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Level of Trace Elements (Copper, Zinc, Magnesium and Selenium) and Toxic Elements (Lead and Mercury) in the Hair and Nail of Children with Autism

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Abstract Autism is a multi-factorial pathology observed in children with altered levels of essential and elevated levels of toxic elements. There are also studies reporting a decrease in nutritional trace elements in the hair and nail of autistic children with healthy controls; moreover, bioelements have been shown to play an important role in the central nervous system. Therefore, the purpose of the present study was to assess the levels of trace elements like copper (Cu), zinc (Zn), magnesium (Mg), and selenium (Se) and toxic elements like mercury (Hg), and lead (Pb) in the hair and nail samples of autistic children and to evaluate whether the level of these elements could be correlated with the severity of autism. The subjects of the study were 45 autistic children with different grades of severity (low (LFA), medium (MFA), and high (HFA) functioning autism) according to Childhood Autism Rating Scale, $n=15$ children in each group and 50 healthy children (age and sex matched). The boys and girls ratio involved in this study was 4:1, and they were 4-12 years of age. The study observed a valid indication of Cu body burden in the autistic children. The children with different grades of autism showed high significance ($p<0.001$) in the level of copper in their hair and nail samples when compared to healthy controls. The level of Cu in the autistic children could be correlated with their degree of severity (more the Cu burden severe is autism). The study showed a significant elevation ($p<0.001$) in the levels of toxic metals Pb and Hg in both hair and nail samples of autistic children when compared to healthy control group. The elevation was much pronounced in LFA group subjects when compared among autistic groups MFA and HFA. The levels of trace elements Mg and Se were significantly decreased ($p<0.001$) in autistic children when compared to control. The trace element Zn showed significant variation in both hair and nails of LFA group children when compared to control group and other study groups. The significant elevation in the concentration of Cu, Pb, and Hg and significant decrease in the concentration of Mg and Se observed in the hair and nail samples of autistic subjects could be well correlated with their degrees of severity.

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Introduction

Autism is a behaviorally defined disorder, characterized by qualitative impairments in social communication, social interaction, and social imagination, with a restricted range of interests and often stereotyped repetitive behaviors and mannerisms [1]. In spite of decades of research and investigation, the etiology of autism remains undefined, since the disorder is complex and heterogeneous with varying degrees of severity [2]. Progress in understanding the causes, nature, and treatment for autism requires ever-increasing integration between concepts, genetic findings, advances in cognitive neuroscience and clinical observations [3]. The pathophysiological etiologies which precipitate autism symptoms remain elusive and controversial in many cases, but both genetic and environmental factors (and their interactions) have been implicated. One environmental factor that has received significant attention is the burden of mercury, lead, and other toxic metals [4]. Adams et al. [5] have analyzed the level of 39 toxic metals and essential minerals in hair samples of children with ASD and their mothers in Arizona and reported some interesting findings with reference to the influence of low levels of iodine and lithium in young children with autism and their mothers.

The aim of the present study is to analyze the level of toxic metals like mercury and lead in the hair and nail samples of autistic children with different grades of severity and compare the data with age- and sex-matched normal healthy children. The study also makes an attempt to investigate the possible relationship of the severity of autism to the level of toxic metals which indicates body burden suffered by autistic children. Also, it has become apparent that many metallic elements play an important role in the maintenance of human health and that an imbalance in trace elements may be a significant factor in a wide variety of physical and psychiatric conditions. The imbalance may occur due to excess of a toxic metal or deficiency of an essential metal [6]. Hence the level of trace elements like copper (Cu), zinc (Zn), magnesium (Mg), and selenium (Se) are also being studied in the hair and nail samples of autistic children and compared with age- and sex-matched normal healthy children. Along with the essential role of trace metals in maintaining good health, they are also known for their interaction with toxic metals and chelating them to reduce the body's burden of toxic metals.

Among various biological specimens, we chose hair and nail samples for the metal analysis as they serve as a best bio-indicator. For at least 50 years hair has been recognized as a potential repository of all the elements that enter the body, providing, if has been claimed, not just a glimpse of a passing state but also a chemical calendar [7]. Hair mineral analysis may indicate the mineral composition accumulated over a long time span, which is proportional to the level of elements in the body. The diagnostic usefulness of hair analysis is confirmed by many authors, who have proven the correlation between the concentration of basic elements in hair and their concentrations in the body, both in the physiological and pathological states [8–10]. Nails also indicate metal body burden [11]. The hair and nails in which trace minerals are sequestered and/or stored can be used to effectively monitor the highest priority toxic trace metals [12]. Hair and nails are recording filaments that can reflect metabolic changes of many elements over long periods of time. The advantages of hair and nail tissue analysis over other diagnostic samples is that trace metal concentrations are not subjected to rapid fluctuation due to diet, air, and water; hence, there is long-term stability over nutritional status [13].

Subjects and Methods

Selection of Subjects

Autistic children presently attending a special school called V-excel Educational Trust at Chennai, India and few other centers for autistic children were the subjects of this study. The institution used Check of Autism in Toddlers to assess autism in children. Autistic children were classified according to the method adopted from Childhood Autism Rating Scale (CARS) as low functioning autism (LFA), medium functioning autism (MFA), and high functioning autism (HFA). Age- and sex-matched healthy normal children served as control subjects. Each group comprised of 15 children and 50 normal children were considered as controls for the study. The boys and girls ratio involved in this study was 4:1, and they were 4-12 years of age. The clinical history is shown in Table 1.

CARS

This is a 15-item scale which aids in the identification of children with autism and which distinguishes them from other children with compromised development but without autism. Its importance is based on its ability to differentiate mild to moderate from severe autism [14]. It is brief and is appropriate for use with any child over the age of 2 years. It was developed over a 15-year period on the basis of 1,500 autistic children. The scale incorporates diagnostic criteria based on the work of Kanner (1943), Creak (1961), Rutter (1978) and Ritvo and Freeman (1978), and from the 1980 Diagnostic and Statistical Manual of Mental Disorders [15]. The scale evaluates behavior in 14 domains that are generally affected in autism, plus a single category for general impression of autism [16]. These 15 items are as follows: relating to people, imitation, emotional response, body use, object use, adaptation to change, visual response, listening response, taste, smell, and touch response and use, fear or nervousness, verbal communication, nonverbal communication, activity level, level and consistency of intellectual response, and finally, general impressions. The scores assigned to each domain vary from 1 (within the limits of normality) to 4 (severe autistic symptoms). The total score varies from 15 to 60 and the cutoff point for autism is 30 [15].

Table 1 Clinical History of Autistic Children

Characteristics	Numbers
Total number of autistic children	45 (15 in each group)
CARS value (15-60)	LFA=46-60; MFA=31-45; HFA=15-30
Male/female ratio	36/9
Age in years (range)	4-12
Children with gluten sensitivity	LFA=12/15; MFA=10/15; HFA=5/15
Children with special talents(dancing, humming, drawing, assembling jumbled picture)	LFA=3/15; MFA=7/15; HFA=11/15
No. of children with low muscle tone	37/45
No. of children with ear infection	2/45
No. of children with sleep disturbance	23/45
No. of children on antibiotic treatment	None
No. of parents given their cooperation	40/45
No. of parents appreciated the study	38/45

Collection of Hair and Nail Samples

With the concurrence of parents of the children, hair and nail samples were collected. Parents of the autistic and normal children were provided with clean, sterile polyethylene bags with zip-lock to collect the hair and nail samples. They were also provided with sterile rust-free scissors to cut hair sample from the nape of the neck and also sterile rust-free clippers to cut finger nails. The study protocol has been approved by the Institutional Ethical Committee.

Processing of Sample

The hair and nail samples were cut into small pieces prior to washing, pre-washed with non-ionic detergent, and soaked in de-ionized water for 10 min. This was followed by soaking in acetone to remove external contamination, rinsing alternately with deionized water and again with acetone three times as per the IAEA advisory group [17]. Then, the samples were dried at 110°C for 1 h and stored in desiccator [18]. The dry weight of the samples ranged from 0.5 to 1.0 g.

Metal Analysis by Atomic Absorption Spectroscopy

Reagents and Apparatus

All the reagents such as HNO_3, & H_2O_2 were purchased from MERCK. Millipore water was used for all analytical work and the entire digestion vessel. Polyethylene bottles (sample container) Micro Pipette tips and others were washed with 10% HCl, rinsed with de-ionized water before preparing standards, reagents, and samples [19].

Sample Preparation

A Multiwave 3000 micro oven system (from Anton Paar, USA) with 16 position teflon vessels with capping is being used for digestion process. The digestion vessels were provided with a controlled pressure, temperature, and release valve. Before use, all Teflon vessels were soaked with 10% HNO_3. The system was initially programmed by giving gradual rise of 20%, 40%, and 50% power for 5, 15, and 20 min, respectively, for the due warming up. The powdered samples were being used without any further treatment for sample preparation. 0.5 to 1.0 gram of sample is weighed into the Teflon vessels followed by digestion with mixture of HNO_3, & H_2O_2 in the ratio of 3:1.

The resulting solution after microwave digestion was filtered through Whatman #40 filter paper (if necessary) and diluted to 10 ml with Millipore water. A sample blank containing only acid mixture was prepared at the same time. The method of standard addition was generally adapted to calibrate the instrument before going for the observation of the samples.

Determination of Metals

All the atomic absorption measurements were carried out with Perkin Elmer model 400/HGA900/AS800 coupled with Mercury Hydride System-15 (MHS-15). Electrodeless discharge lamp for Pb, Hg, Se, and hollow cathode lamp for Zn, Cu, Mg analysis

were used as a light source to provide specific wavelength of the elements to be determined and high purity (99.999%) acetylene was used to provide constant thermal energy for atomization process and argon gas was used for carrier gas purging purposes for Graphite furnace for the analysis of As and Hg by Mercury Hydride System (MHS-15).

Calibration of Instrument

More then three working standard solution of elements to be determined was prepared, covering the concentration range as recommended by the manufacturer of the instrument for the elements to be determined. Before the analysis of samples, the instrument was calibrated with prepared working standard solution. Calibration of the instrument was repeated periodically during operations and blanks were carried out with each set of 10 samples or aspirate any one of the prepared working standard for every 10 samples to check the instrument drift and to validate analytical procedures and performance. Reagent blank reading was taken and necessary correction was made during the calculation of concentration of various elements.

Zn, Cu, Mg, and Pb Analysis by AAS Graphite Furnace

After calibrating the instrument with prepared working standards, the digested liquid sample solutions was subjected to analysis of Pb, Cu, Zn, and Mg in AAS graphite furnace with specific instrumental conditions as given by instruments manufacturer. The solution was introduced into the flame, the readings were recorded, using the mean of the three reading and quantified the concentration of the metals in the given samples against the standard calibration curve obtained from concentration vs. absorbance of the prepared known concentration on the day of the analysis.

Hg Analysis by Cold Vapor Method Using MHS-15

After calibrating the instrument with prepared working standard, the 10 ml of digested liquid sample was pipetted out to a specific container of mercury hydride system analyzer followed by adding 10 ml 1.5% of HCl as diluent for each flask and blank, 3% of NaBH4 solution in 1% of NaOH in reaction flask and digested samples were run through the reaction flask to quartz cell with out heating. The readings were recorded using the mean of the three reading and quantified the concentration of the metals in the given samples against the standard calibration curve obtained from concentration vs. absorbance of the prepared known concentration on the day of the analysis.

Wavelength of analytes;
Cu=324.8, Zn=213.9, Mg=285.2, Pb=283.3, Hg=253.7, Co=240.7, Se=196.0

Statistical Analysis

The trace element levels in hair and nail were expressed as arithmetic mean in microgram per gram with standard deviation and tabulated to illustrate concentration profile over each group. The statistical significance of mean values between different groups was determined by applying Student's t test and the p value <0.05 was considered as significant. The parameters were also analyzed by Spearman test and the r_s values were calculated to find the correlation.

Results

The mean concentration of trace elements Cu, Zn, Mg, and Se, and toxic elements Pb and Hg in the hair and nail of autistic children compared with age- and sex-matched normal children are given in Tables 2 and 3, respectively. The level of Cu in the hair and nail of autistic children showed a significant elevation when compared to age- and sex-matched normal children. Among three different grades of autistic groups, the elevation of Cu was found very significant in LFA and also the level of significance could also be well correlated with the degree of severity. The level of Cu observed in this study may be considered as valid indication of Cu toxicity in autistic children. LFA group children showed a significant decrease in the concentration of zinc in their hair and nail samples when compared with normal healthy children. We observed significantly lower levels of magnesium and selenium in the hair and nail samples of autistic children compared to healthy age- and sex-matched subjects. The level of toxic elements Pb and Hg were found to be high in the hair and nail samples of autistic children when compared with control group.

Internal comparison of the trace metals Cu, Zn, Mg, and Se and toxic metals between LFA and HFA was also done. Interestingly, the severity of autism could be well correlated with the level of accumulation of toxic elements like Pb and Hg. LFA showed significantly higher accumulation of toxic elements like Pb and Hg in the hair and nail when compared with HFA. LFA also showed a significant decrease in the concentration of trace metals like Mg and Se in the hair and nail when compared to HFA.

Table 4 presents the associations between variables as determined by Spearman's correlation coefficient. The results clearly show that the level of trace elements and toxic elements are well correlated with the degree of severity of autism in children.

Discussion

There is a growing interest in unveiling the mystery behind metal metabolism in autistic children suspecting that to be a key factor in contribution to the etiology of autism. For the

Table 2 Mean Concentration Level (μg/g) of Copper, Zinc, Magnesium, Selenium, Lead, and Mercury in the Hair Samples of Autistic Children Compared with Age- and Sex-matched Normal Children

Metal concentration (μg/g)	Mean			
	Control (n=50)	Autistic children (n=15 in each group)		
		LFA	MFA	HFA
Copper	12.31±1.47	36.62±4.39*	23.16±2.77*	12.35±1.48$^{NS,\ *}$
Zinc	171.68±20.60	130.46±15.65**	172.81±20.73^{NS}	171.92±20.63$^{**,\ NS}$
Magnesium	63.84±7.66	20.17±2.42*	49.73±5.96**	57.82±6.93$^{NS,\ *}$
Selenium	3.37±0.40	0.57±0.06*	1.98±0.23*	2.55±0.30$^{**,\ *}$
Lead	1.56±0.18	17.97±2.15*	3.24±0.38*	2.04±0.24$^{**,\ *}$
Mercury	0.37±0.04	3.09±0.37*	1.10±0.13*	0.65±0.07$^{*,\ *}$

Results were expressed as mean±SD

NS non-significant (control vs. MFA and HFA; LFA vs. HFA)

*p<0.001 (control vs. LFA, MFA, and HFA; LFA vs. HFA)

**p<0.01 (control vs. LFA, MFA and HFA)

Table 3 Mean Concentration Level (μg/g) of Copper, Zinc, Magnesium, Selenium, Lead, and Mercury in the Nail Samples of Autistic Children Compared with Age- and Sex-matched Normal Children

Metal Concentration (μg/g)	Mean			
	Control (n=50)	Autistic (n=15 in each group)		
		LFA	MFA	HFA
Copper	9.62±1.15	28.85±3.46*	16.36±1.96*	10.01±1.20^{NS,*}
Zinc	193.98±23.27	150.83±18.09**	192.02±23.04^{NS}	187.44±22.47$^{**,\ NS}$
Magnesium	454.36±54.52	174.02±20.88*	202.21±24.26*	236.31±28.35$^{*,\ **}$
Selenium	5.70±0.68	1.73±0.20*	2.93±0.35*	4.67±0.56***,*
Lead	16.2±1.94	26.38±3.16*	17.68±2.12^{NS}	16.48±1.80^{NS,*}
Mercury	2.87±0.34	5.12±0.61*	4.37±0.52*	2.57±0.30**,*

Results were expressed as mean ± SD

NS non-significant (control vs. LFA, MFA and HFA; LFA vs. HFA)

*p<0.001 (control vs. LFA, MFA, and HFA; LFA vs. HFA)

**p<0.01 (control vs. LFA, HFA; LFA vs. HFA)

***p<0.05 (control vs. HFA)

purpose, hair and nail are considered ideal for metal analysis. This is because of the unique property of hair and nail in providing a history of the mineral status of an individual possibly acting as recording filaments. Also, sample collection is non-evasive and does not readily deteriorate at room temperature on storage until analysis [13].

Though certain essential trace elements are required in trace amounts for various physiological processes, but at higher concentrations, these micro nutrients tend to be toxic and derange various physiological processes, leading thereby to diseases [20].

Table 4 Rank Correlation between CARS and Trace Elements in Different Groups of Autistic Children (n=45)

Elements	r_s	p
Copper(hair) vs. CARS	+0.942	<0.001
Copper(nail) vs. CARS	+0.958	<0.001
Zinc(hair) vs. CARS	+0.850	<0.001
Zinc(nail) vs. CARS	+0.843	<0.001
Magnesium(hair) vs. CARS	−0.872	<0.001
Magnesium(nail) vs. CARS	−0.948	<0.001
Selenium(hair) vs. CARS	−0.913	<0.001
Selenium(nail) vs. CARS	−0.891	<0.001
Lead(hair) vs. CARS	+0.820	<0.001
Lead(nail) vs. CARS	+0.827	<0.001
Mercury(hair) vs. CARS	+0.748	<0.001
Mercury(nail) vs. CARS	+0.764	<0.001

Based on the critical values of the rank correlation (Spearman's rho) null hypothesis of no correlation was rejected and it was concluded that the aforementioned level of trace elements had correlation with the severity of autism

Similarly, deficiency of essential elements may also lead to significant health concerns [21]. Therefore, it is important to determine the metal concentrations in humans to monitor and assess their impact on health [20]. Recent evidences reveal that many children with autism have multiple medical problems including increase in toxic metal burden [22].

The present study focuses on the level of few trace elements like Cu, Zn, Mg, and Se and toxic elements Pb and Hg in the hair and nail samples of autistic children with different grades of severity and age- and sex-matched healthy children. The data obtained from the present investigation showed significantly elevated level of Cu in the hair and nail of autistic children when compared to control group. It is well known that copper is one of many metal ions that are required for essential body functions but are toxic in excess quantity [23]. Potential neurotoxic effects of this metal include depression, irritability, nervousness [24], and learning behavioral disorders in children [25]. Increased concentration of Cu in hair and nail is likely to be a valid indication of the body burden.

There are studies reporting that in autistic children, there is often a failure to convert glutamate to γ-aminobutyric acid (GABA) in sufficient amounts. This results in an imbalance that lead to neuronal excitation (which can lead to "stimming" behaviors) and too little GABA calming (which can lead to speech impairment). One of the frequent findings in autistic children is the presence of a chronic viral infection (like measles). Viral infections are known to inhibit the conversion of glutamate to GABA. Excess copper, a common finding in 85% or more autistic individuals, also interfere with the function of GABA. Excess glutamate can damage and even kill neurons. It does this by generating free radicals in the cells that it over-excites. Glutamate also stimulates the production of glucose, the chief energy source for the brain. When glucose level is low, the brain has difficulty in clearing the excess glutamate; and excess glutamate depletes glutathione, a potent antioxidant that protects neurons from oxidative damage and toxic metal poisoning [26]. Also, increased levels of copper and ceruloplasmin (copper-containing enzyme) inhibit the functioning of the enzyme hydroxytryptophan decarboxylase which decreases the production of the neurochemical transmitter serotonin. A state of hypercopremia appears associated with depression and perceptual disturbances in schizophrenias [27]. Lesser [28], has noted that excess copper can cause depression, feelings of alienation, irritability, and even paranoia. Pfeiffer [29] has documented that elevated copper levels could be associated with paranoid and hallucinatory schizophrenia, autism, childhood hyperactivity, and depression. Copper toxicity has a powerful effect on the mind. Depending on the severity of the toxicity and the susceptibility of the person, copper can affect the mind moderately or very severely. This statement is proven by the data obtained in the present study where LFA group showed higher level of Cu in both hair and nail samples when compared to MFA and HFA. This result could suggest that higher the level of Cu toxicity severe is the autism in children.

The study also shows significant decrease in the concentration of Zn in hair and nail of LFA group children when compared with control group. The reported level of zinc indicates the copper/zinc imbalance. As copper and zinc are antagonists in function, the reported level of zinc indicates its insufficiency to excrete excess copper which results in copper toxicity. Protein intolerance which is observed in autistic children is a result of high copper and low zinc [30]. Physically, the copper build-up interferes with proper conversion of thyroid hormone at the cellular level. It is very interesting to correlate the report by Adams et al. [5] who have also stated that low iodine levels and abnormal thyroid functions to be the likely contributors of defective speech and cognitive skills in autistic children. Excess copper also disturbs zinc imbalance, interfering with adrenal hormone production and this

weakens the immune system, which is observed in autism. Adams et al. [5] have also quoted that low lithium levels observed in autistic children and their mothers could have affected their neurological and immunological development. The antagonism between copper and zinc warrants special concern because zinc is centrally involved in the functions of over 80 different enzyme system functions, including most events relating to cell division and nuclear acid synthesis [31]. Considering the importance of zinc in human physiology, it is not surprising that zinc deficiency is associated with numerous mental, physical, and reproductive disorders [31].

Decreased level of magnesium is observed in the hair and nail samples of autistic children when compared with the age- and sex-matched normal children. Magnesium is essential to the body's utilization of vitamin B6 and numerous recent studies have demonstrated that autistic children showed marked improvement when given a large daily supplement of vitamin B6 and magnesium [32]. Because of the beneficial 'calming' effect of magnesium, symptoms resulting from a deficiency in the mineral may include anxiety, depression, hyperactivity [33], agitation, hallucination, irritability, nervousness [24], aggression, chronic stress [34], learning disability, and memory impairment [35]. There is also evidence suggesting significant lower level of magnesium in the hair of autistic children when compared to normal controls [36]. There is also evidence showing that children with regressive autism have consistently elevated levels of oxidative stress as compared to normal children. Individuals with Mg and Se deficiency resulting to reduced glutathione antioxidant capacity will be under oxidative stress and will be more vulnerable to toxic compounds that act primarily through oxidative damage [37].

Selenium is an important component of an antioxidant enzyme, glutathione peroxidase which acts to prevent the decay of cellular function, and appears to offer protection from the effects of the toxic metals lead, mercury, and cadmium [38]. The present investigation shows a significant decrease in the level of selenium and increased level of lead and mercury in the hair and nail samples of autistic children when compared to normal healthy children. According to the present study, it is observed that the low level of selenium is not enough to provide protection against the excess toxic metals hence, leading them to metal body burden. Toxic metals (lead and mercury) have been found to have synergistic negative effects on childhood development and cognitive ability [39]. Mercury is known to accumulate in endocrine organs such as the pituitary gland, thyroid, and hypothalamus and to alter hormone levels and endocrine system development during crucial periods of development [40]. Such effects are usually permanent and affect the individual throughout their life. Some of the documented effects of exposure to toxic metals include significant learning and behavioral disabilities, mental retardation, autism, etc. It is also stated that the incidence of neurological conditions in children such as autism has increased over 200% in the last decade [41] and mercury has been found to be a factor in most of those tested [42]. High lead levels have been found to be associated with attention deficit hyperactivity disorder, impulsivity, and inability to inhibit inappropriate responding [43].

Evidences show that autistic children show an increased build-up of toxins which may not arise simply from excessive exposure (because the autistic children selected for this study were from the metal pollution-free living areas), but from a marked inability to process and eliminate toxins from the body. Such a mechanism could lead to a back-up of toxic heavy metals and chemical toxins and increases free radical activity in the body. These toxic and oxidized molecules could penetrate into regions of the brain and damage neurons, receptors, synapses, and enzymes [44]. A recent review has suggested that autistic children have been found to have significantly higher exposure to mercury than controls, and autistic children had significantly increased body burdens of mercury resulting from

biochemical and genomic susceptibilities within detoxification pathways [45]. There is also evidence offering relationship between the severity of autism and a biomarker related to heavy metal toxicity, which found that elevations in urinary porphyrins (associated with mercury or lead and mercury toxicity) were significantly associated with CARS. The present investigation also supports the evidence by providing data that shows increasing order (LFA>MFA>HFA) of toxic metals (Pb and Hg) concentration in the hair and nail samples and their correlation with degree of severity. Also, it is notably important that the level of essential trace elements like Mg and Se are decreased in the order of severity which indicates that the lower the level of Mg and Se, the higher is the risk of metal burden and severe is the autism. The limitation of the study is that we have measured the levels of only few trace elements like Cu, Zn, Mg, and Se and toxic elements like Pb and Mg, whereas there are also other essential trace elements which may be just or even more important for life and other toxic elements which may be just or even more threatening to life. So the study can be extended to evaluate the other trace and toxic elements in autistic children with different grades of severity.

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