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Increased excretion of a lipid peroxidation biomarker in autism

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Abstract

It is thought that autism could result from an interaction between genetic and environmental factors with oxidative stress as a potential mechanism linking the two. One genetic factor may be altered oxidative–reductive capacity. This study tested the hypothesis that children with autism have increased oxidative stress. We evaluated children with autism for the presence of two oxidative stress biomarkers. Urinary excretion of 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-isoprostane-F2α (8-iso-PGF2α) were determined in 33 children with autism and 29 healthy controls. 8-iso-PGF2α levels were significantly higher in children with autism. The isoprostane levels in autistic subjects were variable with a bimodal distribution. The majority of autistic subjects showed a moderate increase in isoprostane levels while a smaller group of autistic children showed dramatic increases in their isoprostane levels. There was a trend of an increase in 8-OHdG levels in children with autism but it did not reach statistical significance. There was no significant correlation between the levels of the biomarkers and vitamin intake, dietary supplements, medicine, medical disorders, or history of regression. These results suggest that the lipid peroxidation biomarker is increased in this cohort of autistic children, especially in the subgroup of autistic children.

1. Introduction

Oxidative stress has been implicated in the etiology of neurological, neurodevelopmental, and neuropsychiatric disorders including Parkinson's and Alzheimer's diseases, Down's syndrome, and autism. With respect to autism, the notion of enhanced oxidative stress has been derived from several lines of evidence. First, an increased excretion of oxidative stress biomarkers has been reported. Specifically, nitric oxide, a free radical that can block energy production was found to be increased in autism as compared to age- and sex-matched controls [1]. In addition, elevated nitrite concentrations have been detected in autistic individuals [2] along with thiobarbituric acid reactive substances and xanthine oxidase activity in red blood cells [3]. The elevation of these substances indicates excess free radicals in individuals with autism compared to controls. Consistent with the increased oxidative stress biomarkers, children with autism were found to have increased body burdens of environmental toxins that may generate oxidative stress [4,5].

A second line of evidence that oxidative stress may play a role in autism is suggested by a reduced endogenous antioxidant capacity. Specifically, altered glutathione peroxidase (GPX) [1,6,7], superoxide dismutase (SOD) [6,7] and catalase [3] activities as well as total GSH, and GSH/GSSG and cysteine levels [8] were found in autistic individuals compared to controls.

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Likewise, levels of exogenous antioxidants were also found to be reduced in autism, including vitamin C, vitamin E, and vitamin A in plasma and zinc and selenium in erythrocytes [8].

A third indicator of altered oxidative stress in autism is derived from evidence of impaired energy metabolism. Magnetic resonance spectroscopic study of the brains of individuals with autism showed reduced synthesis of ATP [9]. In addition, higher lactate [10,11] and pyruvate [12] levels in autism may suggest mitochondrial dysfunction in autism [13,14]. One major cause of mitochondria dysfunction is a result of oxidative injury [15].

Finally, improvement in behaviors following antioxidant administration to individuals with autism suggests that oxidative stress may be important in contributing to the etiology of autism. In double-blind, placebo-controlled trials, high-dose vitamin C [16] or carnosine [17] improved the behavior of individuals with autism over baseline observations. Likewise, a 3-week supplement of betaine and folinic acid to 20 children with autism who had lower levels of GSH, GSH/GSSG, cysteine improved their blood levels of the antioxidants [8]. Furthermore, melatonin (an effective $NO^{\bullet}$ and $ONOO^{-}$ scavenger [18] with potential for reducing oxidative stress in both brain and gut [19,20] and increasing expression of GPX [21]) was shown to be effective in the treatment of sleep disorders in autism [22]. Taken together, these lines of evidence support the hypothesis that at least some children with autism exhibit enhanced oxidative stress.

In this study, we determined urinary excretion of 8-isoprostane (8-iso-$PGF_{2\alpha}$), a lipid peroxidation biomarker, and the biomarker of DNA hydroxylation 8-hydroxy-2-deoxyguanosine (8-OHdG), in children with autism and age-matched controls. 8-OHdG is the product of free radical attack on DNA bound guanosine and hence a marker for oxidative damage to DNA. 8-OHdG is the most abundant oxidative product of cellular DNA oxidation [23,24] and is also a potent mutagen [23–27].

Here we report the first observation that the level of one oxidative stress biomarker was significantly increased in a cohort of individuals with autism.

2. Method and materials

2.1. Participants

Thirty-three children with autism were recruited from the UMDNJ Autism Center, and 29 healthy controls were recruited from the Pediatric Ambulatory Center, New Jersey Medical School, UMDNJ, Newark, NJ. Assent and parental consent for these studies was obtained as approved by the Institutional Review Board of the UMDNJ-New Jersey Medical School. The diagnosis of autism was confirmed by Autism Diagnostic Interview-Revised [28], Autism Diagnostic Observation Schedule-Generic [29], or DSM IV [30] criteria. Children with autism whose diagnosis of autism is known (“double syndrome”) were excluded from this study. All subjects, patients of us (XM), were carefully screened for signs of infection or inter-current illness on the day of specimen acquisition, and subjects with acute illness were excluded. The dietary intake history within 24 h of sampling was recorded by parents and confirmed by the investigator (XM), including that of medication, vitamin and/or dietary supplement intake. In addition, medical history and co-morbidity data were collected in the individuals with autism.

2.2. Sample collection

Single spot urine samples were collected between 10:00AM and 4:00PM. Urine specimens were immediately frozen on dry ice and transferred to an $-80\,^{\circ}C$ freezer until assay. The storage time was less than two months, which was within the range of safe storage time for the biomarkers [31].

2.3. Biochemical assays

The determination of urinary 8-iso-$PGF_{2\alpha}$ and 8-OHdG were performed with the laboratory investigators blinded to the identities of the subjects. We used commercially available ELISA kits for both biomarkers (8-iso-$PGF_{2\alpha}$: Oxford Biochemicals, Midland, MI; 8-OHdG: Genox Corporation, Baltimore, MD). Duplicates of each sample were performed with the standards in the same 96-well plates. The results were normalized to urinary creatinine. Creatinine was measured by the picric acid method as modified for a microplate reader using a kit marketed by Sigma Chemical Co. (St. Louis, MO) as previously described [32].

2.4. Statistical analyses

Student’s *t*-tests were used to compare differences across the various sub-groups. Log-scale transformation was used to normalize the distribution. Where appropriate correlation and linear regression methods were employed to examine relationships among continuous variables. Contingency table analysis and associated chi-square tests were used to examine associations among qualitative variables.

3. Results

As shown in Fig. 1, 8-iso-$PGF_{2\alpha}$ levels were significantly higher in children with autism (autism group: 32.92 ± 1.98 ng creat^{-1} M; controls: 5.71 ± 0.98 ng

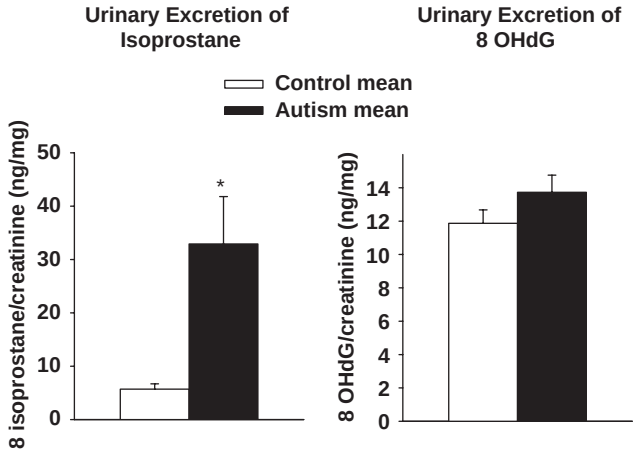

Fig. 1. Urinary excretion of isoprostane and 8-OHdG in children with autism and controls. *$P<0.05$, Student's t-test.

Table 1
Clinical characteristics of participants

Subjects	Control	Autism
Age (yr)	5–16	4–17
Sex	12 F, 17 M	4 F, 29 M
Medications	0	16[a]
Vitamins[b]	11	11
Supplement[c]	0	4

[a]The medicines consumed by these children with autism during sampling were Lamotrigine, Pimozide, Guanfacine, Topiramate, Risperidone, Valproate, Sertraline, Clonidine, Methylphenidate and Gabapentin.

[b]The vitamins used by the participants were generic children's multivitamins.

[c]The dietary supplements used by children with autism were fish oil ($n = 1$), melatonin ($n = 2$), probiotics ($n = 2$).

Table 2
Clinical co-morbidity of individuals with autism

Clinical features	Number of subjects (%)
Epilepsy	7 (22%)
GI disorders	11 (34%)
Sleep disorders	14 (44%)
Other	8 (0%)

None of the control children had any of the above disorders.

creat^{-1} M). Log scale was used to transform to the normal distribution and Student's t-test showed $P = 0.007$. Levels of 8-iso-PGF$_{2\alpha}$ among children with autism showed a greater variability than those of controls (autism SE: 1.98, Controls SE: 0.98). Eight of autistic children had isoprostane values greater than 2SD above the mean of the control group. Five of these children had isoprostane levels ranging from 5- to 46-fold above the control mean (one had his isoprostane level of 46-fold greater and another, 25-fold greater than the control mean). The second group (25 children with autism) had isoprostane levels within 2SD of the control mean.

To examine this group distribution more carefully, we grouped the autism cohort as high or low in terms of isoprostane value (cutoff defined as 2SD above the control mean) and cross-tabulated these with controls stratified along the same cutoff. This gave a significant association (chi-square test, P-value $= 0.02$) between autism and isoprostane values above the cutoff.

There was a trend of an increase in 8-OHdG levels in children with autism but it did not reach statistical significance (autism group: 13.73 ± 1.03 ng creat^{-1} M; controls: 11.87 ± 0.81 ng creat^{-1} M, $P = 0.08$, Student's t-test, Fig. 1). Within the children with autism, a comparison of 8-OHdG level was made between the children with autism whose isoprostane levels were 2 SD above the control mean and the rest of the children with autism (mean 8-OHdG: 14.11 for the former vs. 14.58 for the latter). There is no significant difference of 8-OHdG levels between these two subgroups of autism.

The vitamin intake and dietary history were also recorded in the healthy control children. Tables 1 and 2 summarize the subjects' profiles. Overall there were no differences between the cases and the controls.

Dietary records of the children in this study showed no significant qualitative differences in the type of dietary intake between the controls and the autistic children. Likewise, with respect to vitamin intake, 11 children with autism and 11 controls were taking multivitamins at the time of specimen acquisition. No dietary supplements were noted in the control children while four children with autism were noted to be taking supplements including fish oil ($n = 1$), melatonin ($n = 2$), or probiotics ($n = 2$). One child with autism took multivitamins and multiple supplements. This child's isoprostane level was close to the control mean. Nineteen children with autism did not take either vitamins or supplements. Analysis of vitamin or supplement intake as potential confounding factors contributing the high values of isoprostane levels in children with autism showed no significant difference ($P = 0.681$, Student's t-test).

The medications taken by the subjects in the autism group included Lamotrigine, Pimozide, Guanfacine, Topiramate, Risperidone, Valproate, Sertraline, Clonidine, Methylphenidate and Gabapentin. Analysis of medication intake as a whole against those autistic children who did not take medicine showed no significant association with isoprostane levels in autistic children ($P = 0.284$, Student's t-test). Three children were on Valproate and their isoprostane levels were 6.95, 4.05 and 27.02 ng creat^{-1} M, respectively.

The co-morbid medical disorders in this cohort of autistic children include epilepsy ($n = 5$), GI disorder ($n = 12$), sleep disorder ($n = 12$), and other disorders such as allergy, food intolerance or Tourette's syndrome ($n = 8$). Seven children with autism exhibited no history of medical co-morbidity. Grouping all medical co-morbidity as a whole, analysis of isoprostane levels in autistic children with and without medical co-morbidity showed no significant difference ($P = 0.15$, Student's *t*-test).

Analysis of developmental regression in children with autism against isoprostane levels showed no significant difference between autistic children with and without regression ($P = 0.226$, Student's *t*-test).

4. Discussion

4.1. Experimental assays

Urine is the preferred body fluid for isoprostane and 8-OHdG analyses [23,33]. Both isoprostane and 8-OHdG assays in plasma (isoprostane) or blood cells (DNA, e.g. lymphocytes) are technically difficult being subject to auto-oxidation, there are many interfering agents and the sample work-up for analysis often introduces artifacts [34,35]. If the number of blood samples collected is small and their timing varied, there will be considerable noise in the data. In contrast, urine contains no oxidizable arachidonic acid and therefore there is no need for concern with ex vivo isoprostane generation [23,33]. Urine samples are stable for at least a year when stored at –20 °C. In fact 8-iso-$PGF_{2\alpha}$ is stable in urine for a week at 37 °C [31]. In a recent review, Awad stated 'Urinary isoprostane production is a more practical method for quantifying isoprostane production, in addition to providing a time integrated assessment of the 'oxidative state' of the patient' [23] and the same applies to 8-OHdG [36–38]. Therefore we chose urine as the specimen of choice in this study and we minimized experimental artifacts.

4.2. Potential confounding factors

It is well known that dietary supplements, vitamins, medicines and medical disorders can affect oxidative stress [39]. We have comparable number of children in both groups taking vitamins while urine specimens were acquired. Our study showed that vitamin or dietary supplement did not contribute to the higher levels of isoprostane in children with autism.

Some medications such as valproic acid are associated with increased lipid peroxidation [40–42]. The isoprostane levels in the three autistic subjects who were taking valproic acid suggesting no consistent impact of valproic acid on isoprostane levels in these children. No consistent effects of medication in contributing to the higher isoprostane levels in the autism group were found (see result). However further studies of recruiting medication naïve subjects will elucidate the impact of medication on isoprostane levels.

Likewise, medical disorders such as epilepsy, allergy and inflammation are associated with increased oxidative stress [43]. Similarly we failed to find an association of medical disorders with the increased lipid peroxidation.

Many children with autism exhibit regression during their course of development [44]. Unlike Chauhan et al. [45], we did not find that history of developmental regression is a risk factor for increased lipid peroxidation (see result). However, this could result from the different cohort of autistic children, specimen differences (urine vs. plasma) or use of different biomarkers of lipid peroxidation (isoprostane vs. malondiadlehyde), as well as different criteria for developmental regression. Future studies will control dietary, vitamin, medication and other factors to confirm the inherent enhanced oxidative stress in autism.

4.3. The increased susceptibility in a subgroup of children with autism

This is the first report of increased urinary isoprostane levels in a subgroup of children with autism. Our study is in agreement with the report of Chauhan et al. [45] that lipid peroxidation is increased in a subgroup of children with autism. We believe that urine is a preferred specimen than plasma to represent "whole body". We did not identify any confounding factor associated with the increased isoprostane levels in this cohort of autistic children. Our study showed that a subgroup of children with autism have a dramatically increased lipid peroxidation. This subgroup of children with autism may have increased susceptibility to oxidative stress, possibly caused by a genetic factor. However, this subgroup of children with autism remains to be further defined in terms of other phenotypic, genotypic or biological traits. The observation of elevated oxidative stress biomarkers in autism may help shed light on the underlying etiology and/or the identification of therapeutic or preventative strategies.

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