p
Total thiol (µmol/l)[a]	359.16±68.81	274.00±72.34	t=5.395	<0.001
Native thiol (µmol/l)[a]	190.41±52.18	150.96±62.15	t=3.074	0.003
Dynamic-disulphide (µmol/l)[a]	84.37±21.37	61.51±21.46	t=4.772	<0.001
Reduced thiol ratio (%)[a]	52.72±9.08	54.24±13.38	t=0.595	0.553
Oxidized thiol ratio (%)[a]	23.63±4.54	22.87±6.69	t=0.595	0.553
Thiol oxidation reduction ratio (%)[a]	47.40±15.55	47.63±23.37	t=0.052	0.958
TOS (µmol/l)[b]	15.87±4.44	5.75±2.07	z=7.506	<0.001
TAS (mmol/l)[b]	1.39±0.22	1.83±0.18	z=6.584	<0.001
OSI (%)[b]	8.71±2.51	3.18±1.23	z=7.409	<0.001

TOS: total oxidant level, TAS: total antioxidant level, OSI: oxidative stress index; [a] independent samples t-test; [b] Mann-Whitney U test.

Table 3. Regression findings: binary logistic regression results predicting anxiety disorder

Predictor	B	SE	Wald χ^2	p	Odds ratio (Exp (B))	95% CI for exp (B)
TOS (µmol/L)	1.703	0.770	4.891	0.027	5.49	[1.21, 24.84]
Dynamic-disulphide (µmol/l)	0.116	0.063	3.462	0.063	1.12	[0.99, 1.27]
Constant	-25.311	11.832	4.576	0.032	0.00	–

TOS: total oxidant status; B: unstandardized regression coefficient; SE: standard error of B; CI: confidence interval.

Total oxidant status was identified as a significant predictor (B=1.703, SE=0.770, Wald=4.891, p=0.027), with an odds ratio of 5.49 (95% CI=[1.21, 24.84]), indicating that elevated TOS levels were strongly associated with an increased likelihood of AD. Although dynamic-disulphide level did not reach conventional levels of statistical significance (B=0.116, SE=0.063, Wald=3.462, p=0.063), its inclusion contributed to the overall predictive capacity of the model. The Hosmer-Lemeshow test confirmed an excellent model fit (χ^2 (8)=0.178, p=1.000), indicating no significant discrepancies between observed and predicted classifications.

DISCUSSION

In our study, we aimed to compare the parameters reflecting the total oxidant/antioxidant status and TDH between children with AD and HC, matched for age and gender. Based on previous studies, we can state that this is the first empirical examination to simultaneously measure the effects of parameters related to total oxidant-antioxidant status and TDH in children. The results indicated that higher TOS and OSI, and lower TAS in children with AD. On the other hand, there was no significant difference in reduced thiol, oxidised thiol and thiol oxidation-reduction ratios.

Despite extensive research on oxidative stress markers in AD amongst adults, studies focusing on paediatric patients are relatively scarce. Based on our study results, children with AD exhibit higher levels of TOS and OSI compared to HC, while their TAS levels are lower. Likewise, this suggests that in AD, the oxidative imbalance shifts towards oxidation. These results align with those observed in earlier studies. According to the results of a study in children with AD not taking psychotropic drugs, it was found that TOS and OSI values were higher compared to HC, indicating impairment in favour of oxidation. However, unlike in our study, it was found that there was no significant difference in TAS values between the two groups (10). Also, in one preliminary study, Demirdögen et al. compared TAS, TOS, and OSI levels in groups of 25 children with AD and HC. The study identified a significant increase in OSI levels within the AD group. However, no significant differences were observed in TOS and TAS levels (9). Moreover, studies conducted in adult populations have also demonstrated an increase in TOS and OSI levels, with TAS levels remaining unchanged (6,27). A possible explanation for the differences between our results and those reported in previous studies might be that TOS and TAS values are cumulative and may be influenced by prior infections or psychiatric conditions experienced by children. For example, a child with prolonged illness might exhibit elevated levels of both TOS and TAS. However, the presence of similar antioxidant status between groups might suggest that antioxidant levels could increase in response to oxidative stress over time. Conversely, the OSI highlights the balance between oxidant and antioxidant status, typically maintained in homeostasis. In cases where TOS increases (e.g., due to elevated antioxidant levels), OSI may not necessarily rise. Closely related to this, OSI serves as a more appropriate marker for indicating oxidative stress. Unlike the aforementioned studies, our application of logistic regression analysis provides a quantitative assessment of the independent contributions of oxidative stress markers while developing a predictive model to differentiate AD from HC. This method surpasses descriptive approaches by offering statistically robust insights into the diagnostic utility of these biomarkers.

Another important issue to consider is whether TDH parameters can effectively distinguish between AD and HC. It is somewhat surprising that the levels of total thiol, native thiol, and dynamic disulphide were higher in children with AD, but there were no statistically significant differences between the two groups concerning the proportions of reduced thiol (Native Thiol/Total Thiol), oxidised thiol (Dynamic Disulphide/Total Thiol), and thiol oxidation-reduction (Dynamic Disulphide/Native Thiol) ratios. In the current study, the findings differ from previous studies conducted on adult samples, which ascertained that oxidised thiol levels and thiol oxidation-reduction ratios –indicators of oxidation– were higher in patients with generalised anxiety disorder (GAD) and panic disorder compared to HC (18,19,28). The disruption favouring oxidation signifies an escalation in the oxidation process of thiols found in proteins. This oxidation of thiols leads to the formation of disulphide bonds. Due to their reversible nature as covalent bonds, disulphide bonds are subject to reduction back to thiols through antioxidant mechanisms, establishing a dynamic equilibrium between disulphides and thiols (24). Accordingly, in our study, the elevated levels of total thiol, native thiol, and dynamic disulphide in children with AD may be secondary to children's previous illnesses, diets, and stressful life events experienced in the past. Also,

children may experience a distinct trajectory of AD compared to adults, potentially accounting for the differences observed in the results of the current study. The divergence in findings may be attributed to a possible decline in oxidative defence mechanisms with growing age. There was no significant difference between the two groups in terms of the reduced thiol ratio, oxidised thiol ratio, and thiol oxidation/reduction ratio, the latter of which more accurately reflects current thiol oxidation. In short, it can be inferred that there is no significant oxidative imbalance through current thiol oxidation in children with AD compared to HC. Bearing in mind that the oxidative load, indicated by the imbalance in total oxidant-antioxidant status, may still result from lipid and DNA damage. A study on childhood AD found that the level of lipid hydroperoxides (LOOH), indicating lipid peroxidation, was higher compared to HC, while there was no statistically significant difference in the levels of lipid-based antioxidants such as paraoxonase and arylesterase (29).

Having noted the contribution of the current study and the importance of its findings, several limitations of this study need to be acknowledged. Foremost, the relatively small sample size has potentially limited the generalisability. Second, due to the research design in outpatient clinic conditions, medical conditions had to be based on self-report, which is inherently limited. Third, it is noteworthy that this study utilised the earlier version of the K-SADS-PL (DSM-IV-T), whereas the more recent K-SADS-PL-DSM-5-T (30) incorporates updated diagnostic criteria, which may enhance both the reliability and validity of assessments. Due to budget constraints, the study was unable to include oxidative stress measurements beyond total oxidant-antioxidant status and TDH. Adding to this, our study uses a cross-sectional design, establishing a causal relationship is challenging. It remains uncertain whether elevated levels of oxidants initiate AD or if AD leads to elevated oxidant levels. The effect of treatment on oxidative stress parameters has not been investigated due to ethical considerations, which could be addressed in studies with more complex designs. Furthermore, children with AD were not grouped based on disorder subtype. Extensive longitudinal studies involving larger samples are needed to explore the diverse subtypes of AD, particularly considering the impact of antidepressants, which have been shown to decrease oxidative stress (31).

Despite these limitations, the homogeneity and reliability of the study were enhanced by matching the sample by age and gender, and by focusing on treatment-naive children. Furthermore, excluding any comorbid psychiatric disorders from the AD group enhanced the reliability of our findings. Finally, the method used to assess oxidative stress –TOS/TAS– is simple, reliable, and fully automated, providing a rapid, sensitive, and specific measure of the cellular redox environment. Its primary advantage lies in assessing the overall redox state rather than targeting specific molecules.

To conclude, this study deepens and expands the research on the role of oxidative stress in the pathophysiological mechanisms of AD. To the best of our knowledge, this is the first study to provide valuable empirical evidence on parameters measuring total oxidant-antioxidant status and TDH simultaneously in children with AD. Specifically, higher TOS and OSI values and lower TAS values suggest an impaired oxidative balance in AD. By applying logistic regression analysis, this study reveals new dimensions of TOS relevance in AD, highlighting it as the most robust predictive marker for AD, offering a refined approach to understanding its diagnostic potential. However, the lack of significant differences in TDH, specifically in the reduced thiol ratio, oxidised thiol ratio, and thiol oxidation-reduction ratio, could indicate that other underlying mechanisms of oxidative stress, aside from TDH, may be at play in children with AD. In any circumstance, rectifying the oxidative imbalance in AD could potentially yield therapeutic benefits, regardless of the specific underlying processes involved.

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Ethics Committee Approval: Ethical approval for the study was obtained from the Ondokuz Mayıs University Ethics Committee (Approval No: 2020/599; Date: October 20, 2020).

Informed Consent: Written informed consent was obtained from both the parents and the children.

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