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Biochemical Studies of Autism Spectrum Disorder Patients in Mosul City

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Abstract

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder. It includes five diagnostic subtypes. The current study was carried out among 2-12 years old children with ASD. Thirty seven subjected to ASD (31males and 6 females) were selected from psychiatric research unit -Mosul University. Thirty healthy children were enrolled in this study (20 males and 10 females) with the same age as control group. ASD patients were classified into three groups according to symptoms severity grade. The results indicated a significant ($p \leq 0.05$) decrease in the serum melatonin (-81.8%), neuroglobin (-84.9%), antioxidant activity (-69.8%) and glutathione (-63.3%) in severe ASD group compared to control group. At the same time a significant ($p \leq 0.05$) increase in the serum thiobarbituric acid reactive substances (+82.6%), advanced oxidation protein products (+120.6%) and nitric oxide (+85.5%) in severe ASD patients group compared to control group were observed. Also, a positive significant ($p \leq 0.01$) correlation between antioxidant activity and melatonin, neuroglobin, glutathione was shown, while a negative significant ($p \leq 0.01$) correlation between antioxidant activity and thiobarbituric acid reactive substances, advanced oxidative protein products and nitric oxide was indicated in the current study.

Keyword: Autism spectrum disorder, oxidative stress, biochemical parameters.

Introduction

Autism spectrum disorder (ASD), is a severe neuro developmental disorder with onset before the age of 3 years[1]. The term autism spectrum disorder or pervasive developmental disorders (PDD) represents group of disorders which includes five diagnostic subtypes[2]. The susceptible gender ratio is 4-5 boys to 1 girl[3].

Investigators suggested that ASD might result from interaction between genetic, environmental and immunological factors with oxidative stress as a mechanism linkage of these risk factors[4]. Behavioral abnormalities limitations, sensory processing abnormalities and impaired ability to communicate are the main issues in this manifaceted disorder[5]. Children are more vulnerable than adults to oxidative stress, particularly, because of their naturally low glutathione (GSH) levels from conception through infancy and might be to high vulnerability to oxidative stress during the early of neurodevelopment. This might result in neuro developmental disorders such as autism[6]. Mercury induces glutathione depletion, increases oxidative stress and apoptosis[7]. It induced oxidative modifications of DNA, protein and lipids, as well as, inhibition of the crucial enzymes in the brain development[8]. So, mercury compounds play an important role in the pathophysiology of autism[9]. In addition, GSH is depleted by increase level of nitric oxide (NO)[10], which is a toxic free radical that can block energy production and was found to be increased in ASD[11]. Excess NO in brain would be a serious matter, so, it increases apoptosis and causing a leaky blood brain barrier, increases inflammation and intestinal permeability[12]. Oxidative stress in case of ASD is associated with increase plasma levels of thiobarbituric acid reactive substances (TBARS) and lipid peroxidation biomarkers[13]. An advanced oxidation protein products (AOPP), is a novel marker of inflammation and protein oxidation[14]. Most of AOPP found to increase levels of myeloperoxidase which is released from activated phagocytes[15].

Neuroglobin (NGB), first globin of nerve cells, expressed in vertebrate brain and retina[16]. NGB, as a respiratory protein, binds reversibly O_2 with high affinity, scavenge NO and could alleviate oxidative stress by elimination of ROS[17]. NGB can preserve mitochondrial functions and resist apoptosis[18]. On the other hand, sleep disorders were reported in patients with ASD suggesting that the neuroendocrine functions involved in the circadian sleep-wake cycle might be altered in these disorders[19].

During the last years, studies showed an abnormal decrease in melatonin secretion in children subjected to ASD[20]. As well as, melatonin in autistic individuals have a characteristic circadian rhythm. As a result, melatonin is used therapeutically to treat sleep disturbances, immune dysfunction and abnormalities of the central nervous system[21].

Material and Methods

Subject: Children with autism spectrum disorder (37 cases) were enrolled in this study (31 males and 6 females) aging 2-12 years from psychiatric research unit in College of Medicine,

University of Mosul. In addition, 30 children with the same age (20 males and 10 females) were involved as control group.

The diagnosis of ASD is applied by a specialist psychiatric doctor, and the criteria of autism spectrum disorder as defined in the diagnostic and statistical manual of disorder, fourth edition (DSM-IV) were undertaken.

Patients group were classified into three subgroup, mild, moderate and severe according to symptoms severity grade.

Blood samples: Venous blood samples were collected from both groups in antiseptic plain tubes. Serum was separated by centrifugation at 3000 rpm after blood coagulation for 15 min. Serum samples were divided in small aliquot tubes and stored at -20°C for subsequent analysis.

Biochemical analysis: Glutathione (GSH) a simple and accurate method for the determination of serum GSH was used as described by Sedlack J. and Lindsay R.H., Tietz N.V.[22,23]. Ellman reagent (DTNB) was considered as a reagent for thiol group.

Thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically[24]**.**

Advanced oxidation protein products (AOPP) were determined in serum using spectrophotometric method[25]. The results were calibrated with chloramine-T as standard AOPP concentration expressed as micromoles per liter chloramines –T equivalents.

Nitric oxide (NO) a colorimetric assay was used which provided an accurate, convenient measurement of total nitrite[26].

Anti-oxidant activity (AOA) was measured spectrophotometrically[27].

N**euroglobin (NGB)** was assessed based on a sandwich Enzyme-Linked Immuno Sorbent Assay (ELISA) technique using CUSABIO kit (China)[28]. The analysis was performed in the immunity laboratory in Al-Salam hospital in Mosul city using BIO-TEK instruments, INC.USA.

Melatonin (MEL) Early morning blood were collected from children and used for estimation of serum melatonin. Melatonin concentration was estimated by sandwich Enzyme-Linked Immuno Sorbent Assay (ELISA) technique using CUSABIO kit (China)[29]. The analysis was performed in the immunity laboratory in Al-Salam hospital in Mosul city using BIO-TEK instruments, INC.USA.

Statistical analysis: The data obtained in the current study was analyzed using Statistical Package for Social Sciences (SPSS) version 11.5. i. Standard statistical methods were used to determine the mean and standard error. ii. One way Anova (Duncan test) is used to compare between more than two parameters. iii. Pearson correlation was performed to identify the relationship between different biochemical parameters.

Results and Discussion

Statistical analysis of all used biochemical parameters on mild, moderate and severe autism spectrum disorder compared to control group are listed in table 1 as mean ± S.E for each parameter.

Table 1 summarizes a significant ($P \leq 0.05$) decrease in the serum melatonin, neuroglobin, antioxidant activity and glutathione in ASD patients group compared to control group while, there was a significant ($P \leq 0.05$) increase levels of thiobarbituric acid reactive substances, advanced oxidation protein products and nitric oxide in moderate and severe ASD patients as compared to control and mild groups. Also table 1 demonstrates the change percentage of each parameter.

The data in table 2 indicate the correlation between antioxidant activity with the other biochemical parameters in patients and control groups. Pearson correlation analysis was performed to study the association. A positive significant correlation between AOA with glutathione, melatonin and neuroglobin of ASD patients (to the left) compared to control group (to the right) as illustrated in figure 1 and negative significant correlation with thiobarbituric acid reactive substances, advanced oxidation protein products and nitric oxide of ASD patients group (to the left) compared to control group (to the right) as indicated in figure 2.

The present study is an attempt to examine the oxidative stress and status of the protective antioxidant under condition of stress due to ASD. Oxidative stress is the deregulation of oxidant – antioxidant system which causes tissue damage through the production of reactive oxygen species, lipid peroxidation and protein oxidation[30].

Reactive oxygen species have been suggested as important mediators in traumatic brain injury, strokes, neurodegenerative diseases[31] and psychiatric disorder [32] and in some individual with ASD[33].

Purkinje cells are especially sensitive to ischemia and hypoxia[9]. The decrease in Purkinje cells number is likely to affect the neuronal communication and could contribute to autistic behavior, learning, cognitive and emotional processes[34]. Several studies showed that oxidative stress, lipid peroxidation, DNA and protein oxidation are increased in autism spectrum disorder[35]. Multiple oxidative stress biomarkers have been identified in ASD children[36]. Glutathione is required to maintain the normal reduced state of cells and to counteract all the deleterious effects of oxidative stress. GSH is said to be involved in many cellular processes including the detoxification of endogenous and exogenous compounds[37].

There is evidence that GSH, itself is a neurotransmitter or neuromodulator and any change in either GSH levels turnover rates or oxidation state would adversely affect central nervous system activity[38]. The results in table 1 showed a significant ($p \leq 0.05$) decrease in serum GSH levels in ASD patients groups (-36.0%, -52.4%, -63.3%) which indicated oxidative stress. Glutathione contains thiol group and considered the strongest endogenous cellular antioxidant[39]. So, autistic children would be expected to have difficulty resisting infection, resolving inflammation and detoxifying environmental toxins. Indeed patients diagnosed with ASD were reported to suffer from a recurrent infection, neuroinflammation, gastrointestinal inflammation and impaired antioxidant and detoxification capacity[4]. Low levels of GSH might be attributed to sulphuration pathway impairment[40]. Sulphuration pathway is linked to methylation and folic acid pathway and any perturbation of these pathways will affect the production of GSH[40]. A study reported a significant correlation between biomarkers of oxidative stress and severity of ASD[41]. The present study gave directevidence that reduced levels of antioxidant like melatonin and glutathione might be represent risk factors that can promote ASD incidence. The current results are in a good agreement with those reported by other investigators[1,35]. Lipid peroxidation reflects oxidative deterioration of poly unsaturated fatty acids, important constituents of biological membranes. Higher levels of thiobarbituric acid reactive substances, a marker of oxidative stress, have been reported in the ASD patients[1]. Increased lipid peroxidation is well reported in neurodegenerative diseases[42]. Similarly, a significant ($p \leq 0.05$) increase in the levels of serum TBARS in ASD patients, especially moderate and severe groups compared to control (+26.1%, +82.6%) respectively were observed in table 1. It was suggested that ROS attacks double bonds producing free radicals. This will increase lysosomal enzymes causing tissue and membrane damage with lipid peroxidation leading to loss of membrane function and integrity[43].

Table-1
Concentration of some biochemical parameters as mean ± S.E control group, mild, moderate and severe autism spectrum disorder patients

Biochemical parameters	Control Mean ±S.E	Autism spectrum disorder patients					
		Mild Mean ±S.E	% change	Moderate Mean ±S.E	% change	severe Mean ±S.E	% change
Melatonin pg/ml	d 36.34±2.50	c 27.06±1.18	-25.5	b 15.60±1.02	-57.1	a 6.60±0.51	-81.8
Neuroglobin ng/ml	c 51.97±4.88	b 29.68±3.39	-42.9	a 12.86±0.49	-75.3	a 7.86±0.44	-84.9
Antioxidant activity µmol/L	c 38.52±2.1	c 38.83±1.66	+0.8	b 24.18±0.76	-37.3	a 11.63±1.24	-69.8
Glutathione µmol/L	c 4.58±0.2	b 2.93±0.11	-36.0	a 2.18±0.04	-52.4	a 1.68±0.06	-63.3
Thiobarbituric acid reactive substances µmol/L	a 0.69±0.04	a 0.66±0.02	-4.3	b 0.87±0.02	+26.1	c 1.26±0.05	+82.6
Advanced oxidation protein products µmol/L	a 38.82±1.32	a 39.39±2.0	+1.5	b 58.58±1.42	+50.9	c 85.62±2.09	+120.5
Nitric oxide µmol/L	a 10.33±0.23	a 9.93±0.2	-3.9	a 11.83±0.19	+14.5	b 19.16±3.14	+85.5

S.E = Standard Error .. Different letters horizontally **a, b, c, d** indicate that the mean is different significantly at $p \leq 0.05$,

Table-2
Correlation between antioxidant activity and other measured biochemical parameters of control and ASD patients groups

		AOA	GSH	MEL	NGB	AOPP	TBARS	NO
Control	r	1.0	0.308	0.069	0.005	-0.447*	0.357	0.439
	P	Nil	0.098	0.710	0.977	0.013	0.053	0.069
ASD patients	R	1.0	0.823**	0.890**	0.705**	-0.791**	-0.626**	-0.587**
	p	nil	0.001	0.001	0.001	0.001	0.001	0.004

*Correlation is significant at $P \leq 0.05$, **Correlation is significant at $p \leq 0.01$

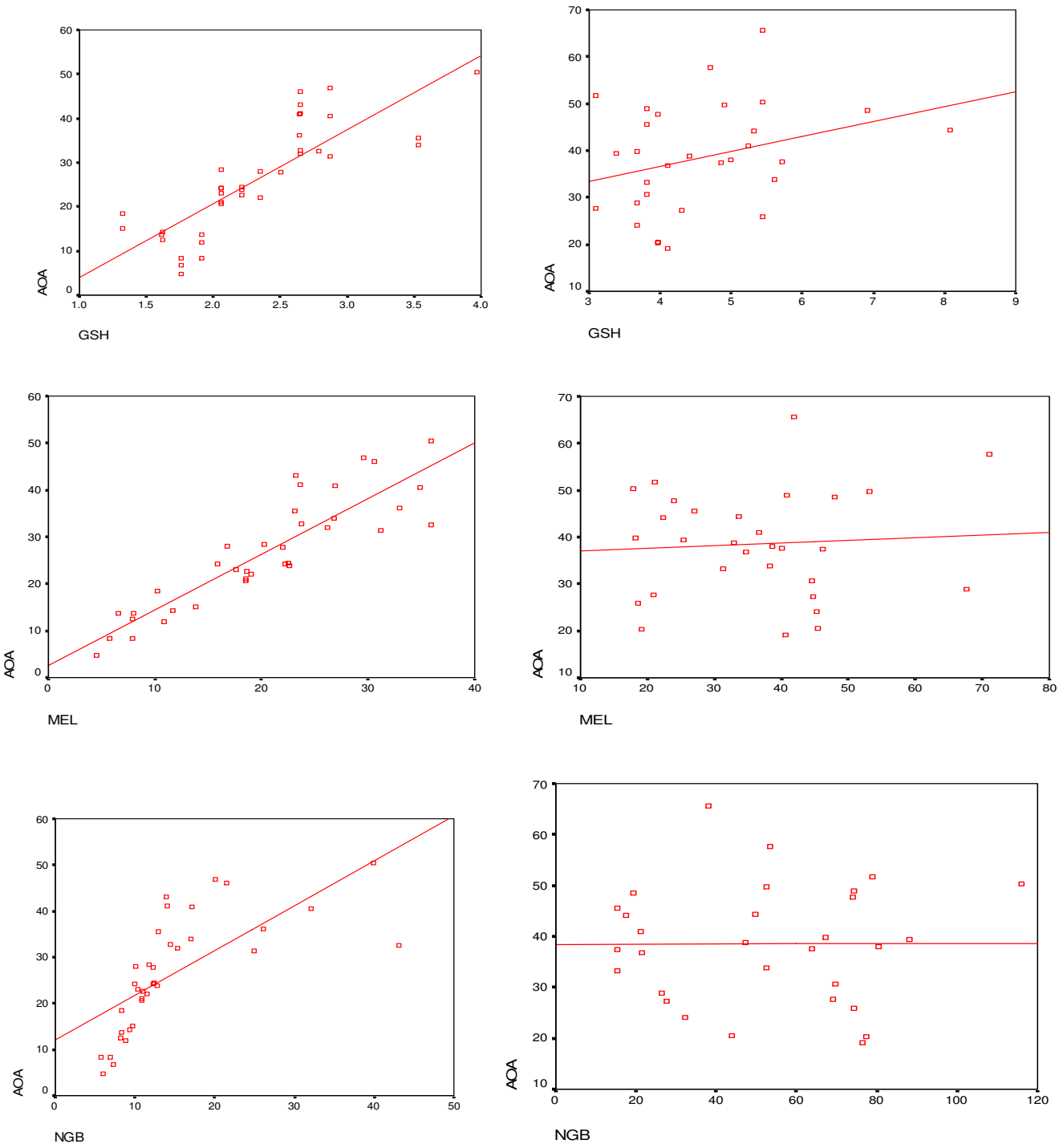

Figure-1
Positive correlation between antioxidant activity and GSH, MEL and NGB in ASD patients to the left compared to control group to the right

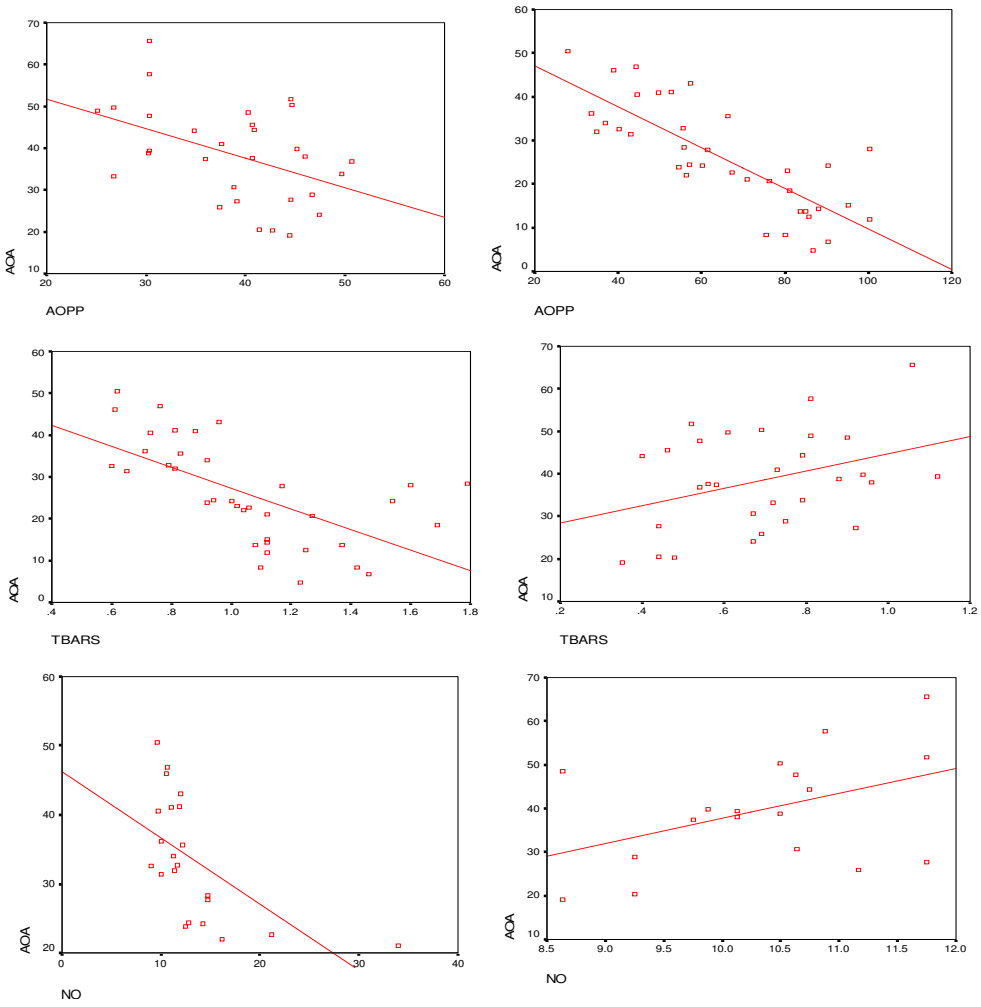

Figure-2
Negative correlation between antioxidant activity and AOPP, TBARS and NO in ASD patients to the left compared to control group to the right

Oxidative modification of proteins has been also implicated in ASD patients through advanced oxidation protein products determination. AOPP has been shown as an inflammatory marker in many diseases[15]. However, there are no reports for AOPP roles in ASD. AOPP might contribute to disorder by activating monocytes and providing pro-inflammatory cytokine production[30]. Acute inflammation that occurs during severe autism spectrum disorder might accelerate AOPP formation. In the present study, AOPP levels increased significantly (P≤0.05) in severe group (+120%) compared to mild, moderate and control group. AOPP high levels in those patients might suggest an active role of macrophage activation and inflammation in the oxidative stress through myeloperoxidase-derived hypochlorous acid (HOCl). It might cause tissue damage, lipid and protein oxidation[44]. Oxidative stress occurrence is further documented by increase concentration of nitric oxide (NO) in ASD, measured as nitrite[45,46]. Nitric oxide is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity[47]. NO has been recognized as biological neural messenger molecule, although it is known to affect development and function of the central nervous system[6,48]. Furthermore, NO block energy production and was found to be increased in autism as compared to age and sex-matched controls[39]. NO has been shown to damage the blood brain barrier. Excess NO in ASD is localized to specific organs or tissues. The brain and gut are plausible sources as both are often abnormal in ASD to gross, behavioral and gastrointestinal symptoms predominate. The current results indicated a significant (P≤0.05) increase level in severe group (+85.5%) which agreed with a previous study[45]. This indicates a possible role of NO in the pathogenesis of autism[49]. Too much NO deplete antioxidant defense, depressing levels of GSH, low GSH in turn increase NO[45]. So, in the present study,

thiobarbituric acid reactive substances, advanced oxidative protein products and NO found to be higher in ASD patients and give negative significant correlation with antioxidant activity.

Some studies reported ASD children have a higher prevalence of sleep abnormalities than typically developing children with a percent ranging from 40% to 86%[50]. Our results revealed that the mean melatonin levels among ASD groups were significantly ($p \leq 0.05$) decreased as compared to control group in early morning by (-25.5%, -57.5%, -81.8%) as indicated in Table 1. These data might be due to the melatonin production in ASD patients seems to be lower than in controls. These results concied with the previous studies which indicated a significant correlation between melatonin levels and ASD symptoms[50]. So, causes might be attributed to abnormalities in ASMT gene that codes for acetyl serotonin methyl transferase, an enzyme involved in melatonin synthesis in some people with ASD[19]or reduced levels of Tryptophan[51]. Lower ASMT is associated with hyper activity, sleep disorder in ASD patients and ASD severity[52]. These studies suggested that melatonin metabolism is directly or indirectly related to certain autistic behavior [50].

A study indicated that neuroglobin, novel globin type, is an endogenous neuroprotection molecule against hypoxia (inadequate oxygen supply) and ischemic insult in the brain (inadequate blood supply) [53]. It has been demonstrated that NGB could alleviate oxidative stress, eliminate reactive oxygen species[54], preserve mitochondrial function and resist apoptosis[18]. Although, currently no literature showing a connection between ASD and NGB, the present study revealed a severity dependent significant ($p \leq 0.05$) in NGB levels in ASD patients (-42.9%,-75.3%,-84.9%) in comparison with control group as indicated in Table 1. The reduction of NGB levels might cause increase oxidative stress and free radical production which accompanied with antioxidant depletion. These finding might affect brain tissue and stimulate lipid, protein oxidation besides neuroinflammation. In addition, the results might reveal the important role of NGB in purkinje cells survival and ASD pathophysiology. It was reported that, administration of antioxidant might protect purkinje cells against oxidative stress[5]. Many studies suggest that NGB acts as NO scavenger because NGB can directly bind to NO with high intrinsic affinity and a low dissociation rate[54]. Our results indicate low levels of NGB and high levels of NO in ASD patients in accord with the previous studies that explained the effect of oxidative stress on some biochemical parameters in autistic children.

Finally, the term antioxidant denotes a compound which can delay or inhibit the oxidation of biomolecules by inhibiting the initiation or propagation of oxidative chain reactions and thus prevents damage done to the body's cell by oxygen, i.e. reactive oxygen species (ROS)[55]. Overproduction of such free radicals can cause oxidative damage to biomolecules, eventually leading to many chronic diseases[56]. So, heavy metals are known to generate toxic ROS such as H_2O_2, O_2^-, OH^-,…etc. which degrade important cellular components by inducing oxidative stress[57]. The current results demonstrated that antioxidant activity level is significantly ($p \leq 0.05$) decreased in ASD group especially moderate (-37.3%) and severe (-69.8%) compared to controls as illustrated in table 1. These finding suggested an increase oxidative stress occurrence and the oxidative compounds turns into toxic substances. When ROS effects cannot be prevented with endogenous and exogenous antioxidants, causing damage to the basic structural cellular elements including lipids, protein and nucleic acids, the process results in cell death via necrosis or apoptosis[58].

Conclusion

The results of the present study might conclude that oxidative stress with environmentaltoxicants exposure promotes autism spectrum disorder. Oxidative stress affects some biochemical parameters concentration and alterations might induce pathophysiology of ASD. Our team would like to advise the administration of specific antioxidants to prevent or to alleviate ROS effect. However, further in depth studies is required for the understanding ASD pathophysiology.

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