

Possible Mechanisms Of 25-Hydroxyvitamin D Depletion And Methylation Capacity Dysregulation In Autism

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Abstract: Autism is behaviorally defined neurodevelopmental disorder usually diagnosed in early childhood that is characterized by impairment in reciprocal communication and speech, repetitive behaviors and social withdrawal. The present study was undertaken to investigate the possible mechanisms of vitamin D depletion, methylation capacity dysregulation in association with oxidative stress in the progression of autism. Fifty (50) children with autism diagnosed according to the DSM-IV criteria of the American Psychiatric Association were recruited for this study. The mean age \pm standard deviation (SD) of the patients was 6.3 ± 1.8 years. Thirty (30) age-matched healthy children of the same socioeconomic status were randomly selected as controls (mean age \pm SD was 6.7 ± 1.1 years). Serum level of 25-hydroxyvitamin D (25(OH) D) and plasma levels of S-adenosylhomocysteine (SAH), total glutathione (GSH), folate, vitamin B12, malondialdehyde (MDA) and paraoxonase activity were measured in all subjects. Autistic children had significantly lower 25(OH) D serum level than healthy controls. Similarly, significant decreases in serum GSH, folate and vitamin B12 levels were detected in children. Additionally, serum paraoxonase activity showed significant decrease in autistic children as compared to the healthy controls. In contrast, children with autism had significantly higher SAH as well as MDA serum levels than their levels in healthy controls. The results of the present study shed light on the proposed mechanisms for the contribution of vitamin D depletion, methylation capacity dysregulation in association with oxidative stress in the progression of autism. This indicates that the targeted nutritional intervention with a combination of vitamin D, folic acid, methylcobalamin plus the antioxidant therapy may be of clinical significance in improving autistic symptoms.

Key words: Autism, 25-hydroxyvitamin D, methylation metabolites, oxidative stress.

INTRODUCTION

Autism is a complex neurodevelopmental disorder that is usually diagnosed between the ages of 2 and 10 with peak prevalence rates observed in children aged 5-8 years (EL-Ansary *et al.*, 2011). Autism is a heterogeneous disorder, both etiologically and phenotypically. It is a behaviorally defined disorder and is classified under the pervasive developmental disorders (PDDs). PDDs are a group of disorders that involve a combination of impairments in communication, reciprocal social interactions, and stereotyped patterns of interest/behavior (Chauhan and Chauhan, 2006).

Worldwide, the rate of autism diagnosis has been steadily rising and the rate of increase of this disease is real and frightening. Thus, autism is increasingly being recognized as a public health problem (Main *et al.*, 2010). There are several environmental factors in concert with genetic susceptibilities that are contributing to this rise (Currenti, 2010). This increased prevalence of autism has enormous future public health implications and has stimulated intense research into potential etiologic factors and candidate genes (James *et al.*, 2004).

Autism is considered a multifactorial disorder that is influenced by genetic, environmental and immunological factors as well as an increased vulnerability to oxidative stress. No single gene has been found to be associated with autism, and involvement of multiple genes has been postulated (Sung *et al.*, 2005). Environmental factors, such as mercury, lead, measles, rubella virus, retinoic acid, maternal thalidomide, valporic acid and alcohol use during pregnancy have been suggested to be involved in the etiology of autism (London, 2000). In addition to behavior impairments, gastrointestinal disturbances (White, 2003) and epilepsy (Tuchman and Rapin, 2002) have been described in some patients with autism. Immune (Pardo *et al.*, 2005), autoimmune (Ashwood and Van de Water, 2004) and infectious factors (Yamashita *et al.*, 2003) have also been suggested to play role in the etiology of autism. Later studies have been shown that autistic children may be experiencing increased inflammation and oxidative stress. Altered immune regulation may be one contributing factor to inflammation and oxidative stress in autistic patients (Suh *et al.*, 2008).

Vitamin D is common denominator of a group of sterols with a crucial role in calcium and phosphorus metabolism. Aside from the skeletal health affection, Vitamin D has a role in the regulation of immune function which was first proposed after the identification of vitamin D receptors in lymphocytes. It has since been recognized that the active form of vitamin D has direct effects on naive and activated helper T cells, regulatory T cells, activated B cells and dendritic cells (Mostafa and AL-Ayadhi, 2012).

Vitamin D deficiency has been implicated as a potential environmental factor triggering some autoimmune disorders, including multiple sclerosis and systemic lupus, erythematosus (Zhang and Wu, 2010; Hamza *et al.*, 2011). Autoimmunity may have a role in the pathogenesis of autism in a subgroup of patients and there is also an increase in the frequency of autoimmune disorders among autistic families (Mostafa and Kitchener, 2009; Mostafa and Shehab, 2010).

Vitamin D is also important in neuronal growth and neurodevelopment since vitamin D deficiency in early life affects neuronal differentiation, axonal connectivity, dopamine ontogeny and brain structure and function (Eyles *et al.*, 2012).

Some investigators reported reduced serum 25-hydroxyvitamin D in autistic children and proposed vitamin D deficiency as a possible environmental risk factor for autism. These studies could classify the autistic children as being "Vitamin D inadequate" which lends support to the hypothesis that autism is a vitamin D deficiency disorder (Maguid *et al.*, 2010; Kočovská *et al.*, 2012).

Methylation handling in our body is an important process in which the methyl groups can be added to proteins both for synthesizing new types of proteins and for determining how certain proteins behave. Additionally, methyl groups can be added directly to the DNA molecule and this process can determine the way DNA is transcribed to RNA, a fundamental first step in the process of gene expression.

Methylation events play a critical role in the ability of growth factors to promote normal development. Neurodevelopmental toxins such as ethanol and mercury interrupt growth factor signaling, raising the possibility that they might exert adverse effects on methylation capacity (Waly *et al.*, 2004).

There is no convincing human evidence supporting a theory of the presence of methylation problem in autism. However, methylation can theoretically affect gene function or protein interaction in the brain, and hence, it has been hypothesized that a methylation defect might underlie the causation of autism. It has been theorized that treatment of autism could be helped by augmenting the availability of methyl groups in the body to improve methylation capacity and protect the brain from damage.

The purpose of this study was to explore the possible mechanisms of vitamin D depletion, methylation impairment in association with oxidative stress in the progression of autism among Egyptian children. These goals could be achieved through assessment of serum 25-hydroxyvitamin D level and evaluation of some metabolites in methylation pathway as well as biomarkers of oxidant/antioxidant status in plasma of autistic children in order to generate hypotheses for future research in the direction of nutritional prevention and intervention for at risk and autistic patients.

MATERIALS AND METHODS

Participating children were referral patients recruited from the Autism Clinic of the Children with Special Needs Care Centre in the Institute of Postgraduate Childhood Studies, Ain Shams University, Cairo, Egypt. Patients were fulfilling the criteria of diagnosis of autism as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association, 1994) and the Childhood Autism Rating Scales (CARS). Exclusion criteria included current infection or fever, chronic seizures, rare genetic diseases associated with symptoms of autism (e.g. fragile X and Rett syndrome), neurological diseases (such as cerebral palsy and tuberous sclerosis), severe gastrointestinal symptoms, metabolic disorders (for example, phenylketonuria) and use of high-dose vitamin or mineral supplements. Also, autistic patients on casein-free diet were not included. The autistic children were 50 (36 males and 14 females). The control group comprised 30 healthy children (20 males and 10 females) with no history of chronic disease, autism or other neurologic disorder.

All subjects were studied during summer to avoid the effect of seasonal variation on serum 25-hydroxyvitamin D level. All studied children had not received calcium and/or vitamin D therapy in the past 6 months. In addition, none of them had a concomitant infection, photosensitivity or treatment known to affect serum 25-hydroxyvitamin D level (such as anti-epileptic drugs, corticosteroids and other immunosuppressive drugs). The study was approved by the Ethics Review Committee of the National Research Centre, Cairo, Egypt. In addition, an informed written consent of participation in the study was signed by the parents of the studied subjects.

Clinical Evaluation Of Autistic Patients:

This was based on clinical history taking from caregivers, clinical examination and neuropsychiatric assessment. In addition, the degree of the disease severity was assessed by using the Childhood Autism Rating Scale (CARS) (Schopler *et al.*, 1986) which rates the child on a scale from 1 to 4 in each of 15 areas (relating to people, emotional response, imitation, body use, object use, listening response, fear or nervousness, verbal communication, non-verbal communication, activity level, level and consistency of intellectual response, adaptation to change, visual response, taste, smell and touch response and general impressions). According to

the scale, children who have scored 30 to 36 have mild to moderate autism (n= 15), while those with scores ranging between 37 and 60 points have a severe degree of autism (n= 35).

Biochemical Analysis:

Fasting blood samples were collected from autistic and healthy control children into 2 different tubes one without anticoagulants for serum and one with EDTA for plasma. The first tubes were left to clot then were centrifuged at 4000 x g for 10 min at 4 °C. While, EDTA tubes were immediately centrifuged at 4000 x g for 10 min at 4 °C. Serum and plasma aliquots were transferred into cryostat tubes and stored at -80 °C until analysis. All samples were analyzed within one month of receipt.

Serum 25-OH Vitamin D level was detected by ELISA technique using the kit purchased from Immundiagnostik, Bensheim and Biomedica, Wien (Germany) according to the method described by Scharla *et al.*, (1996). This test kit based on the competition of 25-OH vit D present in the sample with 25-OH vit D tracer, for the binding pocket of vitamin D binding protein (VDBP, Gc-globulin). Since all circulating 25-OH vit D is bound to VDBP *in vivo*, samples have to be precipitated with precipitation reagent to extract the analyte. The supernatant can be used without further treatment within the test. 25-OH vit D present in the sample compete with the tracer, coated on the well for the specific binding site of the binding protein and the VDBP-antibody is bound to the vitamin binding protein. Hence, with increasing concentrations of 25-OH vit D in the sample, the amount of binding protein, immobilized to the well *via* the tracer, is reduced. After a washing step, the quantitation of VDBP is achieved by incubation with a host specific peroxidase labeled antibody using TMB (tetramethylbenzidine) as enzyme substrate. An acidic stop solution is then added to stop the reaction. The color converts to yellow and its intensity is indirectly proportional to the concentration of 25-OH vitamin D in the sample.

Plasma S-adenosylhomocysteine (SAH) level was determined by reversed phase HPLC according to the method described by Melnyk *et al.*, (2000). Separation of SAH in plasma was accomplished by HPLC, Agilent 1100 series with a reversed-phase C₁₈ column (3 µm bead size; 3.9 x 150 mm). The mobile phase consisted of 40 mmol/L sodium phosphate monobasic, monohydrate: 8 mmol/L heptanesulfonic acid and 18% (v/v) methanol adjusted to pH 3.1 and then filtered through a 0.45-µm membrane filter. The isocratic elution was performed using a flow rate of 1.0 mL/min at 40 °C. The detector wavelength was at 260 nm.

The determination of total glutathione in plasma was performed using HPLC, with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C18 column (5 µm; 4.6 x 150 mm) according the method of Rose *et al.* (2008). 50 µL freshly prepared 1.43 mol/ L sodium borohydride solution containing 1.5 µmol/L EDTA, 66 µmol/L NaOH and 10 µL isoamyl alcohol was added to 200 µL plasma to reduce all sulfhydryl bonds and incubated at 40°C in a shaker for 30 min. To precipitate proteins, 250 µL ice cold 10% metaphosphoric acid was added, mixed well, and the sample was incubated for an additional 30 min on ice. After centrifugation at 18,000 x g for 15 min at 4°C, the supernatant was filtered through a 0.2 µm nylon membrane filter (PGC Scientific, Frederic, MD) and a 20 µL aliquot was injected into the HPLC system using Beckman Autosampler (model 507E). Total glutathione was quantified using a model 5200A Coulochem II and CoulArray electrochemical detection systems (ESA, Inc., Chelmsford, MA) equipped with a dual analytical cell (model 5010), a 4-channel analytical cell (model 6210) and a guard cell (model 5020). The concentration of total glutathione was calculated from peak areas and standard calibration curves using HPLC software.

Plasma folate and vitamin B12 levels were determined using SimulTRAC-SNB Radioassay kit, purchased from ICN Pharmaceuticals Inc. (USA), according to the manufacturer's instructions. The unlabeled vitamin B12 or folate competes with its labeled species for the limited number of available binding sites on its specific binder, thus reducing the amount of labeled vitamin B12 or folate bound. Therefore, the level of radioactivity bound is inversely related to the concentration in the patient sample. Levels of vitamin B12 and folate are determined simultaneously in a single tube. The two tracers, [⁵⁷Co] for vitamin B12 and [¹²⁵I] for folate produce energies at levels which can be easily separated by many commercial two-channel counters.

Plasma malondialdehyde (MDA) level was determined colorimetrically according to the method described by Ruiz-Larrea *et al.*, (1994). Malondialdehyde forms a 1:2 adduct with thiobarbituric acid which can be measured by spectrophotometry.

Plasma paraoxonase activity was measured spectrophotometrically using phenylacetate as a substrate according to the method described by Watson *et al.*, (1995).

Statistical Analysis:

In the present study, all results were expressed as Mean ± S.D. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups (Armitage and Berry, 1987). Difference was considered significant when P value was < 0.01.

Results:

The data in Table (1) show that autism in children produces significant decrease by -42.02% in serum 25-hydroxyvitamin D level in comparison with the healthy controls.

Table 1: Basic clinical data and serum 25-hydroxyvitamin D level in autistic cases and controls

Variable	Healthy controls*	Children with autism*
Number	30	50
Age (Years)	5.9±1.2	6.1±1.1 (3.39 %)
Sex (male/female)	20/10	36/14
25 (OH) D (ng/mL)	38.206±2.68	22.15±2.43* (-42.02 %)

* Means ±SD

Abbreviation: 25 (OH) D: 25-hydroxyvitamin D

Table (2) demonstrates that children with autism show significant increase by 33.14% in plasma S-adenosylhomocysteine level associated with significant decrease by -33.95% in plasma glutathione level in comparison with the healthy controls.

Table 2: Plasma S-adenosylhomocysteine and glutathione levels in autistic cases and controls

Variable	Healthy controls*	Children with autism*
SAH(nmol/L)	19.37±1.64	25.79±3.356* (33.14 %)
GSH(μmol/L)	8.01±0.78	5.29±0.42* (-33.95 %)

* Means ±SD

Abbreviations: SAH: S-adenosylhomocysteine; GSH: glutathione

Table (3) shows that autistic cases have significant decrease by -9.01 and -33.71% in plasma folate and vitamin B12 levels respectively in comparison with the healthy controls.

Table 3: Plasma folate and vitamin B12 levels in autistic cases and controls

Variable	Healthy controls*	Children with autism*
Folate (ng/mL)	17.64±1.53	16.05±2.03* (-9.01 %)
Vitamin B12 (pg/mL)	284.98±43.52	188.9±31.77* (-33.71 %)

* Means ±SD

The data in Table (4) show that children with autism have significant increase by 108.25% in plasma MDA level in concomitant with significant decrease by -35.92% in plasma paraoxonase activity in comparison with the healthy controls.

Table 4: Plasma MDA level and paraoxonase activity in autistic cases and controls

Variable	Healthy controls*	Children with autism*
MDA (nmol/mL)	1.09±0.54	2.27±0.67* (108.25 %)
Paraoxonase (U/L)	271.4±20.28	173.9±29.7* (-35.92 %)

* Means ±SD

Abbreviations: MDA: malondialdehyde

Discussion:

Autism is a behaviorally defined disorder that is thought to be influenced by genetic and environmental factors (Paşca *et al.*, 2006). The interplay between genetic and environmental factors has become the subject of intensified research in the last several years (kočovská *et al.*, 2012).

Vitamin D deficiency has recently been proposed as a possible environmental risk factor for autism spectrum disorder (ASD). However, systematic data obtained by different research groups provide some albeit very limited support for the possible role of vitamin D deficiency in the pathogenesis of ASD. There are two main areas of involvement of vitamin D in the human body that could potentially have direct impact on the development of ASD: 1) the brain (its homeostasis, immune system and neurodevelopment and 2) gene regulation (kočovská *et al.*, 2012).

The current study revealed significant ($p < 0.01$) depletion in serum level of 25-hydroxyvitamin D in autistic children (-42.02%) versus healthy controls. This finding is in accordance with that of Meguid *et al.*,

(2010) and Mostafa and AL-Ayadhi, (2012). The study of Humble, (2010) and Bakare *et al.*, (2011) suggested that vitamin D availability and metabolism may have notable effects on mental health. The mechanisms through which these effects could occur is not fully understood as yet, but animal studies have shown that low prenatal vitamin D in utero can produce abnormal brain development characterized by increased brain size, enlarged ventricles, reduced brain content of nerve growth factor and distortion in brain shape (Eyles *et al.*, 2009). Neuro-imaging findings in autism, though not diagnostic, have consistently revealed enlargement in cerebral volume that affects both gray and white matter as well as enlarged ventricles (Lainhart, 2006). These structural abnormalities resemble those found in animals with prenatal exposure to vitamin D deficiency. Moreover, Cannell, (2010) and others (Grant and soles, 2009; Meguid *et al.*, 2010) have presented evidence that vitamin D deficiency in utero and early childhood is associated with an increased risk for autism. Evidence for an etiologic role of vitamin D deficiency in autism includes, e.g., a higher prevalence of autism in populations born at higher latitudes, urban areas, or regions with intense air pollution and high precipitation—all environments where vitamin D deficiency is likely to be more common because of reduced levels of UVB radiation essential for endogenous vitamin D production (Kinney *et al.*, 2010).

Molloy *et al.*, (2010) stated that ASD could potentially put a child at greater risk of vitamin D deficiency secondary to dietary restrictions or decreased exposure to sunlight. Children with ASD may limit their own diet because of sensory aversions or restricted interests. The diet may also be restricted by parents to eliminate exposure to certain dietary proteins, such as the milk protein casein, in an attempt to treat the ASD symptoms (Marcason, 2009). Children with ASD may have decreased exposure to sunlight because their after-school hours are often devoted to table-based therapies, they do not commonly participate in organized outdoor sports and their preferred leisure activities often involve video game, computer or TV screens in an indoor setting (Molloy *et al.*, 2010).

Genetic polymorphism of cytochrome P450 enzymes have also been linked to autism, specifically CYP27B1 that is essential for proper vitamin D metabolism. Defects in metabolism or deficiency of vitamin D have been implicated in autistic individuals (Currenti, 2010).

Activated vitamin D acts as a molecular switch like most steroid hormones, activating more than 200 target genes, thereby regulating gene expression through multiple mechanisms (Dusso *et al.*, 2005). Vitamin D therefore may play a major role in the etiology of autism by influencing expression of genes related to autism (Palmieri *et al.*, 2010).

Vitamin D plays important roles in repairing DNA damage and protecting against oxidative stress- a key cause of DNA damage. Factors associated with vitamin D deficiency will thus contribute to higher mutation rates and impaired repair of DNA (Kinney *et al.*, 2010) as a result of increased oxidative stress which was reported in autistic children (Mostafa *et al.*, 2010). Oxidation reactions produce deleterious effects on DNA by a variety of mechanisms, depending on the type of affected nucleotide. Indeed, the threat of oxidation reactions to DNA is so prevalent that most genetic material would be altered by reactive oxygen species (ROS) (Cooke *et al.*, 2003). Several lines of evidence suggest that vitamin D in its active form has significant antioxidant properties as it could increase the levels of a key enzyme in the cell's natural anti-oxidative defenses. Moreover, vitamin D could promote DNA synthesis (Edelson *et al.*, 1994) and promote expression of glutathione, the powerful intracellular antioxidant agent (Kinney *et al.*, 2010).

Indeed, the vitamin D theory of autism does not diminish genetic contributions to autism occurrence. Indeed, without the genetic tendency for autism, severe maternal or early childhood vitamin D deficiency may cause bone abnormalities with no evidence of autism. Maternal and early childhood vitamin D deficiency may allow the genetic tendency for autism to express itself. If this theory is true, the path towards effective prevention and perhaps a treatment of autism is so simple, so safe, so inexpensive, so readily available and so easy (Cannell, 2010).

Most of human genes are controlled by methylation and errors of methylation can affect how genes function or malfunction. Atypical methylation has been detected in autism (James *et al.*, 2004) and abnormal metabolic profile of methionine cycle has been found in children diagnosed with autism. This metabolic profile is consistent with impaired capacity for methylation and increased oxidative stress in children with autism (James *et al.*, 2004; Waly *et al.*, 2004; James *et al.*, 2006; Deth *et al.*, 2008).

On the basis of these studies, a "redox/methylation hypothesis of autism is described, in which oxidative stress, initiated by environmental factors in genetically vulnerable individuals, leads to impaired methylation and neurological deficits secondary to reductions in the capacity for synchronizing neural networks (Deth *et al.*, 2008).

In the present study, plasma concentration of S-adenosylhomocysteine (SAH) was significantly ($P < 0.01$) increased (33.14%). While, plasma level of glutathione was significantly ($P < 0.01$) decreased (-33.95%) in the autistic children relative to age-matched controls. These results are in agreement with those of James *et al.*, (2006). Moreover, Rose *et al.*, (2008) recorded lower plasma glutathione level in autistic children compared to that in age-matched healthy children. It has been reported that the increased SAH is associated with hypomethylation of numerous substances, including DNA and proteins that render neurons more susceptible to

damage and apoptosis (Miller, 2003). One explanation for the significant elevation of SAH in autistic children is a downstream defect in adenosine metabolism. An increase in adenosine is well known to bind to the active site of SAH hydrolase as a product inhibitor resulting in an increase of SAH levels. Consistent with this possibility, previous studies have reported a decrease in adenosine deaminase activity (Stubbs *et al.*, 1982) and a functional polymorphism in the adenosine deaminase gene in some children with autism (Bottini *et al.*, 2001). The increase in SAH in autistic children is of clinical concern because SAH is a potent product inhibitor of most cellular methyltransferases. The increased SAH and adenosine concentrations were shown previously to be associated with reduced cellular methylation capacity (Yi *et al.*, 2000).

The observed decrease in plasma glutathione level in our autistic children could be attributed to the increased SAH concentration due to the inhibition of SAH hydrolase (Brodie and Reed, 1985) as a consequence of increased adenosine, which inhibits glutathione synthesis (Atmaca and Fry, 1996).

The other proposed mechanism for the decreased glutathione plasma level in autistic children is the lowering levels of several metabolic precursors for glutathione synthesis suggesting the insufficiency of glutathione synthesis in these patients (James *et al.*, 2009). Moreover, Vargas *et al.*, (2005) documented the presence of chronic inflammation in the autistic brain that seems mediated by innate microglial activation and proinflammatory cytokines. The inflammatory response is augmented when glutathione concentrations are low and chronic inflammation depletes glutathione further and promotes a self-perpetuating cycle that could exacerbate gastrointestinal and central nervous system inflammation associated with autism.

Many micronutrients and vitamins are critical for DNA synthesis/repair and maintenance of DNA methylation patterns. Folate has been most extensively investigated in this regard because of its unique function as methyl donor for nucleotide synthesis and biological methylation. Cell culture, animal and human studies showed that deficiency of folate induces disruption of DNA as well as alterations in DNA methylation status. Thus, the adverse effects of inadequate folate status on DNA metabolism are mostly due to the impairment of methyl supply. Therefore, it has been suggested that the interaction between a nutritional status with genetic polymorphism can modulate gene expression through DNA methylation, especially when such polymorphism limits the methyl supply (Friso and Choi, 2002).

The most important role of vitamin B12 is that it works synergistically with B vitamin folate in the common metabolic pathway supplying essential methyl groups for DNA, RNA and protein synthesis. This is crucial for neurons regeneration and growth in the treatment of autism. Vitamin B12 acts a co-factor for methionine synthase, the enzyme that re-methylates homocysteine to methionine by using 5-methyl- tetrahydrofolate as methyl donor. Vitamin B12 and folic acid are essential for maintaining a healthy physiological level of homocysteine in the circulation. Therefore, a deficiency of either will result in increased serum total homocysteine.

In the current study, the plasma levels of folate and vitamin B12 were significantly ($P < 0.01$) decreased (-9.01% and -33.71% respectively) in autistic children as compared to their corresponding values in healthy children. These findings are in agreement with those of Kaluźna-Czaplińska *et al.*, (2009) who recorded vitamin deficiencies in many autistic children. Main *et al.*, (2010) stated that children with autism may have altered folate or methionine metabolism which suggests that folate – methionine cycle may play a key role in the etiology of autism. The plausible mechanisms for a role of folate deficiency in neurodegenerative diseases include impaired mitochondrial function due to mitochondrial DNA deletions, reduced availability of methyl groups from folate for neurotransmitter synthesis and reduced proliferative potential of regenerative cells in critical regions of the brain caused by diminished nucleotide synthesis (Chou *et al.*, 2007; Chou and Huang, 2009). Folate deficiency and in turn defective folate transport into the central nervous system have been linked with cerebral folate deficiency, a condition associated with developmental delays (with or without autistic features), providing plausibility for involvement of folate deficiency in the etiology of autism (Djukic, 2007). On the other hand, supplementation with folinic acid led to improved cerebrospinal fluid folate status and remarkable cognitive, motor and neurologic changes in children with low-functioning autism and at least one symptom of cerebral folate deficiency (Ramaekers *et al.*, 2007).

Folate metabolism can also be impaired by 2 polymorphisms of methylene tetrahydrofolate reductase (MTHFR), *MTHFR 677c→T* and *MTHFR 1298A→C*, which lower enzyme activity, reduce DNA methylation (Rogers, 2008) and possibly increase chromosomal instability (Iarmarcovai *et al.*, 2008). MTHFR is a pivotal enzyme that catalyses the reduction of 5, 10- MTHF into 5-MTHF, which is the major circulating form of folate and acts as a methyl donor in the remethylation of homocysteine to methionine. Whereas, Boris *et al.*, (2004) reported a significant association for the homozygote *MTHFR 677C→T* and the compound heterozygote *MTHFR677c→T/1298A→C* and autism.

Indeed, the changes in the concentration of metabolites of the methionine cycle in autistic patients may be driven by abnormalities in folate transport and /or metabolism. Almost all of the genetic association studies that have examined the genes of this metabolic pathway were under powered (Main *et al.*, 2010).

The observed lower plasma level of vitamin B12 in children with autism than in controls in the present study could be attributed to picky eating habits leading to poor dietary B12 intake, dysbiosis in the gut leading

to poor and malabsorption of B12, neurodegenerative damage of the neurons as caused by autoimmune antibodies, neurotoxins and heavy metals poisoning and the severe abnormality of transsulfuration, transmethylation and antioxidant capacity (Ali *et al.*, 2011). Our results are in consistent with those of Ali *et al.*, (2011) and Paşca *et al.*, (2006) who reported that the mean vitamin B12 levels are significantly lower in autism as compared to controls.

Other explanation for vitamin B12 deficiency in children with autism is the failure of intracellular transport of B12 by transcobalamin II which leads to functional B12 deficiency. Previous studies indicate that a common 776 C>G transition in the *TCN2* gene (proline>arginine) decreases in the binding affinity of transcobalamin II for vitamin B12 and reduces the transport of B12 into cells (Afman *et al.*, 2002; Miller *et al.*, 2002). The frequency of the homozygous *TCN2* 776GG variant was significantly increased among the autistic children compared to controls and the GG variant was associated with a 1.7-fold increased risk of autism (James *et al.*, 2006). It has been suggested that the raised levels of either serum methylmalonic acid or homocysteine is associated with low levels of transcobalamin II (Turner and Talbot, 2009) with consequent deficiency in vitamin B12 (Kaluźna-Czaplińska *et al.*, 2011). Vitamin B12 deficiency is well known to adversely affect neurodevelopment during infancy (Graham *et al.*, 1992). Also vitamin B12 deficiency has been associated with developmental regression similar to that observed in ~33% of autistic children (Grattan-Smith *et al.*, 1997).

On the other side, a genetic predisposition to environmental agents or conditions that promote oxidative stress could contribute to the abnormal metabolic profile observed in the autistic children (James *et al.*, 2004). Increased oxidative stress has been recorded in our autistic children as indicated by the significant ($p < 0.01$) increase (108.25%) in lipid peroxidation marker, malondialdehyde (MDA), level in plasma of children with autism as compared to control subjects. This result is in consistent with that of Chauhan *et al.*, (2004) and Chauhan and Chauhan, (2006). A growing body of evidence indicated that some children with autism may become autistic from neuronal cell death or brain damage sometime after birth as a result of insult due to oxidative stress (Kern and Jones, 2006). The brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity, higher energy requirement and higher amounts of lipids and iron (Juurlink and Paterson, 1998). Therefore, neurons are the first cells to be affected by the increase reactive oxygen species (ROS) and shortage of antioxidants and, as a result, are most susceptible to oxidative stress. Antioxidants are required for neuronal survival during the early critical period (Perry *et al.*, 2004). Moreover, children are more vulnerable than adults to oxidative stress because of their naturally low glutathione levels from conception through infancy (Erden-Inal *et al.*, 2002). The risk created by this natural deficit in detoxification capacity in infants is increased by the fact that some environmental factors that induce oxidative stress are found at higher concentrations in developing infants than in their mothers, and accumulate in the placenta. Taken together, these studies suggest that the brain is highly vulnerable to oxidative stress, particularly during the early part of development that may result in neurodevelopmental disorders such as autism (Chauhan and Chauhan, 2006).

Various factors leading to increased oxidative stress in autism are as follows; (1) alterations in antioxidant enzymes, (2) abnormal iron and copper metabolism, (3) imbalance in homocysteine and methionine metabolism, (4) increased nitric oxide due to activation of inducible nitric oxide synthase (iNOS) activity, (5) increased xanthine oxidase enzyme, (6) mitochondrial dysfunction and abnormal energy metabolism, (7) environmental risk factors (lead, mercury, viruses and air pollutants) and finally, (8) genetic susceptibility to autism (Chauhan and Chauhan, 2006). Oxidative stress is known to be associated with premature aging of cells and can lead to tissue inflammation, damaged cell membranes, autoimmunity and cell death (Klein and Ackerman, 2003). A growing body of evidence has shown abnormalities in membrane lipid metabolism and imbalance in immune and inflammatory responses in autism. These abnormalities might contribute to behavioral abnormalities, sleep disorder and gastrointestinal disturbances in autism (Chauhan and Chauhan, 2006).

Recent studies have suggested a deficit in the antioxidant enzymes that play a vital role in the defense mechanism against damage by ROS in autism (Melnyk *et al.*, 2012). for instance, compared to controls, patients with autism showed decreased activity of glutathione peroxidase in plasma (Yorbik *et al.*, 2002) and in erythrocyte (Pasca *et al.*, 2006), decreased catalase (Zoroglu *et al.*, 2004) and superoxide dismutase (Yorbik *et al.*, 2002) activity in erythrocytes.

The present results revealed significant ($P < 0.01$) inhibition in plasma level of paraoxonase activity (-35.92%) in children with autism compared to the control group. Paraoxonase (PON1) is an enzyme with hydrolase activity. It-so named because of its ability to hydrolyse the toxic metabolite of parathion, paraoxon- was also shown early after its identification to manifest arylesterase activity. Although the preferred endogenous substrate of PON1 remains unknown, lactones comprise one possible candidate class. Homocysteine-thiolactone can be disposed of by enzymatic hydrolysis by the serum homocystein-thiolactonase/paraoxonase carried on high-density lipoprotein (HDL) (Yilmaz, 2012). Paraoxonase 1 protects lipoproteins against oxidative stress and makes possible to metabolize lipid peroxides (Li *et al.*, 2003). Several polymorphisms of the gene PON1 have been identified. The most important for enzyme activity seem to be two polymorphisms: in the position 55 (L55M) and in the position 192 (R192Q). For instance the genotype PON1 55MM is connected with low enzyme serum level (Sapian-Raczkowska *et al.*, 2010).

Our finding is in line with that of Paşca *et al.*, (2006) who reported a decreased activity of plasma paraoxonase in children with autism compared to its activity in age-matched healthy children. Indeed, Holven *et al.*, (2008) found that hyperhomocyst-einaemic subjects, and particularly those with extreme hyperhomocysteinemia, were characterized by significant less PON1 activity than healthy controls. It has been reported that hepatic expression of PON1 gene is down-regulated in hyperhomo-cysteinemic mice. Therefore, homocysteine may be involved in diminished serum PON1 activity, leading to impair its antioxidant function (Jakubowski *et al.*, 2009). Mungan *et al.*, (2006) found a significant negative correlation between serum PON1 and MDA levels and serum PON1 activity was also negatively correlated with homocysteine levels in Behcet disease patients. Thus it is possible that the relationship between PON1 and mental development is strongly influenced by the role of PON1 in protection from oxidative stress (Eskenazi *et al.*, 2010). Moreover, the study of Paşca *et al.*, (2006) showed that in children with autism there are higher levels of homocysteine, which is negatively correlated with glutathione peroxidase activity, low PON1 arylesterase activity and suboptimal levels of vitamin B12. These authors also demonstrated that *PON1*₁₉₂ genotype and PON1 enzyme measurements have been associated with childhood autism at least in certain populations (D'Amelio *et al.*, 2005). Thus, PON1 polymorphisms and enzyme activities have been associated with autism spectrum disorder.

Conclusion:

In conclusion, the present study highlights the mechanisms for contribution of vitamin D depletion, methylation capacity impairment in concomitant with the failure of the antioxidant defense systems in the development and clinical manifestation of autism. This indicates that options for targeted nutritional intervention with a combination of vitamin D, folic acid, methylcobalamin plus the antioxidant therapy may be of clinical benefit in improving autistic behavior motor and neurologic symptoms in children who have autism. Randomized and controlled trials are necessary to establish the clinical efficacy of a combination therapy supplementation in ill or at risk subjects.

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