RESEARCH ARTICLE

Some Immunological and Physiological Parameters Levels in Iraq Children with Autism Spectrum Disorders

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ABSTRACT

Some studies suggested that dysfunction of immune and endocrine systems could link with neurodevelopment disorder and immunopathogenesis of ASD. Therefore, the goal of this research is to examine the level of some immunological and physiological indicators in an effort to identify a new biomarker for ASD. In the current study, there were 72 Iraqi kids (both boys and girls), 50 kids with ASD who attended different autism centers in Iraq, and 22 kids without ASD. They were between the ages of 6 and 12. These children's peripheral venous blood was drawn in order to measure the concentrations of several cytokines, such as (TNF-α and IL-6), cortisol, and WBCs indices and platelets/lymphocytes ratio. The study's findings revealed: 1) a significantly higher level of TNF- α but not IL-6 in ASD patients when compared to control; 2) a significantly higher level of cortisol in ASD patients compared to controls; and 3) a significantly higher level of lymphocytes and total WBCs but not neutrophils and monocytes. 4) A significant increase in the platelets/lymphocytes ratio. Furthermore, there were no differences in these parameters based on the gender and severity of the autism. As a result, our findings revealed that cytokine level alterations may be crucial in the development of neurological diseases like autism since microglia release large amounts of TNF-α, which plays a significant role in the so-called neuroinflammatory response. An abnormal HPA axis with high or low concentration of cortisol in more than 22% of the ASD was found in our results, which could explain the different reactions of autistic children when exposed to the same situation. So we thought that cytokines, especially TNF-α, and cortisol, may represent a serological marker for autism.

Keywords: Autism**,** TNF-α, IL-6, Cortisol, White blood cells indices.

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INTRODUCTION

Autism spectrum disorder (ASD) is a complex, widespread, and heterogeneous disease marked by varying degrees of dysfunctional social communication, narrow interests, and repetitive behaviors.[1] ASD sufferers experience anxiety, poor emotional learning, inappropriate emotional responses, and a lack of social connection and communication.[2] The signs and symptoms can range from subtle in social interactions, like failing to control non-verbal behaviors, to severe, like a lack of verbal communication and dysfunctional routines, which seriously impair the person's capacity to cope in social situations or those that diverge from their regular daily schedule.[3] The disease's global prevalence has been estimated to be around 1%, with a male-to-female ratio of 4:1.[4] According to the Centers for Disease Control and Prevention and Autism and Developmental Disabilities Monitoring Network, approximately one in every 44 (2.3%) in children aged eight years hase ASD.[5] There is currently no definitive medical plan for diagnosing and caring for children with ASD. Understanding the pathophysiological factors that drive ASD, including immunological dysfunction, is an important part of such planning.[6] On the other hand, despite over a half-century of research into the pathogenesis of ASD, the etiology remains unknown.[7]

Cytokines are defined as small soluble and secreted proteins.[8] In practically every aspect of human immunology, cytokines operate as messengers of innate and adaptive immunity,[9] so it is responsible for immune response and communication between immune cells and significantly influences infectious disease resistance and infection clearance.[10] Cytokines are classified into several families, often based on structural considerations for the cytokines and their receptors, and signaling methods.[11] Chemokines, lymphokines, interleukins (IL), interferons (IFN), and tumor

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necrosis factors are examples of these messenger molecules.[12] According to studies, the immunological pathogenesis of ASD is mostly caused by disturbance of the cytokine system. Brain tissue analysis reveals an increase in proinflammatory chemokines in ASD patients. Actually, the link between autism and immunity was first noted in the 1970s, when it was stated that newborns with a history of maternal sickness during pregnancy or after exposure to the measles, mumps, and rubella vaccine showed signs of ASD.[6] Immune system dysfunctions in people with ASD have also been described, including immune cell overstimulation, cytokine/chemokine imbalances, and enhanced blood-brain barrier permeability. In addition, according to epidemiological research, immune dysregulation was found to be more common in both mothers and fathers of children with ASD.[13] Tumor necrosis factor-alpha (TNF-α) was first discovered in 1975 to promote tumor hemorrhagic necrosis, which helps classify their subfamily.[14] TNF-α is produced by several types of cells, including macrophages, killer NK cells, monocytes, endothelial cells, white blood cells (neutrophils), smooth muscle cells, activated lymphocytes (T-lymphocytes), astrocytes, and adipocytes.[15] TNF-α regulates various developmental and immunological processes (the cellular immune response), including inflammation, differentiation, cell survival, lipid metabolism, and cell death.[16] Increased TNF-α level in the blood causes many diseases, including neural disease.[17] Interlukine-6 (IL-6), one of the interleukins, is released by a range of immune cells, including macrophages, neutrophils, dendritic cells, lymphocytes (helper CD4 + T lymphocytes), monocytes,[15] and endothelial cells.[18] It is thought that higher proinflammatory cytokine production, such as interleukin IL-6, may be linked to an increased risk of ASD. However, it was shown that one of the most frequent proinflammatory cytokines in autistic brains was TNF-α.[6]

Cortisol, the end product of the hypothalamus-pituitary-adrenal (HPA) axis,[19] is a steroid hormone that the human body produces in response to psychological and physiological stress.[20] Since cortisol produces under control of the paraventicular nucleus of the hypothalamus, any change in the adrenergic nerves during immune response or neurodevelopment disorder could affect cortisol production.[21] Autism patients are regularly observed reacting inappropriately to environmental changes or unfamiliar settings that do not bother others. Studies on the variability of cortisol levels in children with autism have shown conflicting results.[22] Some suggest that autistic children experience higher levels of stress because they have a harder time adapting to new situations and that some environmental pressures may cause exaggerated behavioral reactions to stressful events, which may be accompanied by a concurrent spike in cortisol.[22]

Therefore, the purpose of this work is to investigate the level of some immunological markers, such as some cytokines and WBCs indicese and some physiology barameters such as cortisol and platelets/lymphocytes ratio as a trying to find the causes behind this conflicting results of these parameters, and to find if these parameters could used as a biomarker of ASD.

MATERIAL AND METHODS

In current study, 72 Iraqi children participated, 50 of whom had autism and had visited numerousIraq autism centers. The remaining 22 participants were healthy and of the same gender. The age range of the subjects was 6 to 12. According to final diagnosis given by a specialist physician based on criteria about the clinical symptoms and signs of all children in the ASD group, autism was identified in all of them at various levels, ranging from mild, moderate, and severe. All patients' medical histories, including name, gender, age, disease stage, if they have other illnesses and data of diagnosis, were obtained from their profiles. The control group's healthy participants had no history of illness or other inflammatory diseases.

After getting the approval of the patients' parents, blood samples totaling five milliliters were taken from all individuals (ASD and healthy). Using a hematology analyzer from Ms4s/French, 1-mL of blood was mixed into an anticoagulated tube containing ethylene diamine tetraacetic acid (EDTA). The CBC was then calculated. The remaining 4 mL of blood samples were put into a gel activator tube, where they were allowed to coagulate for 15 minutes at 40°C. After that, the serum was separated from the tubes by centrifuging them at 3000 rpm for 10 minutes. The serum was utilized to measure the levels of TNF-alpha and IL-6 using sandwich enzyme-linked immunosorbent assay (ELISA) techniques using kits from Melsin/China, and the levels of cortisol using Cobas 6000 analyzer from Germany origin.

Results were presented as mean standard error. The data were examined for multiple comparisons using Fisher's test or t-test following one-way analysis of variance ANOVA, using Statview version 5.0. The differences were deemed significant at $p < 0.05$.

RESULTS

Table 1 shows a significant increase ($p < 0.05$) in levels of TNF-α (pg/mL) in the serum of ASD children (8.255 ± 0.516) as compared to children without ASD (4.877 ± 0.868). On the other hand, regarding to the level of IL-6 (pg/mL), there was no significant ($p < 0.05$) differences in the serum of ASD children (12.135 ± 0.364 pg/mL) compared with its level in the control group (11.725 ± 0.327 pg/mL). There is a significant increase ($p < 0.05$) in levels of cortisol (nmol/l) in the serum of ASD children (298.089 ± 16.244) compared to the children without ASD (241.936 ± 14.935).

Table 1: The levels of TNF-α, IL-6 and cortisol in the serum of the ASD and control group

Groups	*TNF-α (pg/mL) Mean ± SE*	*IL-6 (pg/mL) Mean ± SE*	*Cortisol (nmol/l) Mean ± SE*
Control	4.877 ± 0.868	11.725 ± 0.327	241.936 ± 14.935
Patient (ASD)	8.255 ± 0.516*	12.135 ± 0.364	298.089 ± 16.244*
p-value	0.0008	0.4838	0.0349

*Significantly different between ASD children vs. control groups

Table 2: The White blood cell indices in the blood of the children with ASD and the control groups

Groups	*Total WBC (cell x10^3/ml) (Mean ± SD)*	*Lymphocytes (cellx10^3/mL) Mean ± SD*	*Monocytes (cellx10^3/mL) Mean ± SD*	*Neutrophils (cellx10^3/mL) Mean ± SD*	*(P/L) Mean ± SE*
Control	6.910 ± 0.372	2.301 ± 0.243	0.580 ± 0.038	4.553 ± 0.467	103.952 ± 8.741
Patient (ASD)	8.045 ± 0.325*	3.033 ± 0.138*	0.592 ± 0.029	4.186 ± 0.273	127.761 ± 7.075*
P-value	0.0400	0.0068	0.8124	0.4903	0.0385

*Significantly different between ASD children vs. control groups

Table 3: The levels of TNF- α, IL-6, , and Cortisol levels in the serum of the ASD groups depended on gender

Gender	*TNF- α(pg/mL) Mean ± SE*	*IL-6(pg/mL) Mean ± SE*	*Cortisol (nmol/l) Mean ± SE*
Male	8.351 ± 0.659	11.557 ± 0.401	296.855 ± 15.863
Female	7.970 ± 0.626	12.018 ± 0.585	301.792 ± 45.891
p-value	0.7531	0.5119	0.8970

According to Table 2, there was a significant increase ($p < 0.05$) in the total WBC and lymphocyte counts (x10^{3}cell/mL) in the serum of ASD children when compared to the control group (8.045 ± 0.325 and 3.033 ± 0.138 x10^{3}cell/mL, respectively). while there were no significant ($p > 0.05$) differences in the levels of monocytes and neutrophils (x10^{3}cell/ml) between the ASD group (0.592 ± 0.029 and 4.186 ± 0.273 x 10^{3}cell/mL, respectively) compared to control group (0.580 ± 0.038 and 4.553 ± 0.467 x 10^3 cell/mL, respectively). Interestingly, there was significant ($p < 0.05$) increase in the (P/L) ratio of ASD children compared to control group (127.761 ± 7.075 and 103.952 ± 8.741, respectively).

However, there were no significant *($p > 0.05$)* variations in TNF-α (pg/mL), IL-6 (pg/mL), and cortisol (nmol/l) levels in the male group when these parameters were studied in the ASD depend on the gender 8.351 ± 0.659, 11.557 ± 0.401 pg/mL, and 296.855 ± 15.863 nmol/l, respectively compared to the female 7.970 ± 0.626, 12.018 ± 0.585 pg/mL and 301.792 ± 45.891 nmol/l, respectively in Table 3.

Depending on the ASD severity shown in Table 4, these variables were also investigated in ASD. Additionally, there were no notable variations among TNF- α, IL-6, and cortisol in mild ASD 8.354 ± 0.800, 12.672 ± 0.598 pg/mL, and 259.276 ± 21.921 nmol/l, respectively, moderate ASD 8.270 ± 0.823, 12.022 ± 0.544 pg/mL, 326.080 ± 25.093 nmol/l, respectively and severe ASD (7.959 ± 0.978, 11.232 ± 0.733 pg/mL, and 319.020 ± 30.394 nmol/l, respectively).

DISCUSSION

For the first time in more than 40 years, an association between immune system dysfunction and ASD was suggested. A probable link between the autistic phenotype and various features of impaired immunity, such as cytokine levels, has been hypothesized in several studies. Immune system abnormalities have recently been discovered in ASD patients.[13] In recent years, research have shown that ASD is associated with a strong inflammatory state, which is frequently connected to immune system malfunction.[23] Experimental investigations have shown that cytokines, such as TNF- α, are crucial for the emergence of ASD and are linked to its identification.[24,25] In our investigation, we discovered a considerably higher level of TNF- α serum levels in children with ASD compared to controls. This finding backs up previous research that found higher level of some cytokines including TNF-α levels in serum,[25,26] plasma, CSF, and brain of autistic subjects,[27] while in early study show that the level of TNF-α in autism patient was not different from control group,[28] however, the researchers found that the majority of autistic children in the study, particularly those with elevated TNF- α, had overactive or poorly regulated innate immune responses.[24] Furthermore it has been demonstrated that too much TNF- α plays a role in autistic traits including decreased social interaction and repetitive actions.[29] Moreover, we did not find any significantly different in IL-6 serum levels of children with ASD compared to controls which agreed with the studies of Suzuki *et al.* (2011) and Singh, 1996 who found no significant differences in serum IL-6 levels between ASD children and the control group,[28,25] while some study showed increasing level of Interleukin-6 in the ASD subjects.[23,29] This variation in cytokine levels between studies is probably due to certain constraints, including sample quality, cytokine profile alterations over time, statistical adjustments,[23] and inflammatory response.[29] Ricci *et al.*, 2013 showed no correlation between the cytokine level and autism severity, which agreed with our results as there was no difference in the level of both cytokines depending on the autism severity. On the other hand, our data shows there is no significant difference in the level of the pro-inflammatory cytokines TNF-α and IL-6 between males and females with autism and this agreed to other studies conducted on autistic children that shows there is no correlation between the level of this cytokines and gender.[30] It is thought that the blood-brain barrier is compromised in ASD, which is accompanied by a rise in neuro-inflammatory processes. The barrier membrane's increased permeability permits proinflammatory signaling chemicals generated by

Table 4: The levels of TNF-α, IL-6 and cortisol in the serum of the ASD related to ASD degree (mild, moderate and severe)

Groups	*TNF- α (pg/mL) Mean ± SE*	*IL-6(pg/mL) Mean ± SE*	*Cortisol (nmol/l) Mean ± SE*
Mild	8.354 ± 0.800	12.672 ± 0.598	259.276 ± 21.921
Moderate	8.270 ± 0.823	12.022 ± 0.544	326.080 ± 25.093
Severe	7.959 ± 0.978	11.232 ± 0.733	319.020 ± 30.394
p-value	L vs. M= 0.9421 L vs. S=0.8343 M vs. S=0.8646	L vs. M=0.4210 L vs. S=0.2726 M vs. S=0.5304	L vs. M=0.0586 L vs. S=0.2891 M vs. S=0.8958

circulating monocytes to enter freely, altering neural function. Several types of brain and immunological cells are involved; Mast cells are stimulated to release IL-6 and TNF-α, which leads to microglia and localized brain inflammation are then activated and proliferate, which disrupts neural plasticity and result in deficits in social interaction, communication, and behavior.[23] Therefore, peripheral cytokines can pass across the blood-brain barrier and signal to the brain, and can play a crucial role in brain development.[29] D'Mello and Swain, 2009 suggested the cellular mechanism used by peripheral cytokines and their mediated signals to reach the brain is as follows: TNF- α and other cytokines may drive microglia to recruit monocytes into the brain (perhaps by increasing the synthesis of monocyte chemoattractant protein).[31] TNF-α, on the other hand, is a proinflammatory cytokine that affects the central nervous system's homeostasis and pathophysiology. In pathological conditions, microglia create large levels of TNF- α; this de novo synthesis of TNF- α is a key part of the so-called neuroinflammatory response associated with several neurological illnesses.[32] Thus, TNF-α has a role in the manifestation of ASD symptoms more than IL-6, which may explain the increase in TNF-α but not IL-6 levels in the ASD group in our work.

Despite the fact that numerous studies have discovered elevations in inflammatory cytokines in ASD, few have isolated and stimulated circulating monocytes to uncover dynamic responses of these cells in children with ASD. These studies' findings revealed that children with ASD have more monocytes than usual[33,34] and neutrophiles[35,36] refuting the conclusions of Ellul *et al.*, 2022 and the current investigation, which found no differences between the monocytes from ASD and controls as well neutrophils. But compared to the control group, our findings showed that the ASD group had more total WBCs and lymphocytes.[37] These findings corroborate those of Ashwood *et al.*, 2011, and Harutyunyan *et al.*, 2021, who found that ASD patients had higher median lymphocyte and total WBC counts than control persons.[38,39] Robinson-Agramonte *et al.* 2022 also found that children with ASD had a reduced lymphocyte response to mitogens and showed that lymphocyte subsets were involved in the pathogenesis of ASD.[40] Meanwhile, Ellul *et al.*, 2022 advocated taking into account the disparity in results between research that was linked to a number of causes of heterogeneity. First, variations in staining techniques can contribute to the heterogeneity of results. Second, due to the inherent heterogeneity of the ASD population, more homogeneous subgroups must be studied.[37] However, we believe the results were homogeneous, with the lymphocyte count rising in correlation with the TNF- α level rising and the monocyte count declining in correlation with the IL-6 level declining. This leads us to believe that the innate immune response of ASD subjects is not as affected as the humoral immune response. Therefore, the calculation of the platelets\lymphocyte ratio is important to prove this fact and as expected, because of the role of platelets in the immunity response, our results found an increase in this ratio in the ASD subject compared to control. To our knowledge, there are only one work that studied PLRs in ASD, which was similar in comparison to healthy controls.[36] This study disagreed with ours, but they did not explain their results, so this result needs more research to prove it.

It is widely known that cortisol often exhibits a strong rise in the morning hours, followed by a steady drop throughout the day until it reaches its nadir during nighttime sleep.[41] Any departure from this pattern is indicative of dysregulation of the HPA axis. Numerous investigations that have assessed cortisol's diurnal regularity and responsiveness have suggested that there is more circadian disruption in ASD compared to normally developing (TD) healthy controls.[7] In our study, afternoon cortisol concentrations were measured. The results showed that children with ASD had considerably higher cortisol blood levels than the controls, but the mean concentration of cortisol in both groups was within the normal value (55–386 nmol/l in the afternoon determination). Numerous research studied serum cortisol levels. However, the findings were contradictory. Some studies agreed with our results, which demonstrated a significantly higher cortisol level in the serum[7,22] and in urine, salivary,[22] and hair[42] of children with autism than in healthy children, which could make ASD children more anxious and exhibited than control children.[42] Contrary to our findings, another investigation using morning plasma cortisol collection found lower cortisol levels in ASD children than in controls.This pattern of hormone fluctuation could indicate autism-related abnormalities in the HPA axis. On the other hand, disruption of the HPA axis has been connected to both excessive and insufficient cortisol responses.[43] Interestingly, when we calculated the case frequency of the ASD subjects has higher cortisol concentration compared to the normal value the results was 18, 4% decreased cortisol level and 78% showed normal level while the case frequency of control subject has a higher level of cortisol was 4.545 and 95.45% normal cortisol. These results could suggest that ASD subjects have abnormal HPA axis with high or low concentrations of cortisol in more than 22% of the patients but not all. This finding could explain the inconsistent results of the cortisol concentration in different studies. Many studies showed no significant between cortisol concentration and autism severity,[7,44,22] which agreed with our results. The results of other studies that compared urinary and serum cortisol levels across genders and between people with and without autism found no statistically significant differences between males and females, supporting the findings of our study that there is no statistically significant difference in the level of cortisol between males and females.[22]

CONCLUSION

The current study's findings suggest that ASD participants underwent a number of alterations, including the following.;

- Increase the level of TNF-α but not IL-6 in autistic children; this increasing TNF-α is an important component of the so-called neuroinflammatory response associated with several neurological disorders. Thus, TNF-α has a role in the manifestation of ASD symptoms more than IL-6. Therefore, a thorough evaluation of peripheral cytokine

levels may offer a novel strategy to enhance the prognosis of autistic individuals.

- Increase the count of lymphocytes and P/L ration. Taken together, point 1 and 2 could lead us to believe that the innate immune response of ASD subject is not as affected as the humoral immune response. 3) Increase the level of cortisol in autistic children, but the mean concentration of the cortisol in both groups was within the normal value. When the case frequency of abnormal cortisol levels was calculated, abnormal HPA axis with a high or low concentration of cortisol in more than 22% of the ASD but not all was found which could explain the different reactions of autistic children when exposed to the same situation.

Moreover, neither gender nor the severity of the autism was related to cytokines or cortisol levels. These, however, require additional investigation because ours have an uneven number of patients with various levels of autism severity. These measures' evaluation may serve as predictive biomarkers of clinical symptoms, offering crucial direction for upcoming treatment approaches to reestablish physiological homeostasis.

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