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Oxidative stress in autism: Increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin - the antioxidant proteins

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Abstract

Autism is a neurological disorder of childhood with poorly understood etiology and pathology. We compared lipid peroxidation status in the plasma of children with autism, and their developmentally normal non-autistic siblings by quantifying the levels of malonyldialdehyde, an end product of fatty acid oxidation. Lipid peroxidation was found to be elevated in autism indicating that oxidative stress is increased in this disease. Levels of major antioxidant proteins namely, transferrin (iron–binding protein) and ceruloplasmin (copper–binding protein) in the serum, were significantly reduced in autistic children as compared to their developmentally normal non-autistic siblings. A striking correlation was observed between reduced levels of these proteins and loss of previously acquired language skills in children with autism. These results indicate altered regulation of transferrin and ceruloplasmin in autistic children who lose acquired language skills. It is suggested that such changes may lead to abnormal iron and copper metabolism in autism, and that increased oxidative stress may have pathological role in autism.

Keywords: Autism; Ceruloplasmin; Lipid peroxidation; Oxidative stress; Pervasive developmental disorders; Transferrin

Introduction

Autism is a severe neurological disorder with onset before the age of 3 years. It is associated with severe impairment in language, cognition and socialization (Lord et al., 2000). Autism is classified under

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the pervasive developmental disorders (PDD), a group of disorders that involve a combination of impairments in communication, reciprocal social interactions and stereotyped patterns of interest / behavior. PDD includes autism, Asperger's disorder (an autistic condition not associated with language delay or general intellectual impairments), childhood disintegrative disorder, and a range of atypical and milder forms (PDD - not otherwise specified).

Extensive studies have demonstrated that oxidative stress plays a vital role in the pathology of several neurological diseases such as Alzheimer disease (Christen, 2000), Down syndrome (Kannan and Jain, 2000), Parkinson disease (Bostantjopoulou et al., 1997; Torsdottir et al., 1999) and Schizophrenia (Herken et al., 2001; Akyol et al., 2002). Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide, and the antioxidant capacity of the cell. Stress and injury to cells occur when redox homeostasis is altered, and ROS generation overpowers the biochemical defenses of the cell. Lipid peroxidation reflects a chain reaction between polyunsaturated fatty acids and ROS producing lipid peroxides and hydrocarbon polymers that are both highly toxic to the cell (Tappel, 1973; Jain, 1984; Horton and Fairhurst, 1987). Currently, the studies on oxidative stress in autism are limited (Yorbik et al., 2002; Sogut et al., 2003). Sogut et al. (2003) reported low activity of plasma antioxidant enzymes, namely glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in autism. Yorbik et al. (2002) on the contrary, observed similar activities of these enzymes in autism as compared to controls. We report here that peroxidation of lipids is increased in the plasma of autistic children as compared to their developmentally normal non-autistic siblings suggesting an increased oxidative stress in autism.

Ceruloplasmin and transferrin are major antioxidant proteins that are synthesized in several tissues including brain. Ceruloplasmin is an α2-serum glycoprotein that transports 95% of copper in blood. It has a major role in the metabolism of copper to which it binds reversibly. It also acts as ferroxidase and SOD, and it protects polyunsaturated fatty acids in red blood cell membranes from active oxygen radicals (Sass-Kortsak, 1965; Arnaud et al., 1988). Transferrin is present principally in serum, but is also found at lower concentrations in other body fluids. Its main function is the transport of iron to proliferating cells, and it is also an important growth factor (Loeffler et al., 1995). Ferrous ion contributes to oxidative stress by catalyzing the conversion of hydrogen peroxide to highly toxic hydroxyl radicals by Fenton reaction. Transferrin acts as an antioxidant by reducing the concentration of free ferrous ion. We compared serum ceruloplasmin and transferrin levels in the autistic children and their non-autistic developmentally normal siblings, and report that levels of both these antioxidant proteins are reduced in autism.

Materials and methods

Subjects

Members of 19 families (Group A), i.e., children with autism, and their developmentally normal (non-autistic) siblings were studied to compare ceruloplasmin and transferrin levels, and another 11 families (Group B) were studied for lipid peroxidation studies. Siblings were taken as controls because variations such as race, diet, socio-economic status and genetic background are similar between autistic children and their normal siblings. Therefore, the alterations noted were because of autism and were not

influenced by the factors described above. Autism was diagnosed based on the Autism Diagnostic Interview-Revised (ADI-R) criteria (Lord et al., 1994), and by direct observation of the child using the Autism Diagnostic Observation Schedule-Generic (ADOS-G) criteria (Lord et al., 1989). In Group A, 15 children were diagnosed with autism, 3 with PDD-not otherwise specified, and 1 had disintegrative disorder. Two of these children had a prior history of siezures, and one was receiving treatment with fluoxetine. Fourteen autistic children were of same sex as their sibling, while five autistic children were of opposite sex compared to their sibling. In Group B, 9 children were diagnosed with autism, and 2 with PDD-not otherwise specified. None of these children was treated with any medication. The nutritional status of the autistic children and their normal siblings was similar except that five autistic children of Group A and two of Group B were on gluten-free diet. The mean (S.E.) age of the affected children in Group A was 4.4 (0.27) and of normal siblings was 6.0 (0.9). In Group B, the mean age of autistic children was 6.0 (0.45) and that of siblings was 6.8 (0.87).

The blood samples from 11 families of Group B were collected in tubes containing sodium citrate as anticoagulant for the preparation of plasma in order to assess lipid peroxidation. The blood samples from 19 families of Group A were collected in the absence of anticoagulant for the preparation of serum. The sera were analyzed to measure ceruloplasmin and transferrin levels. Plasma and serum samples were obtained by separating supernatants upon centrifugation of blood samples at 2,500 g for 10 minutes.

Approximately 1/3 of children with autism undergo a regression during early childhood, having lost previously acquired language and social skills, and this subgroup may be at increased risk for seizures or epileptiform activity (Tuchman and Rapin, 1997). Of the 19 children assessed for ceruloplasmin and transferrin, 12 (63%) were reported by their parents to have lost previously acquired skills. These subgroups were then compared with each other.

Lipid peroxidation in plasma

Malonyldialdehyde (MDA) is an end product of peroxidation of polyunsaturated fatty acids and related esters, and is a marker of lipid peroxidation (Jain, 1984, 1989; Esterbauer et al., 1984). The measurement of MDA is the most widely used method for assaying lipid peroxidation. We compared status of lipid peroxidation in the plasma of autistic children and their non-autistic siblings by measuring MDA (Fig. 1) as described previously (Chauhan et al., 2002). MDA reacts with thiobarbituric acid (TBA), and forms a colored complex with a maximum absorbance at 532 nm (Jain, 1989). In brief, 1 ml of plasma sample from each subject was mixed with 2 ml of 0.37% (w/v) TBA–15% w/v trichloroacetic acid (TCA)–0.25 M HCl. The samples were heated in a boiling water bath for 15 minutes, and then kept at room temperature for cooling. The samples were centrifuged at 1,000 g for 10 minutes, and absorbance noted at 535 nm. MDA content was calculated using a molecular extinction coefficient for MDA of 2.56×10^5.

Ceruloplasmin and transferrin levels in serum

Ceruloplasmin and transferrin levels were analyzed in the serum samples of 19 children with autism and their 19 non-autistic siblings using the Minineph nephelometer and the assay kits (The Binding Site Inc. CA). The determination of soluble antigen concentration by nephelometric method involves a reaction with specific antiserum to form insoluble complexes. When light is passed through the

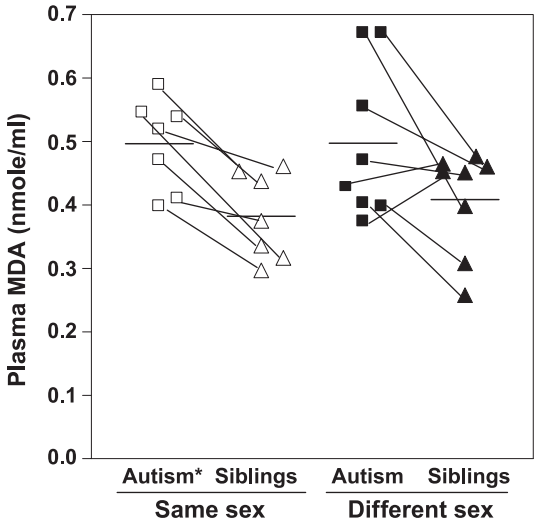

Fig. 1. The scattered plot of MDA levels in the plasma samples of children with autism and their non-autistic siblings of same or different sex. The bar represents the average MDA content in each group. *$p < 0.005$ in autism as compared to non-autistic sibling group of same sex; paired t-test; $p < 0.02$, unpaired t-test. Additionally, when entire autism group was compared with entire sibling group of same and different sex ($n = 15$), $p < 0.005$ (paired t-test and unpaired t-test).

suspension formed, a portion of the light is scattered and detected by a photodiode. For each assay, a scattered reading was taken at the beginning of the antibody/antigen reaction (blank) followed by a second scattered reading at a fixed time. The standards for ceruloplasmin and transferrin were also run. Ceruloplasmin levels were monitored using 40 μl of diluted serum, and 40 μl of human ceruloplasmin antiserum in total reaction volume of 480 μl. Transferrin levels were measured in 30 μl of diluted serum using 40 μl of human transferrin antiserum and 400 μl of reaction buffer.

Statistical analysis

Lipid peroxidation status, ceruloplasmin and transferrin levels in the autistic and non-autistic sibling groups were analyzed statistically by two-tailed unpaired t-test and paired t-test using the GraphPad StateMat Program. Comparison was made between (a) autism and sibling groups of same sex, (b) autism and sibling groups of different sex, and (c) entire autism group and entire sibling group (same sex plus different sex as compared to autism). Additionally, the two sub-groups of children with autism (with and without a history of regression) were compared with independent sample t-tests using separate estimates of variance for each group.

Results

Increased lipid peroxidation in the plasma of autistic children

We compared the MDA contents in the plasma of children with autism and their non-autistic siblings (Fig. 1). The MDA contents in plasma were significantly ($p < 0.005$, paired t-test; $p < 0.005$,

unpaired t-test, n = 15) higher in autism (Mean ± S.E. = 0.4969 ± 0.025 nmole / ml) as compared to non-autistic siblings (Mean ± S.E. = 0.396 ± 0.019 nmole / ml); indicating that lipid peroxidation is increased in autism. The data was also significant when (a) autistic children (Mean ± SE = 0.4966 ± 0.027) were compared with their siblings of same sex (Mean ± S.E. = 0.3822 ± 0.026, $p < 0.005$, paired t-test; $p < 0.02$, unpaired t-test), (b) male autistic children were compared with their male siblings ($p < 0.05$, paired and unpaired t-test), and (c) female autistic children were compared with their female siblings ($p < 0.05$, paired t-test). However, there was no significant difference between autism and siblings of opposite sex.

Reduced serum ceruloplasmin levels in autism

Fig. 2 illustrates the levels of ceruloplasmin in 19 children with autism and their 19 non-autistic siblings of same or different sex. 13 of these 19 autistic children were observed to have reduced levels of ceruloplasmin as compared to their normal siblings (Fig. 2). Statistical analysis showed that ceruloplasmin levels were significantly decreased in (a) whole autism group (Mean ± S.E. = 0.2996 ± 0.0138 mg / ml) as compared to whole non-autistic siblings group of same sex plus different sex compared to autism (Mean ± S.E. = 0.3296 ± 0.0182 mg / ml, $p < 0.02$, paired t-test, number of pairs = 19), and (b) autism group of 14 children (Mean ± S.E. = 0.2951 ± 0.0162) as compared to siblings of same sex (Mean ± S.E. = 0.3329 ± 0.0218, $p < 0.05$, paired t-test, number of pairs = 14). The data was however, not significant when above groups were compared by unpaired t-test, and when autistic children (Mean ± S.E. = 0.3124 ± 0.0281, n = 5) and their siblings of different sex (Mean ± S.E. = 0.3206 ± 0.0356) were compared.

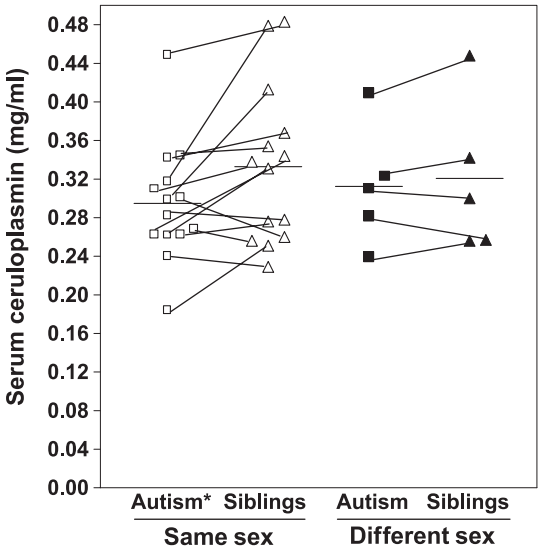

Fig. 2. Reduced serum ceruloplasmin levels in autism as compared to non-autistic siblings. Ceruloplasmin levels were analyzed in 19 pairs of autistic children and non-autistic siblings. Connecting lines represent each paired group of autistic and his / her non-autistic sibling. Horizontal line represents average ceruloplasmin level in each group. *$p < 0.05$ in autism as compared to non-autistic siblings of same sex, paired t-test (no. of pairs = 14). Additionally, $p < 0.02$ (paired t-test) when entire autism group was compared with entire sibling group of same and different sex (n = 19).

Reduced serum transferrin levels in autism

The levels of transferrin in serum samples of 19 children with autism, and their 19 non-autistic siblings are shown in Fig. 3. The transferrin levels were observed to be lower in 16 out of 19 autistic children as compared to their non-autistic siblings. Statistical analysis of whole data (same sex plus different sex, n = 19) by both two-tailed unpaired t-test ($p < 0.05$) and paired t-test ($p < 0.005$) indicated significant decrease in autism (Mean ± S.E. = 2.456 ± 0.0664 mg / ml) as compared to non-autistic siblings (Mean ± S.E. = 2.699 ± 0.0932 mg / ml). The data was also significant ($p < 0.05$, n = 14, paired t-test) when the autistic children (Mean ± S.E. = 2.441 ± 0.0706 mg / ml) were compared with their siblings of same sex (Mean ± S.E. = 2.635 ± 0.0963 mg / ml). It was however, not significant when autistic children (Mean ± S.E. = 2.497 ± 0.1716 mg / ml) were compared with their siblings of opposite sex (Mean ± S.E. = 2.876 ± 0.2311 mg / ml, n = 5).

Relationship between ceruloplasmin / transferrin levels and lost acquired language skills in autism

The analysis of serum ceruloplasmin and transferrin levels in autism suggests that these effects are seen most strongly in children who had shown a loss of previously acquired language skills (Fig. 4, Fig. 5) as shown in the box and whisker plots [Mean ± S.E. (box) and ± 95% Confidence Interval

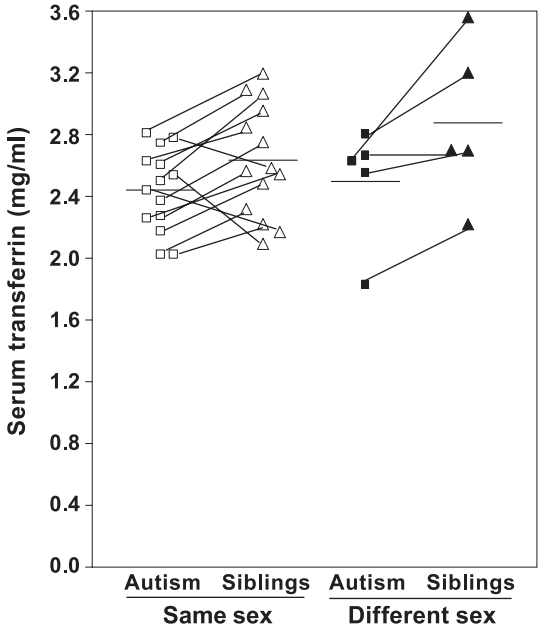

Fig. 3. Reduced serum transferrin levels in autism as compared to non-autistic siblings. Transferrin levels were measured in 19 children with autism and their non-autistic siblings. Connecting lines represent each paired group of autistic and his / her non-autistic sibling. Horizontal line represents average transferrin level in each group. *$p < 0.05$ in autism as compared to non-autistic siblings of same sex, paired t-test, n = 14. Additionally, when entire autism group was compared with entire sibling group of same and different sex (n = 19), $p < 0.005$ (paired t-test) and $p < 0.05$ (unpaired t-test).

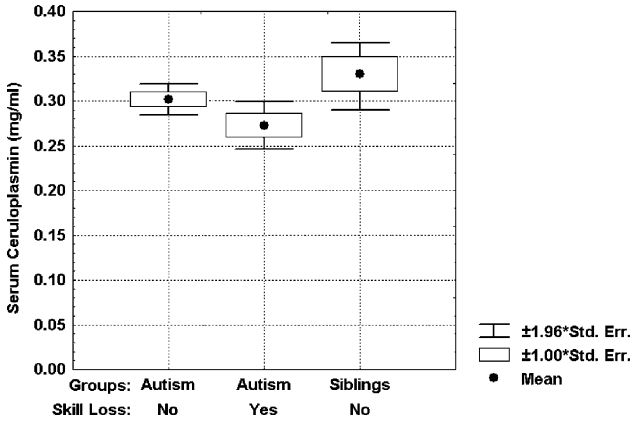

Fig. 4. Relationship between serum ceruloplasmin levels and lost language skills in autism subjects. Whisker plot showing serum ceruloplasmin levels versus lost language skills in autism subjects and non-autistic siblings.

(whisker)]. Children who had not lost language skills had levels similar to that seen in the non-autistic siblings.

Discussion

Autism is a severe neurodevelopmental disorder (Lord et al., 2000) that affects approximately 6 per 1000 live births (Fombonne, 2003). Although this disease is behaviorally defined, its biochemical defects, diagnostic markers and biochemical targets for its treatment are unknown.

Lipid peroxidation caused by oxidative stress is a well-established mechanism of cellular injury (Tappel, 1973; Horton and Fairhurst, 1987; Kannan and Jain, 2000). This process results in the production of lipid peroxides and their by-products that lead to the loss of membrane functions and

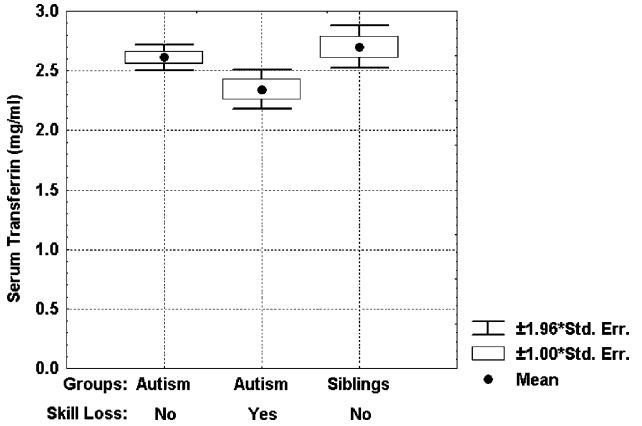

Fig. 5. Relationship between serum transferrin levels and lost language skills in autism subjects. Whisker plot showing serum transferrin levels versus lost language skills in autism subjects and non-autistic siblings.

integrity (Turrens et al., 1982; Jain, 1984). It is well recognized that overload of oxygen free radicals, and / or peroxides is associated with cell damage in inflammatory diseases, autoimmune diseases (e.g. rheumatoid arthritis), atherosclerosis and neurodegenerative diseases (Alzheimer and Parkinson diseases) (Bostantjopoulou et al., 1997; Torsdottir et al., 1999; Kannan and Jain, 2000; Herken et al., 2001; Akyol et al., 2002). Our results indicate that lipid peroxidation in plasma is significantly increased in children with autism as compared to their non-autistic siblings. This suggests that there is an increased oxidative stress in autism, which may be an integral part of the development of autism or it may be secondary to other alterations in autism.

In this study, the content of MDA, a marker of lipid peroxidation, was measured by reaction with TBA. A good relationship has been established between MDA measurement by TBA reaction and other methods of measuring lipid peroxidation such as diene conjugation, chemiluminescence, oxygen uptake, high performance liquid chromatography (HPLC) and lipid hydroperoxide content (Slater, 1984). The limitation of using TBA method is that certain drugs and high sucrose concentration can interfere with this assay. Out of 30 autistic children in this study, only one child was taking medication. Therefore, it is not likely that the observed increase in MDA content in autism is a result of interference by other substances.

The exact mechanism of oxidative stress in autism is not known. It may be due to increased production of pro-oxidants, or deficiencies of antioxidants or both. Our results suggest that the levels of ceruloplasmin (copper-transporting protein) and transferrin (iron-transporting protein), which are the major antioxidant proteins in the blood, are significantly reduced in children with autism as compared to their non-autistic siblings. It was of particular interest to observe that the levels of ceruloplasmin and transferrin were reduced more effectively in autistic children who had lost acquired language skills. It is not clear whether these antioxidant proteins affect the intellectual behavior of children in early age of development or loss of language skills results in reduced levels of these proteins, or if both phenomena are related to a third unknown but correlated variable. The plasma ceruloplasmin and transferrin levels have also been reported to be lower in the patients with Alzheimer and Parkinson disease than in their controls (Logroscino et al., 1997; Torsdottir et al., 1999). Furthermore, Loeffler et al. (1996) observed increased concentration of ceruloplasmin in different regions of brain of patients with neurodegenerative diseases (Alzheimer, Parkinson and Huntington). In Parkinson disease, evidence also exists of higher iron concentration in the substantia nigra that has been suggested to lead to increased lipid peroxidation and neuronal death (Logroscino et al., 1997; Hirsch and Faucheux, 1998). Further studies are warranted to investigate whether the regional brain concentrations of ceruloplasmin, transferrin, iron and copper are altered in autism.

The data on increased oxidative stress and reduced ceruloplasmin and transferrin levels in autism was statistically significant when compared with siblings group regardless of their sex, or with siblings of same sex (as autistic child). Significance was however, not established when autism group was compared with siblings of different sex. It may be attributed to smaller size of this sub-group.

Ceruloplasmin protects polyunsaturated fatty acids in the red blood cell membranes from active oxygen radicals (Sass-Kortsak, 1965; Arnaud et al., 1988). It acts as a ferroxidase and superoxide dismutase. Superoxide ($O_{2\cdot}^{-}$) is the first reduction product of molecular oxygen. In addition to its intrinsic toxicity, superoxide is an important source of hydroperoxides and deleterious free radicals (Fridovich, 1986). Hydrogen peroxide and $O_{2\cdot}^{-}$ react, catalyzed by transition metals to form the deleterious hydroxyl radical ($OH_{\cdot}$), $O_{2\cdot}^{-} + H_2O_2 \rightarrow OH\bullet + O_2 + OH^{-}$ (McCord and Day, 1978). Most toxic effects are due to

hydroxyl radical formation, which also initiates lipid peroxidation (McCord and Day, 1978). SOD inhibits lipid peroxidation by catalyzing the conversion of superoxides into hydrogen peroxide and oxygen ($2O_{2\cdot}^{-} + 2H^{+} \rightarrow O_2 + H_2O_2$) (McCord and Fridovich, 1969; Kellogg and Fridovich, 1975; Gutteridge, 1977; Beyer et al., 1991).

Transferrin acts as an antioxidant by reducing the concentration of free ferrous ion that catalyzes the conversion of hydrogen peroxide to highly toxic hydroxyl radical by Fenton reaction. In addition, Fe^{3+}-protoporphyrin (heme) group is also present in the four protein subunits of catalase enzyme that plays a vital role in defense mechanism against damage by ROS (Chance, 1954). Catalase converts hydrogen peroxide to water and molecular oxygen, thereby reducing the amount of free hydroxyl radical formation ($2\ H_2O_2 \rightarrow O_2 + 2H_2O$) (Chance, 1954).

Recently, we have reported that phospholipid composition of erythrocyte membrane is altered in autism (Chauhan et al., 2004). While the major phospholipids: phosphatidylcholine (PC) and sphingomyelin (SPG) remained unchanged, the levels of phosphatidylethanolamine (PE) were significantly decreased and phosphatidylserine (PS) were increased in the children with autism as compared to their non-autistic developmentally normal siblings (Chauhan et al., 2004). During oxidative stress both in vivo (Jain, 1985) and in vitro (Jain, 1984), asymmetry of biological membrane is lost, and PS is externalized. It is suggested that increased oxidative stress in autism may lead to externalization of PS and alteration of aminoglycerophospholipids (AGP) i.e., PS and PE in autism.

In conclusion, we found that MDA levels were higher while ceruloplasmin and transferrin levels were lower in groups of autistic children as compared to their normal siblings. Increased levels of lipid peroxidation together with decreased levels of serum ceruloplasmin and transferrin suggest that children with autism are under increased oxidative stress. It may be due to abnormal metabolism of pro-oxidant metal ions and / or decreased anti-oxidant proteins.

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