THE RESEMBLANCE OF FLUOROSIS PATHOLOGY TO THAT OF AUTISM SPECTRUM DISORDER: A MINI-REVIEW

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ABSTRACT: The rising prevalence of autism spectrum disorder (ASD) in recent decades might reflect an increased burden from the synergistic action of new ecotoxicological factors such as aluminum (Al^{3+}), heavy metals, pesticides, fertilizers, endocrine disruptors, and others. ASD pathogenesis is still poorly understood. Although epidemiological studies have identified fluoride (F) as a developmental neurotoxin, F is not included among the ASD risk factors. Millions of people drink fluoridated tap water for the prevention of tooth decay. Currently, about 500 million people live in endemic areas with high concentrations of F in groundwater and in the biosphere. Endemic fluorosis is a progressive degenerative disease. Comparing the fluorosis pathological symptoms with ASD pathological symptoms supports the view that F might play a role in ASD etiology. Mitochondrial dysfunctions, oxidative stress and inflammation, decreased melatonin levels, and decrease of IQ have been observed in both ASD and endemic fluorosis. It has been estimated that, in the United States of America, the total costs of ASD per year will reach 461 billion USD by the year 2025. This significant burden, which has widespread effects from the personal level to the state economy, can be probably lowered by focusing more on ASD prevention, including the elimination of F.

Keywords: Autism spectrum disorder; Fluoride; Fluorosis; Mitochondria; Oxidative stress; Glutathione; Melatonin; Intelligence.

Abbreviations: Aluminum (Al^{3+}); autism spectrum disorder (ASD); calcium (Ca^{2+}); Centers for Disease Control and Prevention (CDC); European Union (EU); fluoride (F); glutathione (GSH); GSH peroxidase (GPx); intelligence quotient (IQ); lipid peroxidation products (LPP); mitofusin-1 (Mfn1); oxidized glutathione (GSSG); reactive oxygen species (ROS); United States of America (U.S.).

INTRODUCTION

Autism Spectrum Disorder (ASD) covers a range of neurodevelopmental disorders that begin in early childhood and tend to persist till adolescence and adulthood. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), people with ASD have some degree of impaired social skills, repetitive behaviors, and difficulties with speech and communication.[1] Persons with ASD usually require specific care to perform activities of normal everyday life. WHO estimates that 1 in 160 children (62 per 10,000) in the world has ASD.[2] Autism has attracted the attention of researchers and public health agencies, particularly in North America where escalating increases have been reported since the 1990s. According to estimates from the CDC,[3] about 1 in 59 children (169 per 10,000) were diagnosed with ASD in the United States (U.S.) in 2014. The prevalence was 3.63% (95% CI, 3.19%–4.08%) for boys and 1.25% (95% CI, 0.99%–1.51%) for girls. More than 3.5 million Americans currently live with ASD.

However, the etiology of ASD is still poorly understood. Several environmental agents have been considered factors contributing to ASD pathogenesis, including heavy metals, persistent organic pollutants, and many others, such as phthalates,

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bisphenols, and glyphosphate.[4] It is remarkable that F is not the key suspicious risk factor in the autism epidemic, even in the U.S. where a third of children between the ages of 6 months and 4 years get significantly more F than the recommended daily dose and where there was a high prevalence of dental fluorosis (40.6%) in 1999–2004 among teenagers aged 12–15 years.[5] Dental fluorosis refers to changes in the appearance of the tooth enamel caused by the long-term ingestion of F. It is also the first apparent sign of F overload. Wiener et al.[6] recently reported an increase of 31.6% in the prevalence of dental fluorosis, of very mild or above severity, for U.S. adolescents aged 16–17 years, in 2012–2011 compared to the data from 2002–2001.

Currently, about 500 million people live in endemic areas with high concentrations of F (>1.5 mgF/L) in the groundwater and in the biosphere. Fluorosis occurs in at least 25 countries and the number of people affected has reached 200 million people. Endemic fluorosis has been regarded as a severe public issue in China and India since the 1960s. Chronic endemic fluorosis induces extensive damage in the human body with changes in multiple organs and systems. Numerous studies have been published, which have raised the level of concern about the impact of increasing F exposure on the brain.[7] These studies further highlight that it is not the teeth, but the brain which is most impacted by too much F during development. Several studies have shown that F can cross the blood brain barrier, induce neurotoxicity, and affect children's cognitive abilities and mental development.[7,8] Prolonged exposure to F in the prenatal and postnatal stages of development might have toxic effects on the development and metabolism of brain.

The recent meta-analysis of Wang and colleagues[9] of ASD prevalence in China included 44 studies with 2,337,321 subjects in China, covering 30 of the 34 autonomous regions of the country. The pooled prevalence of ASD based on clinical diagnostic criteria was 39.23 per 10,000, which is lower than in other countries worldwide. However, based on screening tools, the prevalence of ASD ranged from 33 per 10,000 to 1853.3 per 10,000 with the pooled figure of 429.07 per 10,000. This meta-analysis found that for children in the age group ≤4 years, the ASD prevalence was 530 per 10,000 in China.

Recently, Hossain et al.[10] published a systematic review on ASD prevalence in eight South Asian countries. The reported prevalence of ASD ranged from 0.09% in India (9 per 10,000) to 1.07% in Sri Lanka (107 per 10,000 or 1 in 93 children). An alarmingly high prevalence of 3% (300 per 10,000) was reported in Dhaka, the capital city of Bangladesh. The huge variation in ASD prevalence between the studies is probably due to the fact, that each survey has unique design features that reflect the local educational and health services infrastructure, and the studies rely on variable sampling methods, screening tools, diagnostic criteria, instruments, and methods. Nevertheless, these data show that the potential danger of ASD in F endemic areas exists.

The comparison of fluorosis pathological symptoms with ASD pathological symptoms supports the view that F might be an important environmental factor in the ASD pathogenesis.[11] Our very great concern is that children worldwide are being exposed to this toxic element which is eroding intelligence, disrupting behaviors, and damaging societies, both in the countries with endemic fluorosis and in those with fluoridated water.

FLUORIDE-INDUCED DAMAGE OF MITOCHONDRIA

F is a metabolic poison capable of altering several enzyme activities and cellular metabolism. The effect of F on energy metabolism has been studied in detail during the last century by biochemists. The use of F in laboratory investigations enabled the identification of the glycolytic and Krebs-cycle pathways.[8] Mitochondria play a vital role in energy metabolism. Whereas mitochondrial disorders are heterogeneous in their pathological expressions, all of them are characterized by impaired energy production, mitochondrial electron transport chain dysfunction, and generation of oxygen free radicals. Metabolic and mitochondrial defects may have toxic effects on brain cells, causing neuronal loss and altering the modulation of neurotransmission systems.[12] Depletion of cellular energy levels increases the sensitivity to excitotoxins, leading to cell death.

It has been pointed out that chronic fluorosis may result in abnormal mitochondrial dynamics. A reduced level of mitofusin-1 (Mfn1) protein and elevations of fission-1 (Fis1) and dynamin-related protein-1 (Drp1) were detected in the cortices of the brains of rats with chronic fluorosis.[13] In addition, the alternations of the mRNA manifestations encoding all of the three proteins were almost the same as the corresponding changes in the protein levels. Furthermore, the mitochondria were fragmented and redistributed away from the axons of the cortical neurons. An *in vitro* study showed that F induced dysregulated mitochondrial fission/fusion manifested by inhibited fission and accelerated fusion, accompanied by mitochondrial heterogeneity and dysfunction such as mitochondrial membrane potential loss and mitochondrial superoxide overproduction in SH-SY5Y cells.[14] The mitochondrial fission inhibition and the subsequent mitochondrial dysfunction may trigger defective autophagy and excessive apoptosis. Interestingly, the apoptosis induced by fluorosis has been recently focused on as a possible toxicological mechanism for the disease. Integrating mitochondrial fragmentation, the procedure of apoptosis associated with chronic fluorosis might be involved in the mitochondria-mediated pathway regulated by anti-apoptotic Bcl-2 families.[15]

F-associated mitochondriopathy and ultrastructural changes were observed in tissue samples from patients in an endemic fluorosis area and have been implicated in the pathogenesis of the F-caused injuries. Quadri et al[16] evaluated F-associated changes of mitochondria in renal cells of a group of 32 children with nephrotic syndrome, aged 4–12 years with a high urinary concentration of F (>1 mg/L). Various degrees of F-associated mitochondriopathy, including mitochondrial swelling, cristolysis, and rupture, were observed in the cells of human renal tubules in children with increased urinary F levels as compared with children with normal urinary F levels. Chronic F toxicity induced mitochondrial edema/swelling and rupture which may lead to renal cell death and renal impairment.

Several studies showed a frequent association of ASD and mitochondrial diseases.[12] Almost 50% of children with ASD may display peripheral markers of disturbances of mitochondrial energy metabolism. If such dysfunction is present at the time of infections or immunizations in young children, the added oxidative stress from immune activation on cellular energy metabolism is likely to be especially critical for the central nervous system, which is highly dependent on mitochondrial function.[17,18]

It has also been demonstrated that suppression of energy generation significantly increases sensitivity to excitotoxicity.[11] Excitotoxicity, as well as inflammation, stimulates the generation of nitric oxide by microglia and astrocytes, and this directly suppresses mitochondrial function. The mitochondria also act as calcium (Ca^{2+}) buffering systems, which, when disturbed, can not only increase excitotoxic sensitivity, but also, by altering essential Ca^{2+} waves, alter neuronal and glial migration. The combination of mitochondrial dysfunction, oxidative stress, Ca^{2+} dysregulation, and excitotoxicity, significantly increases the neuronal and glial sensitivity to F damage.[11]

FLUORIDE-INDUCED OXIDATIVE STRESS

Excessive F induces a high level of oxidative stress of the body. The imbalance between production and elimination of free radicals can induce a wide range of impairments. Oxidative stress, which can stimulate microglial activation and immunoexcitotoxicity, may lead to neurodevelopmental abnormalities in children.[19] Guan et al[20-22] suggested that reactive oxygen species (ROS) and lipid peroxidation products (LPP) may be important mediating factors for the pathogenesis of endemic fluorosis and the main mechanism in the damage to multiple organs or systems. This idea has been subsequently confirmed by a large number of researchers.[23-26] F exposure can reduce the cellular level of glutathione (GSH) and induce oxidative stress in liver, kidney, heart, spleen, and brain. GSH, a radical scavenger, is converted to oxidized glutathione (GSSG) through GSH peroxidase (GPx) and converted back to GSH by GSH reductase (GR). GSH can detoxify hydrogen peroxide (H_2O_2), preventing the formation of ROS and LPP (Figure1). The GSH redox system is important for reducing oxidative stress.

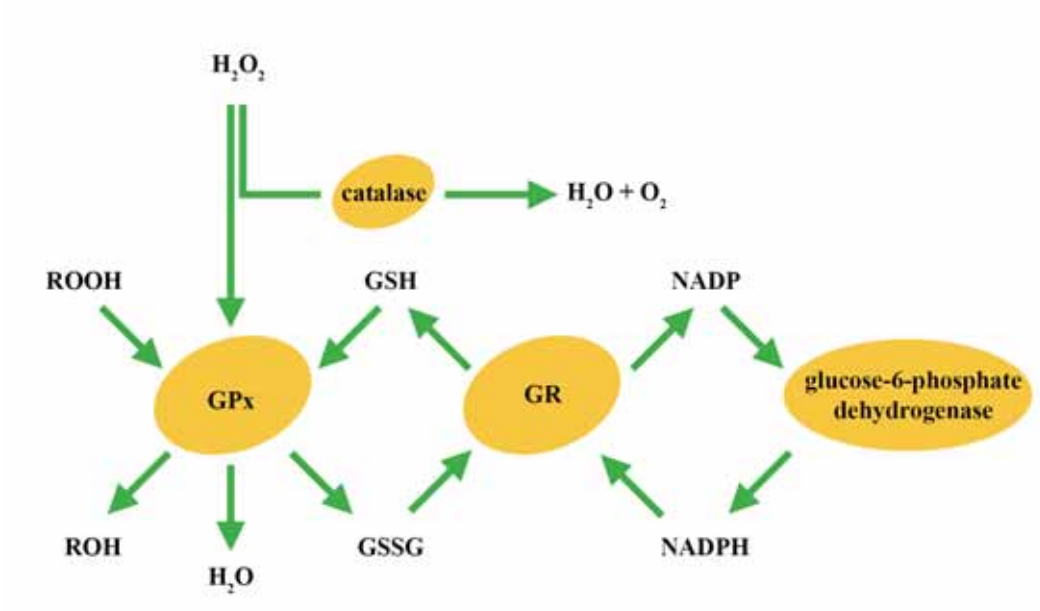

Figure 1. Hydrogen peroxide (H_2O_2), the immediate precursor of the hydroxyl radical, is metabolized via the action of catalase and glutathione peroxidase (GPx). GPx also metabolizes reactive hydroperoxides (ROOH) and oxidizes reduced glutathione (GSH) to its disulfide form (GSSH), which is recycled back to GSH by the action of glutathione reductase (GR). A cofactor for GR is NADPH, which is supplied by the action of glucose-6-phosphate dehydrogenase. F induces oxidative stress as described in the text and increases the activity of glucose-6-phosphate dehydrogenase.

A decreased GSH content and decreased GPx activity was found in the blood of the population living in areas with severe coal-burning endemic fluorosis.[20-22] The activity of superoxide dismutase, GPx, GR, catalase, and GSH transferase were significantly decreased in the blood plasma and erythrocytes of children and adults with intoxication by F.

Oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the oxidants, represents the link between the genetic, immunological, and environmental factors underlying ASD. A decrease in GSH is one of the best documented biochemical changes in ASD.[12] GSH and GSSG levels are significantly changed in autistic individuals, mainly as the consequence of disturbances in processes of transmethylation/transsulfuration.[27,28] Reduced levels of GSH greatly increase the sensitivity of neurons and astrocytes to excitotoxicity and oxidative stress. When combined with reduced mitochondrial energy production, low antioxidant enzymes, low GSH, and a reduced secretion of melatonin, an acceleration of damage to brain elements can be assumed.

Oxidative stress induces the secretion of many vasoactive and proinflammatory molecules, which leads to neuroinflammation. Inflammation in the brain has been reported by several groups in postmortem brain specimens of both young and old individuals with ASD. The initial reaction to inflammation may be activation of microglia.[11] F activates BV-2 microglial cells *in vitro*[29] by induction of oxidative stress. Once activated by F, microglia secrete large concentrations of IL-1ß and TNF-α, which recruit more microglial activation in a vicious cycle that ultimately leads to neurodegeneration. Chen et al.[30] found that F altered the inflammatory status and oxidative stress by inhibiting the Wnt signaling pathway in BV-2 microglial cells. Wnt signaling has been implicated in developmental processes, in axonal remodeling, cytoskeletal organization, and neuronal plasticity.

MELATONIN IN FLUOROSIS AND ASD

Melatonin is a hormone synthesized primarily in the pineal gland during the night. It is a biological signal of light/dark cycles and is considered as a major circadian synchronization system. Circulating melatonin regulates and influences the sleep wake cycle and sexual development, as well as various immune, endocrine, and metabolic functions. Melatonin administration has been used as an effective treatment of sleep disorders.

The human pineal gland avidly attracts F from the bloodstream because the gland calcifies physiologically (even in childhood) as hydroxyapatite.[31] Luke reported that Mongolian gerbils fed higher doses of F excreted less melatonin metabolite in their urine and took a shorter time to reach puberty. The pineal glands of children are now exposed to F at an earlier age and at higher levels. Healthy infants and prepubescent children have the highest nocturnal plasma melatonin concentrations.[31]

People in an endemic fluorosis area often complain about insomnia. A health survey of 2,691 subjects exposed to F in drinking water in villages of Sanganer Tehsil, India, revealed that 25% of adults suffer from insomnia.[32]

Around 30% of adults in the U.S.have some form of sleep disorder, insomnia being the most common one. While the excessive intake of F from fluoridated water has not been recognized as one of the factors contributing most to insomnia, 1.3% of adults

use melatonin. Melatonin also appears to be beneficial for its free-radical scavenging actions beyond its stimulatory effects on antioxidant enzyme systems. Melatonin increases GSH production by stimulating its synthesis. Several authors have reported its protective effect on F-induced oxidative stress.

Decreased levels of melatonin in blood or urine have been reported as a very frequent feature in individuals with ASD compared to typically developing controls.[11,12] Researchers estimate that 40–80% of children with ASD suffer from sleep disorders. Indian researchers found that nearly three fourths of children with ASD have sleep disorders with a possible effect on the behavioral phenotype.[33] Several studies have observed a correlation between the melatonin level and the severity of autistic behaviors. The babies with the lowest melatonin production had the most neurobehavioral problems.[34]

The disruption of the serotonin-N-acetyl serotonin-melatonin pathway (Figure 2) has been suggested as a biomarker for ASD. Pagan et al.[35] assessed plasma melatonin and whole-blood serotonin in 278 patients with ASD, their 506 first-degree relatives (129 unaffected siblings, 199 mothers and 178 fathers) and 416 sex- and age-matched controls. They confirmed a deficit in melatonin in 51% (45–57%) as well as hyperserotonemia in 40% (35–46%) of the ASD patients. Biochemical impairments were also observed in the first-degree relatives of the patients.

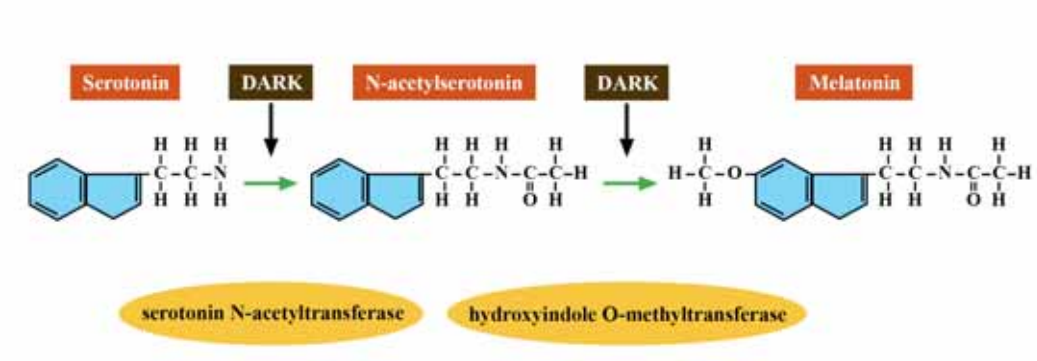

Figure 2. Serotonin is converted to melatonin through the action of two enzymes: serotonin N-acetyltransferase and hydroxyindole O-methyltransferase. F inhibits hydroxyindole O-methyltransferase.

The observed accumulation of N-acetylserotonin in the blood platelets of patients with ASD documents the earlier described inhibitory effect of F on hydroxyindole O-methyltransferase activity.[36] N-acetylserotonin was found to be elevated in 47% of individuals with ASD.[35]

Melatonin has been also recommended in the therapy of autistic persons. In addition, sleeping in a dark room without any permanent light is recommended.

FLUORIDE-INDUCED EFFECTS ON INTELLIGENCE

Studies documenting the neurotoxic effects of F first appeared in China. Du et al.[37] demonstrated that fetal brain is highly susceptible to F poisoning. An elevated F content was found in embryonic brain tissues obtained from required abortions in areas where endemic fluorosis was prevalent. These studies showed a poor differentiation of brain nerve cells and delayed brain development. Higher F concentrations in water have consistently been associated with lower levels of

children's intelligence. A meta-analysis of 16 studies carried out in China between 1998 and 2008 evaluated the influence of F levels on the intelligence quotient (IQ) of children. Tang et al.[38] concluded that children living in an area with a high fluorosis occurrence (and high F levels of ambient air) have five times higher odds of developing statistically lower IQ than those who live in a low F level area. This was further supported by results based on the meta-analysis of 27 studies published in the last 22 years.[39] The results from various geographic regions confirm that the IQ of children living in endemic fluorosis areas is lower than that of children living in low F areas.[7,40-43]

ASD was once considered to be highly associated with intellectual disability (IQ<70). Historically, the prevalence rate of intellectual disability in autistic individuals was around 70%.[44] However, according to the CDC estimates in 2014, only 31% of children with ASD were classified in the range of intellectual disability (IQ <70), 25% of them were in the borderline range (IQ: 71–85), and 44% had IQ scores in the average to above average range (i.e., IQ >85).[3] According to the facts and statistics on autism, 44–52% of autistic people may develop a learning disability. Around a third of people with learning disabilities (IQ <70) are also autistic.

CONCLUSIONS

Many studies by researchers in China and India brought the knowledge that endemic fluorosis induces extensive damage to the human body with the changes in multiple organs and systems. The prolonged exposure to F in the prenatal and postnatal stages of development affects health and intelligence, both in the countries with endemic fluorosis and in the countries with fluoridated water. It seems that the level of F intoxication from artificial water fluoridation is very low in comparison with that in areas with endemic fluorosis and that the millimolar concentrations of F needed for the biochemical effects on enzymes cannot be reached inside the cells of human body *in vivo*. Nevertheless, the serum F concentrations associated with high intakes from drinking water may exceed 50 µmol/L – more than 1,000 times the levels of some other neurotoxicants that cause neurodevelopmental damage.[45]

In addition, as already explained in the authors' previous papers,[46,47] Al^{3+} can amplify the effects of F. Aluminum, once relatively inaccessible in the biosphere, has become ubiquitous. The available literature on this topic clearly shows that the neurotoxicity of Al^{3+} is also reflected in symptoms such as impaired learning, memory, and concentration, speech disabilities, and altered behavior (i.e., confusion, anxiety, repetitive behaviors, and sleep disorders).[48] Given the dangers that Al^{3+} poses to the various organ systems, the exposure to this neurotoxin in food, water, cosmetics, and various medicinal products, including vaccines, should be limited. Al^{3+} plus F forms aluminofluoride complexes, which may mimic or potentiate the action of numerous extracellular signals and significantly affect many cellular responses. These complexes can exert their disrupting activities at several times lower concentrations than F or Al^{3+} acting alone.

Oxidative damage is the major mode of action of F. Melatonin has powerful neutralizing effects on ROS and LPP in the brain. Melatonin is a highly-efficient OH scavenger. Its neuroprotective effects, based on its antioxidant activity, has been observed *in vitro* in several types of cells and *in vivo* in rat's brain.[49,50] It is also an important modulator of mitochondrial metabolism, digestive functions, and

immunity. Melatonin may protect against F-induced oxidative stress in the brain through mechanisms involving the enhancement of the enzymatic and non-enzymatic antioxidant defense system.

The severity and the development of ASD symptoms also depends on genetic susceptibility factors, age, nutrition, immune status and infections, kidney function, tendency to allergies, and many other factors. Pesticides and fertilizers, heavy metals, excitotoxic proteins, and others might have synergistic effects with F in the human body.[51,52] The heterogeneity of their mutual dynamic interactions can explain the clinically heterogeneous symptoms of ASD and contribute to understanding the various responses of any child to the identical environmental neurotoxins, since it is known that each case of autism is unique.

The comparison of fluorosis pathological symptoms with ASD pathological symptoms indicates F as a potential key environmental factor contributing to ASD pathogenesis. With F exposure being so common, it is no longer possible to ignore its potential consequences for human health.[53-56]

While the countries in Asia are trying to control and eliminate F exposure in endemic fluorosis areas, the U.S. CDC advocates that roughly 276 million Americans consume fluoridated drinking water as a safe way of preventing tooth decay. On the contrary, 97% of the European population has been drinking non-fluoridated tap water for several decades. Unfortunately, there is no central recording of ASD cases in any EU Member State. The age specific prevalence rates for autism in the EU can be estimated as varying from 3.3 to 16.0 per 10,000 and it is still considered as a rare disease.[57]

Both ASD and fluorosis are associated with significant functional impairments and long-term health, social, and financial costs for individuals, their families, and society. The CDC informs us that the total costs per year for children with ASD in the U.S. were estimated to be 11.5 – 60.9 billion USD in 2011, including a variety of direct and indirect costs, from medical care, to special education, and to lost parental productivity.[3] In 2005, the average annual medical costs for Medicaid-enrolled children with ASD were 10,709 USD per child, which was about six times higher than the costs for children without ASD. In addition to medical costs, intensive behavioral interventions for children with ASD cost 40,000 to 60,000 USD per child per year. Autism Speaks, the world's leading autism science and advocacy organization, announced the results of new research that estimates autism cost society a staggering 126 billion USD per year in 2012 – a number that has significantly increased since the year 2005. According to the estimates of the Autism Society, the estimated annual cost of autism services, including the direct medical, direct non-medical, and productivity losses due to ASD, in the U.S. in 2015 was around 268 billion USD.[58] It is estimated that this figure will increase to about 461 billion USD by the year 2025.

The escalating increase of ASD has led to a public health crisis of historic proportions. It is devastating a generation of children and their families. The increasing prevalence of ASD since 1990s probably reflects the burden of the synergistic action of several new ecotoxicological factors. Thus, there have been several studies indicating that F is a ubiquitous environmental and food-derived neurotoxin which can exacerbate the pathological and clinical problems of ASD.

CONFLICT OF INTEREST DECLARATION

The authors declare that there is no conflict of interest by any of the authors.

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