Thioredoxin level and inflammatory markers in children with autism spectrum disorders

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Abstract
Background: Autism Spectrum Disorders (ASD) are a group of neurodevelopmental disabilities with unknown etiology. Recent studies suggest the contribution of immune dysfunction and oxidative stress in its pathophysiology. The present study aimed to investigate the serum level of thioredoxin (Trx), as a marker of oxidative stress and some inflammatory cytokines, and to evaluate their role in children with ASD.

Results: Concentrations of Trx, IL-1β, IL-8, and TNF-α were significantly higher in children with ASD compared with matched controls. There were no association between cytokine levels and the severity of clinical manifestations, according to CARS classification of severity.

Conclusion: The present study provides support for the idea that physiological abnormalities, such as oxidative stress and immune dysfunction, may contribute in the pathophysiology of ASD.

Keywords: Autism spectrum disorders, Childhood, Cytokines, Immune dysfunction

Background

Autism spectrum disorders (ASD) are a group of neurodevelopmental disabilities that cause impaired communication and social interactions and narrow patterns of behaviors [1].

The Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), provides diagnostic criteria for ASD, which includes Asperger syndrome, classic autism, childhood disintegrative disorder, and pervasive developmental disorders not otherwise specified. Deficits may appear as early as 6–9 months of age, although most manifest during the second year. Reliable diagnosis can be made around age 2; however, many children do not have final diagnosis until much older [2].

Multiple pathological mechanisms implicated in psychiatric diseases including immune dysregulation, inflammatory processes, oxidative stress, environmental contaminants exposures, and mitochondrial dysfunction [3].

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alpha (TNF-α) and interleukin-1β (IL-1β) are found to have an essential role in neurodevelopment and synaptic pruning [15, 16]. Moreover, emerging evidence involves cytokines in higher neurological functions, including cognition and memory [17, 18]. Thus, abnormal inflammatory activity could contribute to behavioral and neurological dysfunction in ASD [14, 19].

Therefore, the aim of the present study was to investigate the serum level of thioredoxin and some inflammatory cytokines (IL-1β, IL-8, TNF-α), and to evaluate their role in children with autism spectrum disorders.

Methods
A case control study was conducted in the psychiatry outpatient clinic at Al-Azhar University Hospital. Thirty five children with ASD aged from 2 to 10 years were randomly selected.

1. Inclusions criteria
   (a) Newly diagnosed children with autism spectrum disorders who are fulfilling the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) [20].
   (b) Score on childhood autism rating scale (CARS) is above 30 [21].

2. Exclusion criteria
   (a) Children with another psychiatric disorder (e.g., ADHD, anxiety, depression)
   (b) Children with other causes of intellectual disability, other causes of language impairment
   (c) Children with chronic medical co-morbid condition
   (d) Children with proved genetic, metabolic, or neurologic etiology for autistic disorder
   (e) Children receiving antioxidants

Another 35 apparently healthy children with matched age and sex were recruited from the outpatient clinic at Al-Azhar University Hospital and served as a control group. They were the siblings of the children attending this clinic because of a minor illness. The control children were not related to the children with ASD, and demonstrated no clinical findings suggestive of infections, allergic manifestations, and immunological or neuropsychiatric disorders.

Ethical considerations
The study protocol was approved by the institution ethical review board (medical research ethics committee at National Research Centre, Cairo, Egypt), according to Helsinki Declaration 1975 revised in 2008.

Written informed consent was obtained from all parents of both patients and control groups before getting them involved in the study. They were informed by the aim and potential benefits of the study.

All studied children were subjected to the following:

1. Thorough history taking including:
   (a) Personal data: age, sex, and residence
   (b) Prenatal history: Parental age at birth (maternal and paternal), maternal diseases (as diabetes mellitus, hypertension, epilepsy, depression, infections), and maternal drug therapy (as hormonal, antiepileptic, anti-hypertensive, or antipsychotic drugs)
   (c) Natal and postnatal history as gestational age at birth, birth weight, mode of delivery, and birth order

2. Thorough clinical examination which included meticulous general and specific systemic examination.
3. Clinical diagnosis of ASD was based on the criteria for autistic disorder as defined in the DSM-5.
4. Assessment of autism severity using Childhood autism rating scale (CARS): The CARS is a 15-item behavioral rating scale developed to identify autism as well as to quantitatively describe the severity of the disorder [22]. The examiner assigned scores between 1 and 4 for each domain: 1 indicates normal behavior appropriate for age level (no signs of autism), while 4 indicates a severe deviance with respect to the normal behavior (severe symptoms of autism). The scores for the single items are added together into a total score. The maximum CARS score is 60, and the cutoff for autism is 30 [21, 23].

Accordingly, patients with ASDs were classified into three groups:

a. Mild cases group: their (CARS) score ranging from 30 to < 33
b. Moderate cases group: their (CARS) score ranging from 33 to < 37
c. Severe cases group: their (CARS) score at or above 37
5. Anthropometric assessment: Weight, height, and BMI were expressed as weight-for-age Z-score (WAZ), height-for-age Z-score (HAZ), and BMI-Z-score (BAZ) after being calculated according to the WHO standards using the Anthro Plus software of WHO (2009) [24].

6. Laboratory investigations:

(a) Complete blood picture was done automatically by EPX MICRO 60 automated hematological counter.
(b) Five milliliters of venous blood were collected and delivered into a tube and were allowed to clot and after centrifugation, sera were separated and stored frozen at −20°C after careful labeling till the time of assay.
(c) Quantitative determination of serum thioredoxin (Trx), Interleukin 1β (IL-1β), IL8, and tumor necrosis factor α (TNF-α) concentration were done using ELISA technique. Kits were delivered by INOVA.

Principle of the assay
This ELISA kit uses Sandwich-ELISA as the method. The Micro Elisa strip plate provided in this kit has been pre-coated with an antibody specific to thioredoxin (Trx), IL-1β, IL8, and TNF-α standards, or samples are added to the appropriate Micro Elisa strip plate wells and combined to the specific antibody. Then, a horse-radish peroxidase (HRP)-conjugated antibody specific for Trx, IL-1β, IL8, TNF-α is added to each Micro Elisa strip plate well and incubated.

Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain Trx and HRP conjugated Trx antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wave length of 450 nm. The OD value is proportional to the concentration of Trx, IL-1β, IL8, and TNF-α concentration of Trx in the samples can be calculated by comparing the OD of the samples to the standard curve [25].

Statistical analysis
Data were analyzed using the statistical package for social science (SPSS). Computer software package SPSS 16.0 was used in the analysis. For quantitative variables, mean, standard deviation, minimum, and maximum (as measures of variability) were presented. ANOVA, Independent t, Mann-Whitney, and Kruskal Wallis tests were used to estimate differences in quantitative variables. Spearman’s rank correlation test was used to determine the relationship between different numerical variables. For all tests, probability values (p) of less than 0.05 were regarded as statistically significant.

Results
This study was conducted on 35 children with autism spectrum disorders (ASD); 24 males and 11 females with age ranging from 2 to 10 years (mean age = 4.7 ± 2.4 years), 10 of them were mild ASDs, 16 children were moderate ASDs, and 9 children were severe ASDs. An additional 35 healthy children (16 females and 19 males) with mean age of 5.4 ± 2.4 years were served as a control group. Comparison between the two studied groups according to demographic data is shown in Table 1.

No significant differences were found between groups as regards the anthropometric measurements. Hemoglobin concentration was significantly lower in ASD group (p = 0.003), but no significant differences were found in other hematological parameters as shown in Table 2.

Table 3 shows that levels of IL-1β, IL-8, and TNF-α were significantly higher in children with ASD compared with those of controls. In addition, the mean level of thioredoxine in children with ASD was significantly higher as compared with that of controls (170.4 ± 54.2 and 76.9 ± 24.8 pg/mL, respectively p < 0.001).

We then examined whether there were associations between cytokine levels and the severity of clinical manifestations; according to CARS classification of severity, patients were divided into 3 subgroups: mild, moderate, and severe. Comparison of thioredoxin and cytokines levels between ASD subgroups is shown in Table 4. No significant differences were found between subgroups.

Discussion
Evidence is accumulated that ASD is interrelated with certain physiological abnormalities, including oxidative stress and immune dysregulation [26].

Research suggests the involvement of cytokines in neurodevelopment, beside their effect in some neurological functions, including cognition and memory [18, 27]. Hence, disturbances within cytokine levels may result in multiple neurological outcomes related to ASD [28].

In addition, disturbance in the levels of oxidative stress may be relevant to the pathophysiology of ASD [6].

The aim of the present study was to assess the state of immune dysregulation, as estimated from the serum levels of some cytokine (IL-1β, IL-8, TNF-α) and thioredoxin, as an indicator of oxidative stress in children with ASD.

In this work, it was found that 68.6% of autistic patients were males, and male to female ratio was 2.19:1 respectively. This finding is corresponding to previously reported ratios [29].
Results also revealed that residence in urban areas is not a risk factor of autism. These results are in accordance to the study of [30]. However, [31] stated that patients with ASD were more common in urban areas.

The current study showed that none of maternal or paternal age was a risk factor for ASD. This was previously proved by [30]. However, [32] illustrated an association between advancing paternal age and risk of ASD. On the other hand, [33] showed that high maternal age (> 35 years) may be a predisposing factor for ASD. Moreover, [34] revealed that the risk of ASD was greater with increase of either paternal or maternal age.

Our ASD patients were anemic as their mean level of hemoglobin was (10.37 ± 1.24 g/dl) compared with (11.55 ± 1.27 g/dl) in healthy children. This finding was in accordance with results of [35] that revealed an association between ASDS and iron deficiency anemia.

In addition, [36] reported a frequency of 28.07% of children with ASDs having anemia based on low hemoglobin concentrations; they explained this finding by the high prevalence of diminished iron intake in those children, due to the associated feeding difficulties and food selectivity.

In the present study, the serum levels of IL-1β, IL-8, and TNF-α were significantly higher in patients with ASD in comparison to control subjects (p = < 0.001, 0.04, < 0.001 respectively).

As several pro-inflammatory cytokines are known to be produced by adipose tissue and the serum levels of those cytokines have been correlated with parameters of

| Table 1 | Demographic and clinical characteristics data of autism spectrum disorders (ASD) and control children |
|---------|--------------------------------------------------------------------------------------------------|
| Characteristic | ASD (mean ± SD) | Controls (mean ± SD) | p |
| Number of children | 35 | 35 | NS |
| Age (year) (mean ± SD) | 4.7 ± 2.4 | 5.4 ± 2.4 | NS |
| Sex | | | |
| Males | 24 | 19 | NS |
| Females | 11 | 16 | |
| Residence | | | |
| Urban | 29 | 24 | NS |
| Rural | 6 | 11 | |
| Paternal age at birth (year) (mean ± SD) | 32.5 ± 6.8 | 31.3 ± 3.8 | NS |
| Maternal age at birth (year) (mean ± SD) | 28.2 ± 5.02 | 27.9 ± 3.7 | NS |
| Prematurity | Yes | 5 | 3 | NS |
| No | 30 | 32 | |
| Groups of ASD according to CARS | n (mean ± SD) | | |
| Mild (30–33) | 10 (30.6 ± 0.3) | N/A | <0.001* |
| Moderate (33–37) | 16 (34.6 ± 0.8) | N/A | |
| Severe (37–60) | 9 (44.2 ± 3.6) | N/A | |

NS non-significant, N/A not applicable, CARS childhood autism rating scale  
*p significant p value (p ≤ 0.05)

| Table 2 | Comparison between the two studied groups according to anthropometric and hematological parameters |
|---------|--------------------------------------------------------------------------------------------------|
| Parameter | ASD (mean ± SD) | Controls (mean ± SD) | p |
| WAZ | 0.067 ± 0.47 | 0.11 ± 0.5 | NS |
| HAZ | 0.09 ± 0.4 | 0.1 ± 0.5 | NS |
| BAZ | 0.02 ± 0.6 | 0.1 ± 0.9 | NS |
| Hb (g/dl) | 10.37 ± 1.24 | 11.55 ± 1.27 | <0.001* |
| RBCs (× 10^6/mm³) | 4.81 ± 0.5 | 4.73 ± 0.4 | NS |
| WBCs (× 10^3/mm³) | 7.87 ± 2.26 | 7.77 ± 2.58 | NS |
| Platelets (× 10^3/mm³) | 336.6 ± 71.5 | 311.5 ± 72.2 | NS |

WAZ weight-for-age Z-score, HAZ height-for-age Z-score, BMI body mass index, Hb hemoglobin, RBCs red blood cell count, WBCs white blood cell count,  
NS non-significant  
*p significant p value (p ≤ 0.05)
obesity [37]; therefore, we measured the weight and height of all the participants and body mass index (BMI) was calculated.

Our results showed no significant difference between the two groups as regard WAZ, HAZ, and BAZ. Thus, it is likely that the elevations in serum levels of those cytokines were significantly associated with the diagnosis of ASD.

In accordance with our results, [38] indicated that the levels of many immune proteins in plasma, including cytokines, are altered in ASD. Also, [14] reported high plasma concentrations of pro-inflammatory cytokines, such as IL-1β and IL-8 in autistic patients. The findings of elevated plasma levels of IL-8, a chemoattractant cytokine of important role in inflammation process, coincide to IL-8 elevation reported in the brain and cerebrospinal fluid (CSF) in ASD [39].

Moreover, [40] reported higher levels of IL-8 without similar increases in IL-1β or TNF-α. They suggested that IL-8 is the most sensitive pro-inflammatory mediator in autistic.

On the other hand, [41] found increased TNF-α in the cerebrospinal fluid of autistics. The disturbed immunity was further evidenced by increased levels of pro-inflammatory cytokines in brain tissue of autistics [11, 25, 42].

In addition, histological changes of microglia cells that were found in different brain areas provide an evidence for activation of the immune system [43]. These histological changes were supported by neuroimaging studies as well as by [44].

Synaptic dysfunction is consistently thought to be the main underlying mechanism of ASD. Pro-inflammatory mediators, including cytokines, might be involved in different synaptic processes, such as long-term potentiation (LTP), that is involved in synaptic plasticity in learning and memory processes [45]; cytokines, such as IL-18, IL-1β, and TNF-α, act on many molecular components of LTP in variable fashions [46].

Our results revealed that none of the analytic serum levels of cytokines were correlated with the severity of autistic symptoms. So it was suggested that the elevation of cytokines observed in this study may represent an abnormal steady-state immune response in subjects with ASD.

Oxidative damage is thought to play a central role in the pathogenesis of autism [47]. Different evidences supported the relation between oxidative stress and ASD. For instance, the evidence of diminished antioxidant capacity besides enhanced production of oxidative stress biomarkers that has been found in children with ASD [48, 49]. Previous reports have shown that the serum thioredoxin level is a good indicator of oxidative stress [50].

A major concern of this study was to assess the serum level of thioredoxin in children with autism as compared with that of the controls, and it was interesting to find that there was a highly statistically significant increase in the mean serum level of thioredoxin in ASD group as compared with that of the control group (\( p = 0.001 \)).

These results were in agreement with a study done by [6] who found that the median serum thioredoxin levels were significantly higher in children with ASD as compared with typically developing children.

Conclusions
Overall, the present study provides support for the idea that physiological abnormalities such as oxidative stress and immune dysfunction, may contribute in the etiopathogenesis of ASD. The available data indicate a

| Table 3 | Inflammatory cytokines and thioredoxin levels in the two studied groups |
|---------------------------|---------------------------------|--------------------------|
| Parameter                 | ASD (mean ± SD) | Controls (mean ± SD) | \( p \) |
| Interleukin-1β (pg/ml)   | 54.2 ± 22.3     | 21.66 ± 11.3           | < 0.001* |
| Interleukin 8 (pg/ml)    | 59.4 ± 34.4     | 45.3 ± 18.5            | 0.04*   |
| Tumor necrosis factor-α (pg/ml) | 344.29 ± 51.6 | 285.5 ± 64.6           | < 0.001* |
| Thioredoxin (pg/ml)      | 170.4 ± 54.2    | 76.9 ± 24.8            | < 0.001* |

*significant \( p \) value (\( p \leq 0.05 \))

| Table 4 | The mean serum level of cytokines and thioredoxin in different groups of children with ASD |
|---------------------------|-----------------|-----------------|
| Parameter                 | Mild ASDs (\( n = 10 \)) (mean ± SD) | Moderate ASD (\( n = 16 \)) (mean ± SD) | Severe ASDs (\( n = 9 \)) (mean ± SD) | \( p \) |
| Interleukin-1β (pg/ml)   | 65.6 ± 37.5     | 51.3 ± 10.8     | 46.8 ± 8.2     | NS |
| Interleukin 8 (pg/ml)    | 62.7 ± 29.2     | 59.9 ± 26.5     | 54.8 ± 52.2    | NS |
| Tumor necrosis factor-α (pg/ml) | 336.5 ± 56.5 | 344.7 ± 51.7    | 352.2 ± 50.8   | NS |
| Thioredoxin (pg/ml)      | 168.3 ± 57.1    | 167.5 ± 60.7    | 177.8 ± 42.6   | NS |

NS non-significant
possible relationship between cytokine alterations and autism. However, systematic investigations of the immunological factors in autism are needed and will provide a better understanding of the underlying mechanisms responsible for the pathogenesis of autism.

Abbreviations
ASD: Autism spectrum disorders; BAZ: BMI-Z-score; BMI: Body mass index; CARs: Childhood autism rating scale; CSF: Cerebrospinal fluid; DSM-5: The Diagnostic and Statistical Manual of Mental Disorders, 5th edition; HAZ: Height-for-age Z-score; HRP: Horseradish peroxidase; IFNγ: Interferon gamma; IL-1β: Interleukin-1β; LTP: Long-term potentiation; OD: Optical density; p: Probability values; SPSS: Statistical package for social science; TGFβ1: Transforming growth factor β1; TNF-α: Tumor necrosis factor alpha; Trx: Thioredoxin; WAZ: Weight-for-age Z-score

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Authors’ contributions
NAA was a major contributor in writing the manuscript, OMI provided recent references for the discussion, HAE was responsible for the lab work, and NME and BH participated in the design of the study. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate
The study protocol was approved by the institution ethical review board (medical research ethics committee at National Research Centre, Cairo, Egypt) according to Helsinki Declaration 1975 revised in 2008. Committee’s reference number is 1453. Written informed consent was obtained from all parents of both patients and control groups before getting them involved in the study. They were informed by the aim and potential benefits of the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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