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ORIGINAL PAPER

Oxidative Stress in Adults with Autism Spectrum Disorder: A Case Control Study

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Abstract

Oxidative stress has been proposed as being important in the pathophysiology of autism spectrum disorders (ASD), and heightened levels of oxidative stress has found in children with ASD. Our aim was to investigate, whether this change is temporary or persist into adulthood. We included 89 adult patients with ASD and sex and age matched controls. Plasma levels of antioxidants superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) and pro-oxidant xanthine oxidase (XO) were measured. Individuals with ASD had higher levels of SOD1, which furthermore correlated with autism severity as measured by autism quotient-score. We found no difference regarding SOD2 and XO between ASD group and controls. However, SOD1 and SOD2 were elevated in males compared to females.

Keywords Autism Spectrum Disorder · Superoxide Dismutase · Xanthine Oxidase · Oxidative Stress · Sex

Despite many years of research, the cause of autism remains uncertain (Constantino and Charman 2016). A genetic component is evident with concordance rates around 95% among monozygotic twins compared to 7% among dizygotic (Tick et al. 2016). Likewise, more than 100 genetic loci have been associated with the development of ASD (Nickl-Jockschat and Michel 2011). However, newer studies have increasingly focused on the role of environmental factors and epigenetics in the development of ASD in the search for valid biomarkers for the disorder (Rossignol and Frye 2012). A complex interrelation between genetic predisposition and environmental exposure, resulting in oxidative stress, has been proposed to be involved in early neurodegenerative processes, leading to ASD (Kern et al. 2013). These processes include mitochondrial dysfunction, abnormal methylation cycle and transsulfuration pathway, all of which are involved in the regulation of oxidative stress, and are affected by abnormal levels of oxidative stress. (Deth et al. 2008; Li et al. 2013; Reik and Dean 2001).

Oxidative stress is caused by an imbalance of pro-oxidative and anti-oxidative substances resulting in increased production of free radicals. Free radicals lead to cell damage through lipid peroxidation, DNA strand breaks and, eventually, cell death which have also been reported in patients with ASD (Cortelazzo et al. 2016). One important class of oxidative defense mechanisms is represented by the antioxidant enzymes copper/zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2), which are extraordinarily efficient at catalyzing the dismutation of the superoxide radical into less toxic substances such as hydrogen peroxide (H_2O_2) and oxygen (Fridovich 1978). Conversely, xanthine oxidase is a pro-oxidant enzyme which generates the reactive oxygen species superoxide, the substrate of superoxide dismutase (Harrison 2002).

The significance of these pathways in the etiology of ASD is however still not well understood. Single nucleotide polymorphisms in the SOD gene are associated with ASD

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(Kovač et al. 2014). Regarding SOD activity in plasma and erythrocytes, studies have been heterogenous in design and with conflicting results (Frustaci et al. 2012). However, the concentration and differentiation between the isoforms of SOD in plasma have not been investigated so far. The role of xanthine oxidase in ASD is not well studied, but one study reported increased activity in erythrocytes of patients with ASD (Zoroglu et al. 2004). Furthermore, the studies so far in ASD have focused on children. It is not known whether oxidative stress in ASD is a temporary finding postnatal and in childhood, or a persistent trait marker (Frustaci et al. 2012). We therefore aimed to investigate the levels of classical pro- and antioxidants, the pro-oxidant XO and two isoforms of the antioxidant SOD in adults with ASD.

Methods and Materials

Subjects

The ASD patients group consists of two cohorts: Firstly, a cohort of all patients diagnosed as children at the Department of Child and Adolescents Mental Health in Odense in Region of Southern Denmark, Denmark (the Funen cohort), and secondly, a cohort of patients diagnosed as adults at the Aachen University Hospital in Germany (Aachen cohort). The latter is partly described in another paper (Michel et al. 2010).

The patients diagnosed as children were contacted by letter and asked for their consent to participate. If no answer had been received after 2 months, a second invitational letter was sent. Patients were asked to reply with consent of participation, along with a completed Autism Quotient questionnaire (AQ). Healthy controls were recruited using posters at local schools, a homepage and through social media.

The patients diagnosed as adults were recruited via a specialist clinic for adults with ASD. Patients went into the diagnostic services either through a self-referral, referral by their general practitioner, private practicing psychiatrist, neurologist or other hospitals for assessment with the expert team at the Department for Psychiatry at the Rheinisch Westfaelische Technische Hochschule (RWTH), Aachen University Hospital. They were independently given information about the study and gave informed consent.

All patients included were ≥ 18 years of age and had an ICD-10 diagnosis of childhood autism (F84.0), atypical autism (F84.1), Asperger syndrome (F84.5) or pervasive developmental disorder, not otherwise specified (F84.8). The healthy controls were matched for sex and age respectively.

Demographic Data

Sex, age, diagnosis, Autism Diagnostic Observation Schedule (ADOS) score, Autism Quotient questionnaire (AQ) score, intelligence quotient (IQ) and use of psychopharmacological drugs (antipsychotics, antidepressants, anti-epileptica and central stimulants) were included.

Psychometric Data

ADOS (Autism Diagnostic Observation Schedule)

We have carried out the standardized protocol for observation and scoring of social and communicative disturbances (ADOS) seen in ASD, which poses the current gold standard observational test for assessment of ASD (Lord et al. 1989).

AQ (Autism Quotient)

The AQ consists of 50 statements, in which the participant has to mark either "definitely agree", "slightly agree", "slightly disagree" or "definitely disagree" (Baron-Cohen et al. 2001). An answer to a statement can give either 0 or 1 point, making 50 points the maximum achievable score (Baron-Cohen et al. 2001). A score ≥ 32 is considered clinically significant for autistic traits (Baron-Cohen et al. 2001).

IQ (Intelligence Quotient)

The cognitive assessment used was either the Wechsler Adult Intelligence Scale (WAIS) or Wechsler Intelligence Scale for Children (WISC) respective of patients age of diagnosis childhood.

Biological sample

Venous blood samples were collected at the Research Unit at the Department of Psychiatry Odense, Denmark and at the Aachen University Hospital. All blood samples were taken between 8:00–11:30 in the morning. Plasma was collected in a 10.0 ml k2EDTA tube, inverted 8–10 times and immediately refrigerated (5 °C). Time to centrifugation was a maximum of 2.5 h, at which the samples were centrifuged at room temperature (23 °C) at 2000 G for 10 min. Samples were separated into 400 µl aliquots in 2 ml Sarstedt tubes, and stored at −80 °C until analysis. Included samples had gone through no more than one freeze-thaw cycle.

Biological Measures

Plasma levels of Cu/Zn and Mn-superoxide dismutase were analyzed using enzyme-linked immunosorbent assay technique (ELISA). We used Cu/ZnSOD (Human) ELISA Kit (Abnova, Taipei City, Taiwan), Mn-SOD (Human) ELISA kit (Abnova, Taipei City, Taiwan) and Human XDH / Xanthine Oxidase ELISA Kit (Sandwich ELISA) - LS-F6180 (Nordic BioSite, Täby, Sweden). Analyses were carried out in collaboration with the Danish State Serum Institute (SSI).

Statistical Analysis

Wilcoxon rank-sum test was used to analyze group differences in continuous data. Chi-square test was used to analyze binary data. Linear regression was used to test for correlation between continuous data. In all calculations, p values <0.05 were considered significant and p values <0.01 highly significant. Stata 14.2 was used for all statistical analyses.

Ethical Approval

All participants gave informed consent to participate. The study was carried out according to the 2nd Helsinki Declaration. It has been approved by the Regional Committees on Health Research Ethics for Southern Denmark (no. S-20150070) and Aachen University (EK 172/08) respectively and the Danish Data Protection Agency (no. 15/39055).

Role of the Funding Source

The funder of this study had no influence on design, on collection, analysis and interpretation of data, on writing the report or on the decision to submit the paper for publication. The authors had full access to all data in the study and had final responsibility for the decision to submit the paper for publication.

Results

In the group diagnosed as children 605 children had received a diagnosis of ASD from 1994 to June 2015, of which 334 were 18 years or above. Of these, 68 patients gave their informed consent to participate in the study.

In the group diagnosed as adult 139 referred themselves to the clinic, 77 of which received a diagnosis of ASD. Informed consent was given by 40, of which 21 had their blood analyzed.

In total, we included 68 patients diagnosed as children and 21 diagnosed as adult, resulting in a total number of 89 patients, along with 96 sex and age matched healthy controls (Table 1).

Patients with ASD showed significantly higher mean plasma levels of SOD1 compared to controls (274.0 vs.

Table 1 Data from ASD and control group

Data from ASD and control group	ASD $N=89$	Controls $N=96$	P Value
MALE, NO. (%)	69 (77.5)	67 (69.8)	0.23
AGE, YEARS, MEAN (S.E.)	25.9 (0.8)	26.1 (0.8)	0.96
AQ-SCORE, MEAN (S.E.) – *(84 CASES, 67 CONTROL)*	28.4 (1.0)	10.9 (0.7)	<0.0001**
ADOS, MEAN (S.E.) – *(28 CASES)*	10.5 (0.9)		
IQ, MEAN (S.E.) – *(40 CASES)*	99.8 (4.3)		
DIAGNOSIS			
CHILDHOOD AUTISM, NO. (%)	17 (19.1)		
ATYPICAL AUTISM, NO. (%)	10 (11.2)		
ASPERGER SYNDROME, NO. (%)	30 (33.7)		
PERVASIVE DEVELOPMENTAL DISORDER, NOT OTHERWISE SPECIFIED, NO. (%)	24 (27.0)		
UNCERTAIN SUBTYPE, NO. (%)	8 (9.0)		
PSYCHOPHARMACOLOGICAL TREATMENT			
NONE, NO. (%)	49 (55.1)	66 (68.8)	
ANTIPSYCHOTICS, NO. (%)	5 (5.6)	0 (0.0)	
ANTIDEPRESSANTS, NO. (%)	13 (14.6)	1 (68.8)	
ANTICONVULSANTS, NO. (%)	2 (2.2)	0 (0.0)	
CENTRAL STIMULANTS, NO. (%)	3 (3.4)	0 (0.0)	
MISSING, NO. (%)	21 (23.6)	29 (31.2)	

Data from ASD and control group. S.E. = Standard Error, % = Percent. * = $p<0.05$, ** = $p<0.01$

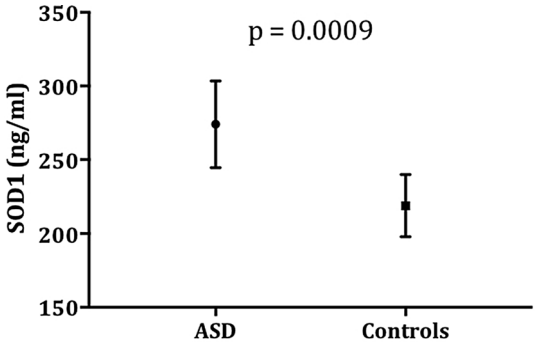

Fig. 1 Mean plasma level of SOD1

218.9 ng/ml, $p = 0.0009$, fig. 1). This was still significant if excluding participants in pharmacological treatment with antipsychotics, antidepressants, anti-epileptica and/or central stimulants along with participants with missing information (40 cases and 30 controls dropped) (297.5 vs. 229.4 ng/ml, $p = 0.0004$). Overall, sex showed strong influence on both SOD1 and SOD2, while the sex differences for XO just reached statistical significance (see Table 2). Stratified analyses showed that males with ASD had highly significant elevated plasma SOD1 compared to male controls (288.8 vs. 234.1 ng/ml, $p = 0.0012$), while females with ASD did not show higher plasma SOD1 compared to female controls (see Table 2).

Furthermore, SOD1 showed a positive correlation with the AQ-score (SOD1 increasing by 2.40539 ng/ml per one point increase in AQ-score, R-Squared = 0.0496, $p = 0.0060$, fig. 2).

SOD1 did not correlate with neither ADOS score nor IQ (see Table 3). No case vs. control significant differences in mean plasma levels of SOD2 and XO were found (Table 2).

Discussion

We showed that adults with ASD have a highly significant higher plasma concentration of the antioxidant SOD1, which persisted when dropping measurements from individuals in psychopharmacological treatment. In addition, SOD1 levels correlated positively with autism severity, as measured by AQ-score. Males generally had higher plasma SOD1 and

Table 2 Plasma concentration with stratifications

	N	First Group	N	Second Group	Z-Value	P Value
ASD VS. CONTROLS		**ASD**		**Controls**		
SOD1, NG/ML, MEAN (S.E.)	89	274.0 (14.8)	96	218.9 (10.6)	2.782	0.0009***
SOD2, NG/ML, MEAN (S.E.)	89	97.67 (4.58)	96	95.23 (5.13)	1.611	0.15
XO, NG/ML, MEAN (S.E.)	89	2.221 (0.541)	96	1.577 (0.311)	1.762	0.22
NO MEDICINE USAGE		**ASD**		**Control**		
SOD1, NG/ML, MEAN (S.E.)	49	297.5 (18.4)	66	229.4 (12.6)	3.524	0.0004***
SOD2, NG/ML, MEAN (S.E.)	49	107.09 (7.02)	66	99.43 (6.30)	1.459	0.14
XO, NG/ML, MEAN (S.E.)	49	1.681 (0.344)	66	1.863 (0.448)	0.376	0.71
MALE VS. FEMALE		**Male**		**Female**		
SOD1, NG/ML, MEAN (S.E.)	136	261.8 (10.0)	49	199.8 (19.7)	4.475	<0.0001***
SOD2, NG/ML, MEAN (S.E.)	136	101.69 (4.04)	49	81.74 (6.19)	2.802	0.0008***
XO, NG/ML, MEAN (S.E.)	136	2.122 (0.414)	49	1.233 (0.098)	1.201	0.05
MALE ASD VS. MALE CONTROL		**ASD**		**Control**		
SOD1, NG/ML, MEAN (S.E.)	69	288.8 (14.9)	67	234.1 (12.4)	2.980	0.0012***
SOD2, NG/ML, MEAN (S.E.)	69	101.56 (5.14)	67	101.81 (6.31)	0.896	0.31
XO, NG/ML, MEAN (S.E.)	69	2.462 (0.693)	67	1.772 (0.443)	0.954	0.40
FEMALE ASD VS FEMALE CONTROL		**ASD**		**Control**		
SOD1, NG/ML, MEAN (S.E.)	20	223.3 (40.0)	29	183.6 (18.8)	0.181	0.46
SOD2, NG/ML, MEAN(S.E.)	20	84.22 (9.63)	29	80.03 (8.20)	1.293	0.54
XO, NG/ML, MEAN (S.E.)	20	1.390 (0.213)	29	1.124 (0.076)	1.426	0.84
TIME OF DIAGNOSIS		**Childhood**		**Adulthood**		
SOD1, NG/ML, MEAN (S.E.)	68	292.5 (17.4)	21	214.3 (23.7)	2.988	0.01*
SOD2, NG/ML, MEAN (S.E.)	68	101.63 (5.57)	21	84.82 (6.57)	0.347	0.30
XO, NG/ML, MEAN (S.E.)	68	2.131 (0.564)	21	2.513 (1.411)	1.063	0.01*

Mean plasma concentration of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and xanthineoxidase (XO) in ASD and control group along with stratification analyses. S.E. = Standard Error. * = p < 0.05, ** = p < 0.01, ***$p < 0.0028$ (Bonferroni correction)

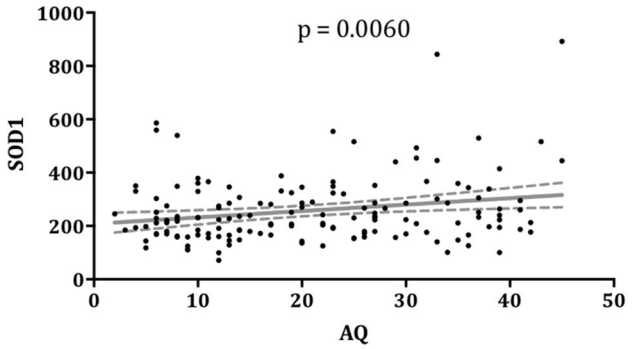

Fig. 2 Linear correlation of SOD1 and AQ-score

SOD2 while XO just reached significance. Previous studies have suggested that an increase in antioxidants might be due to a compensatory up-regulation of this protective enzyme as a response to an increased exposure to oxidative stress and free radicals (Michel et al. 2007). In rats, SOD released by microglial cells has been shown to grant neuroprotection (Polazzi et al. 2013). The result could therefore indicate that the exposure to oxidative stress is not temporary, but persists into adulthood and is correlated with the lifelong symptoms of ASD (Baxter et al. 2015).

When comparing our findings on the levels of the two isoforms of SOD to previous studies, one has to interpret the different results carefully, since earlier studies have shown various results regarding the isoforms of SOD (Frustaci et al. 2012). A meta-analysis found no difference in activity of plasma SOD in autistic individuals versus controls, but also emphasized that differences in assay methods made a comparison difficult (Frustaci et al. 2012). The main part of earlier studies have focused on activity of SOD not concentration like in our study, making a direct comparison with our study difficult. Higher concentration does not necessarily mean higher activity and vice versa. Genetic alterations in the SOD1 gene have been shown, which theoretically could influence the specific activity of the enzyme (Kovač et al. 2014). A change in the concentration of SOD1 could be a regulatory mechanism due to alteration of its specific activity properties. Furthermore, SOD1 can show pro-oxidant activity under certain circumstances (Halliwell and Gutteridge 2007). One of the main biproducts of the detoxification of superoxide by SOD is H_2O_2, which can react and damage cell walls and DNA (Fridovich 1978). Some cell types, for example oligodendrocytes and myelin are especially vulnerable for hydrogen peroxide induced damage (Fridovich 1978). In mice, overexpression of SOD1 has resulted in abnormal neuromuscular junction, altered serotonin metabolism, increased angiogenesis in response to growth factors and neurological defects characteristic to Down's syndrome (Halliwell and Gutteridge 2007). H_2O_2 is also the substrate of glutathione peroxidase (GPx), which in the process oxidizes two reduced glutathione (GSH) to reduced glutathione (GSSG). A reduced GSH to GSSG ratio has been associated to oxidative damage in the autistic brain compared to controls (Rose et al. 2012). It has even been proposed as being diagnostic of ASD (Howsmon et al. 2017). The heightened level of SOD found in the present study, could lead to a higher level of hydrogen peroxide, which would skew the GSH/GSSG ratio.

In contrast to earlier studies this study differentiated between the isoforms of SOD making our results more specific compared to studies measuring SOD1 and SOD2 cumulatively. This is especially relevant if only one of the enzymes is significantly affected, as in our study (Frustaci et al. 2012). Only one previous study has investigated the concentration of SOD, and found lower serum levels in Chinese children with ASD compared to healthy controls. However, the specific isoform of the measured enzyme is not stated (Yui et al. 2017). Furthermore, only one other study has included adults and did not show any difference in SOD1 activity in erythrocytes. The study had a relatively small sample size (13 cases) and may therefore have lacked statistical power to detect a difference (Torsdottir et al. 2005).

Regarding xanthine oxidase, one study found increased erythrocyte activity in children with ASD (Zoroglu et al. 2004). However, in our study, we did not find any difference XO levels between ASD and controls. This could mean that XO differences are a temporary finding only found in children, perhaps prompting an activation of antioxidative pathways.

Indications of increased levels of oxidative damage and defense have been found in post-mortem brain tissue from ASD patients (Rose et al. 2012). It has been shown, that SOD1 is predominantly located in astrocytes, especially in

Table 3 Linear regression analysis

Regression Analysis	N	Coefficient	Standard Error	T	R-Squared	P Value
AQ	151	2.40539	0.8627604	2.79	0.0496	0.0060**
ADOS	28	2.45	4.006163	0.61	0.0142	0.5461
IQ	40	−0.0407058	0.5521255	−0.07	0.0001	0.9416

Linear regression analysis between plasma level of superoxide dismutase 1 and respectively AQ-score, ADOS and IQ in separate analyses. * = $p < 0.05$, ** = $p < 0.01$

the cytosol, while SOD2 is mainly found in neurons with a mitochondrial location (Lindenau et al. 2000). Interestingly, increases in markers of astrocytes have also been found post-mortem in the brains from autistic patients, mainly in the prefrontal cortex, associated with cognitive and social functioning, which could indicate an increased number of astrocytes (Edmonson et al. 2014). Immune dysregulation have been shown to result in damage to the astrocytes in post-mortem brains of patients with ASD (DiStasio et al. 2019). An increased number of astrocytes, exposed to oxidative stress could result in an "outflux" or up-regulation of SOD1 which, together with a damaged blood-brain-barrier, could create a higher plasma level as seen in our study (Fiorentino et al. 2016).

We found a higher SOD1 plasma level in patients with ASD compared to controls, even when excluding individuals with psychiatric comorbidity such as schizophrenia and depression, which have been associated with oxidative stress (Michel et al. 2012; Michel et al. 2011). These disorders, like other major psychiatric and neurodegenerative disorders, have a higher prevalence among individuals with ASD compared to controls without ASD (Croen et al. 2015). This suggest that oxidative stress might be a common risk factor for neurodevelopmental and neurodegenerative diseases.

Sex showed a strong influence with males having significantly higher values of SOD1 and SOD2. Oxidative stress is part of the inflammatory process, where sex differences in ASD regarding inflammatory markers have been shown (Schwarz et al. 2011).

The strengths of this study include the large sample size, making some stratification analyses possible, thereby showing that the results were not due to either psychopharmacological treatment, or the comorbidities being treated. The differentiation between the isoforms of SOD furthermore made it possible to make a more specific analysis compared to other studies, showing that only SOD1 is significantly higher in patients with ASD. Lastly, the relatively small time frame (between 8:30–11:30 in the morning) in which all samples have been taken, weakens any circadian influence on biomarker levels.

Before venturing into final conclusions, our results warrant to take some of the studies limitations into account. Relatively few gave their consent to participate in the study, which may influence the generalizability of the results. All had a verbal language, and the mean IQ was 99.8. We furthermore measured the SOD concentrations in plasma, not in cerebrospinal fluid (CSF). It is still uncertain how plasma and CSF levels correlate. However, plasma levels of other proteins involved in neuronal development, such as brain-derived neurotrophic factor (BDNF) have been found to correlate with CSF levels. Equivalent studies have not been done for SOD1, SOD2 and XO, however a similar correlation may exist, and should be investigated in the future. We focused on the antioxidant enzymes SOD1 and SOD2 and the pro-oxidant XO, which are only some of the components of a larger regulatory system of oxidative stress. While the study did find SOD1 to correlate with AQ-score, it did not correlate with ADOS score. Lastly, due to size of study population there was a limit to how many sub-stratification analyses were possible, to avoid too small sample groups.

In conclusion, this study is the first to show that adults with ASD have a higher plasma concentration of the antioxidant superoxide dismutase 1, which is especially pronounced in men with ASD. This might reflect that individuals with ASD are more exposed to oxidative stress than controls, and warrants further studies into the complex interactions between oxidative stress, immune dysregulation and mitochondrial dysfunction.

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Conflict of interest The authors declare that they have no conflict of interest.

Author Contributions All authors meet the four ICMJE criteria for authorship. They have all been essential in the conception and design of the study, and have also been vital in the analysis and interpretation of the data. All authors have been involved in the drafting and revision of the paper, and have given final approval for publication while agreeing to be accountable for all aspects of the work.

Compliance with Ethical Standards

Conflict of interest The authors of this paper have no conflicts of interests to declare. This research included human participants who all gave a written informed consent of participation.

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