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Changes in nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiological mechanisms involved in autism

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Abstract

Background: There is evidence that oxygen free radicals play an important role in the pathophysiology of many neuropsychiatric disorders. Although it has not been investigated yet, several recent studies proposed that nitric oxide (NO) and other parameters related to oxidative stress may have a pathophysiological role in autism. *Methods*: We assessed the changes in superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities and thiobarbituric acid-reactive substances (TBARS) levels in plasma as well as NO levels in red blood cells (RBC) in patients with autism ($n=27$) compared to age- and sex-matched normal controls ($n=30$). *Results*: In the autistic group, increased RBC NO levels ($p<0.0001$) and plasma GSH-Px activity ($p<0.0001$) and unchanged plasma TBARS levels and SOD activity were detected. *Conclusions*: These findings

Abbreviations: AA, arachidonic acid; CARS, Childhood Autism Rating Scale; Cd, cadmium; CNS, central nervous system; eNOS, endothelial nitric oxide syntase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; iNOS, inducible nitric oxide synthase; MDA, Malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; NOS, nitric oxide synthase; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide anion radical; $^{\cdot}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; –SH, thiol; –S–NO, nitroso-thiols; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; XO, xanthine oxidase.

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indicate a possible role of increased oxidative stress and altered enzymatic antioxidants, both of which may be relevant to the pathophysiology of autism.
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Keywords: Autism; Thiobarbituric acid-reactive substances (TBARS); Superoxide dismutase (SOD); Glutathione peroxidase (GSH-Px); Nitric oxide (NO)

1. Introduction

The underlying etiology of autism is unknown. Although it had initially been thought to be a consequence of defective parenting, today autism is generally accepted as a biological disorder. It has been suggested that a wide range of neurotransmitters, neuromodulators and neurohormones should be investigated. The hypothesis that reactive oxygen species (ROS) play an important role in autism as well as other psychiatric disorders remains speculative and there have been studies to test this hypothesis [1]. ROS including superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and nitric oxide ($NO^{\cdot}$) may lead to cellular injury when they are generated extremely or the antioxidant defence systems are destructed. A number of oxygenated compounds, particularly aldehydes including 4-hydroxynonenal and malondialdehyde (MDA), are produced during the attack of free radicals to membrane lipoproteins and polyunsaturated fatty acids (PUFAs). Therefore, assessment of thiobarbituric acid-reactive substances (TBARS) or 4-hydroxynonenal is probably the most commonly applied method for the measurement of lipid peroxidation [2].

Nitric oxide (NO) has chemical properties that make it uniquely suitable as an intracellular and intercellular messenger. It is produced by the activity of nitric oxide synthases (NOS), which are present in peripheral tissues and in neurons. NO is known to be an oxygen radical and a neurotransmitter in the central and peripheral nervous systems. Although NO is described as an atypical neurotransmitter in the nervous system, it seems more appropriate to consider it as a second messenger. On the other hand, NO is known to affect neurodevelopmental processes in CNS [3]. It has been implicated in a number of physiological functions such as noradrenaline and dopamine release, memory and learning, and certain pathologies such as schizophrenia, bipolar disorder, and major depression [4,5].

Superoxide dismutase (SOD) is a potent protective enzyme that can selectively scavenge $O_2^{\cdot -}$ by catalyzing its dismutation to H_2O_2 and oxygen (O_2). The other antioxidative enzyme, glutathione peroxidase (GSH-Px), catalyzes the conversion of H_2O_2 to water by using reduced glutathione (GSH) and reduced NADPH as cofactors. Although it has not yet been investigated, several recent studies proposed that altered activities of antioxidant system might have a pathophysiological role in autism [1,6]. But, there has been only one study in which Golse et al. [7] found that SOD I and GSH-Px activities seem to be abnormal in the RBCs whereas only SOD I activity appears to be abnormal in the platelets. There has been no study about RBC NO and plasma TBARS and lipid peroxidation in the same autistic patient groups. Assessment of the activities of these free radical scavenging enzymes in plasma may help to understand better the changes in antioxidative status in autism. The hypothesis is that the imbalance between oxidant and antioxidant systems might be involved in the pathophysiology of autism like other psychiatric diseases such as schizophrenia, bipolar disorders, etc. Therefore, the aim of the present study was to determine the activities of antioxidant enzymes and the lipid peroxidation end product levels in plasma as well as RBC NO levels of the autistic patients and healthy controls.

2. Materials and methods

2.1. Patients

Twenty-seven patients and 30 healthy controls were included in this study. The autistic children were referred as outpatients to the Department of Child and Adolescent Psychiatry in Gaziantep University Medical School during 1999–2002. The children in the control group were selected from among

the students of a kindergarten and a primary school in Gaziantep. All of the patients and controls were screened for psychiatric disorders by obtaining historical information, performing clinical interviews, and using symptom ratings according to DSM-IV. They were also examined by a pediatrician for medical problems including the measurement of blood pressure and for their dietary patterns. Among all the patients and controls, no abnormal blood pressure level was found and there was no considerable difference between the patients and the controls with regard to the diet. The children were referred by community-based psychiatrists or pediatricians or by family members. Samples from 27 children received the diagnosis of autism, according to DSM-IV diagnostic criteria and Childhood Autism Rating Scale (CARS) score, >30. All diagnoses were made by a child psychiatrist and psychologist independently. No subject had any diagnosed genetic, metabolic, or neurological aetiology for autistic disorder. Patients who had a history of chronic systemic disease, acute and chronic inflammatory disease, or severe head injury were excluded from the study. The patients with autistic disorder were 16 boys and 11 girls ranging in age from 2 to 12 years (4.7 ± 2.7, mean $\pm$ SD). All subjects participated in the study after written informed parental consent had been obtained. All autistic subjects had evidence of mental retardation and all of the children in the patient and control groups were free of any medication. The control group consisted of 16 boys and 14 girls ranging in age from 2 to 13 years (5.1 ± 2.9). The study was approved by the ethical committee of Gaziantep University Medical School.

2.2. Sample collection and preparation

Fasting blood samples of the study and control subjects were taken from cubital vein into heparinized tubes in the morning during routine blood sampling, to prepare RBC sediment and plasma. Hematological parameters were examined by routine laboratory techniques (Coulter STKS, UK). After immediate centrifugation ($1000 \times g$ for 10 min at 4 °C), plasma was removed and stored frozen at -30 °C. After buffy coat was separated carefully, RBC sediment was washed three times with 10-fold isotonic NaCl. At the end of washing, RBC sediment was treated with fourfold ice-cold deionized water to obtain the hemolyzate. The plasmas were used to estimate the TBARS, SOD and GSH-Px levels; RBC sediment was used to measure the NO level.

The plasma TBARS level was determined by reaction with thiobarbituric acid (TBA) at 90–100 °C [8]. MDA or MDA-like substances and TBA react together for production of a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm (Ultraspec Plus, Pharmacia LKB Biochrom, England). A standard curve was prepared using serial dilutions of 1,1,3,3-tetramethoxypropane.

Total SOD (EC 1.15.1.1) activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system (XO) [9]. Activity was assessed in the ethanol phase of the plasma after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of the plasma and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate.

GSH-Px (EC 1.6.4.2) activity was measured as described previously [10]. The enzymatic reaction was initiated by the addition of H_2O_2 to the reaction mixture containing GSH, NADPH, and glutathione reductase. The change in the absorbance at 340 nm was monitored.

The stable oxidation end products of NO, nitrite (NO_2^-) and nitrate (NO_3^-) were used in vitro and in vivo as indicators of NO production [11]. Thus, RBC NO_2^- and NO_3^- levels were estimated as an index of NO production. Lyzate samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured after the reduction of nitrate to nitrite by copperized cadmium granules (Cd) in glycine buffer at pH 9.7 (2.5 to 3.0 g of Cd granules for a 4-ml reaction mixture). Quantitation of NO_2^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthylethylenediamine and sulphanilamide [12]. A standard curve

was established with a set of serial dilutions (10^{-8} to 10^{-3} mol/l) of sodium nitrite.

All the analyses were performed by the same examiners who did not know the groups. All samples were assayed in duplicate.

2.3. Statistical analysis

Data were analyzed by using SPSS® for Windows (ver. 9.0) computing program. Distribution of the groups was analysed with one sample Kolmogrov–Smirnov test. As both groups showed normal distribution, parametric statistical methods were used. One-way ANOVA test was performed intergroup for pairwise comparisons. Bivariate comparisons were examined using Pearson rank correlation coefficients (r). Results are presented as mean ± standard deviation. A $p<0.05$ was considered significant.

3. Results

As to the demographic data (age, sex, etc.), patients and their controls showed homogeneity and there was no significant difference between the groups. Results are summarized in Figs. 1 and 2. There was a statistically significant increase in plasma GSH-Px activity (40.9 ± 11.3 vs. 24.2 ± 6.3 U/l, $p<0.0001$) and RBC NO levels (1.62 ± 0.49 vs. 0.91 ± 0.22 μmol/g Hb, $p<0.0001$) in autistic patients compared to the control group. There were increased TBARS levels in autistic patients vs. controls but were not statistically signifi-

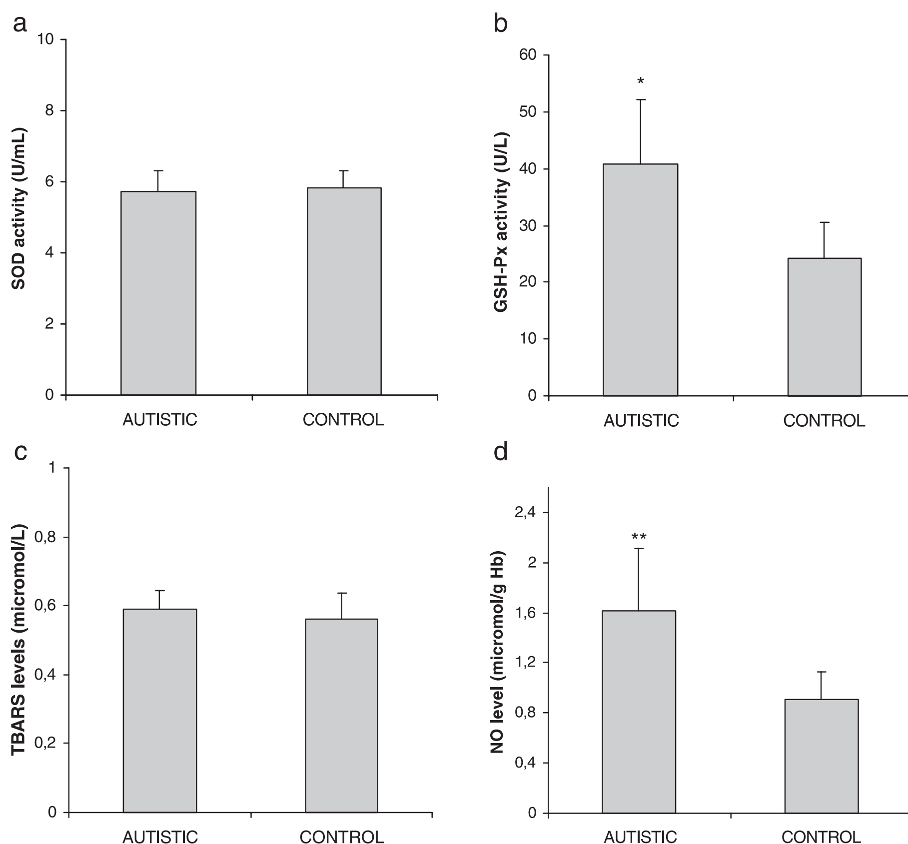

Fig. 1. Plasma superoxide dismutase (SOD) activity (a), glutathione peroxidase (GSH-Px) activity (b), thiobarbituric acid-reactant substances (TBARS) levels (c), and RBC nitric oxide (NO) levels (d) in autistic children and control groups. Bars and error bars represent mean and standard deviation values, respectively. *$p<0.0001$ and **$p<0.0001$ compared to control. One-way ANOVA test was performed intergroup for pairwise comparisons.

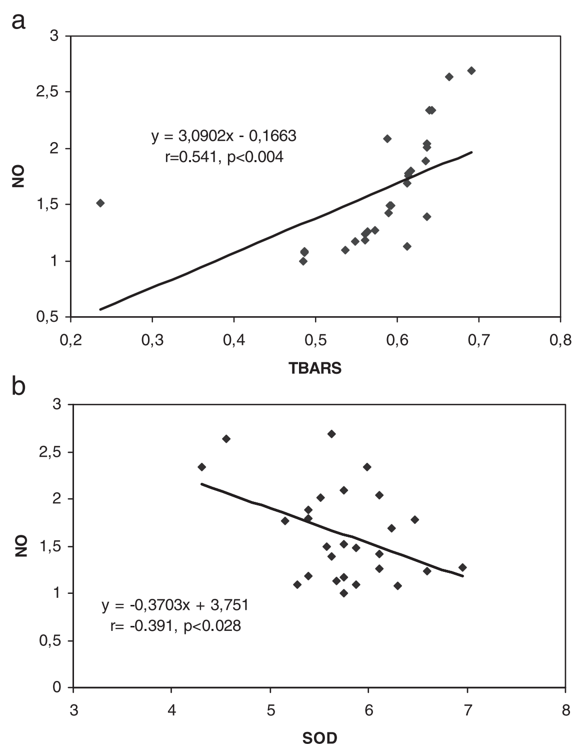

Fig. 2. Correlation analysis (r values) between RBC nitric oxide (NO) and plasma thiobarbituric acid-reactant substances (TBARS) (a) and between RBC nitric oxide (NO) and plasma superoxide dismutase (SOD) activity (b) in autistic children. Two-tailed Pearson correlation analysis method was used to determine the correlation coefficient.

cant (0.59 ± 0.05 vs. 0.56 ± 0.07 μmol/l). We detected no change in plasma SOD activity in autistics compared to controls (5.74 ± 0.56 vs. 5.84 ± 0.46). In the correlation analyses, there was a positive correlation between TBARS and NO ($r = 0.541$, $p < 0.004$; Fig. 2a), and a negative correlation between SOD and NO ($r = -0.391$, $p < 0.028$, Fig. 2b). There were no positive or negative correlations between the parameters studied in the control group.

4. Discussion

Nitric oxide has been recognized as a biological neural messenger molecule although it is best known as a toxic reactive free radical in the CNS. NO or NO-derived nitrogen oxides must interact with neuromodulators in order to modify these modulators, especially monoamines, and thereby change their regulatory action on synaptic transmission [13]. NO is synthesized on demand by the enzyme NO synthase (NOS) from L-arginine. Thiol (–SH) containing enzymes and proteins is a critical target for NO, so it forms relatively stable nitroso-thiols (–S–NO). Reduction of NO production may consequently result in a stimulation of oxidative phosphorylation and increase peripheral oxygen uptake [14]. A critical reaction that NO undergoes in oxygenated biological media is a direct bimolecular reaction with $O_2^{\cdot -}$ yielding peroxynitrite ($ONOO^-$). Peroxynitrite and its further products have been linked to several interactions which may contribute to cellular injury, including lipid peroxidation, nitrosylation of some molecules, and inactivation of sodium channels. Liposomes exposed to XO-derived reactive species in the presence of NO exhibited both stimulation and inhibition of lipid peroxidation, depending on the ratio of the rates of ROS production and NO introduction into reaction system. Therefore, $ONOO^-$ has been shown to oxidize a variety of biological molecules and may be responsible for certain types of NO-mediated toxicity [15]. Taken together, NO or closely related molecules have been reported to be neurodestructive. The positive correlation between NO and TBARS in our findings may show the direct or indirect enhancer effect of NO on lipid peroxidation (Fig. 2a), but TBARS levels were not different in autistics vs. controls. NO, like glutamate, can induce neurological toxicity under conditions of excessive formation [16]. This type of toxicity depends on calcium and since NOS is a Ca-dependent enzyme, the activation of NOS could be involved in NMDA neurotoxicity. It has been suggested that treatment of cortical cultures with NOS inhibitors or removal of L-arginine from medium blocks NMDA neurotoxicity [17].

There may be a question about whether peripheral total nitrite level indicates NO activity in autistic brains or not. Serum total nitrite was increased in a group of demyelinating diseases including multiple sclerosis, inflammatory neurological diseases and AIDS [18]. These studies suggest that peripheral NO metabolites can be used as a marker of CNS-dependent NO changes. The changes in NO levels may be meaningful in autism owing to the aforementioned functions of NO in the nervous system. There was a remarkable increase in total nitrite levels

in patients with autism vs. controls, which indicates a possible role of NO· in the pathogenesis. Increased oxidant end-products by the reactions of NO with other free radicals may probably contribute to the neuropathophysiology, and thereby psychopathology, of autism because of the preferential vulnerability of the brain to oxidative injury. The positive correlation between RBC NO and plasma TBARS (Fig. 2) may support the possible role of NO· and the subsequent molecules such as $ONOO^-$ in the pathophysiology of autism.

The antioxidant enzymes SOD, CAT and GSH-Px have complementary activities in the antioxidative defense system. Hydrogen peroxide, the product of the reaction catalysed by SOD, is also the substrate for CAT and GSH-Px. We found increased GSH-Px activity but unchanged SOD activity in plasma of autistic patients compared to controls. Increased antioxidant enzyme activity may reflect a preceding cellular oxidative stress or serve as a compensatory mechanism. Otherwise, the fact that SOD activity did not change in patient with autism despite oxidative stress may possibly lead to an increase in the activities of the other antioxidant enzyme, GSH-Px. The negative correlation between SOD and NO (Fig. 2) may suggest that SOD is involved in the antioxidant defense system against ROS. GSH-Px may also use lipid hydroperoxides as substrates. So, GSH-Px activity may be increased because of increased lipid peroxidation, but we could not show increased TBARS level possibly because of the nonspecificity of the TBARS assay. Overproduction of H_2O_2 in plasma is another possibility for increased GSH-Px activity. Since H_2O_2 is a neutral and highly liposoluble molecule that can easily pass through tissue membranes, an excess amount of it found in CNS can pass through the membranes into the plasma and thus cause an increase in GSH-Px activity. We have previously found that the brain has moderate GSH-Px (2.0 U/mg protein) and total SOD activity (16.0 U/mg protein) [19]. All of the enzymes functioning in humans are synthesized intracellularly, and most of them carry out their functions within the cells. It is possible to infer the nature of pathological changes in the tissues of body by measuring the activities of certain enzymes in disease conditions. There are several factors that affect plasma enzyme activities and amounts, one of them is the leakage of enzymes from cells and altered enzyme production. Therefore, all the changes of enzyme activities in the cellular level contribute to the levels and/or activities of the same enzymes in the extracellular compartment including plasma [20]. From this point of view, our results may consequently show increased GSH-Px and unchanged SOD activity in CNS in autistic patients.

The fact that plasma TBARS levels in patient with autism were unchanged compared to the controls suggests that unchanged lipid peroxidation and thus oxidative stress were coupled with increased activities of antioxidant enzyme GSH-Px. The other antioxidant enzymes in plasma such as catalase that we did not measure may also change to balance oxidative stress. Vancassel et al. [21] examined the phospholipid fatty acids in the plasma of a population of autistic subjects vs. mentally retarded controls. Their results showed a marked reduction in the levels of 22:6n-3 in the autistic subjects, resulting in significantly lower levels of total (n-3) PUFAs, without significant reduction in the (n-6) PUFAs series, and consequently a significant increase in the (n-6)/(n-3) ratio. Gangliosides, sialic acid-containing glycolipids found in all cells especially abundant in nerve cells and mainly situated on outer membrane surfaces, were measured in CSF from children and adolescents with autism spectrum disorder [22]. Although there is increasing evidence that abnormalities of fatty acid and membrane phospholipid metabolism play a role in a wide range of psychiatric disorder including autism [23], the abovementioned studies concerned with membrane structure mostly showed no membrane defects in autism. Bell et al. [24] reported that the fatty acid compositions of RBC phospholipids from a patient with autistic spectrum disorder (ASD) had reduced percentages of highly unsaturated fatty acids vs. controls. However, arachidonic acid (AA) and docosohexoenoic acid and other longer chain fatty acids have been reported to increase in the RBC membranes in patients with autism [25]. This could account for uncontrolling abnormal electrical discharges from neurones and the abnormal brain size in autistic patients. Our findings with unchanged lipid peroxidation end products and consequently no membrane breakdown are consistent with other findings, at least for plasma used as a biological material to test this hypothesis.

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