Relationship Between Sonic Hedgehog Protein, Brain-Derived Neurotrophic Factor and Oxidative Stress in Autism Spectrum Disorders

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Abstract The etiology of autism spectrum disorders (ASD) is not well known but oxidative stress has been suggested to play a pathological role. We report here that the serum levels of Sonic hedgehog (SHH) protein and brain-derived neurotrophic factor (BDNF) might be linked to oxidative stress in ASD. By using the whole blood or polymorphonuclear leukocytes, we demonstrated that autistic children produced a significantly higher level of oxygen free radicals (OFR). In addition, we found significantly higher levels of serum SHH protein in children with mild as well as severe form of autism. We also found that the serum level of BDNF was significantly reduced in autistic children with mild form of the disorder but not with severe form of the disorder. Our findings are the first to report a correlation between SHH, BDNF and OFR in autistic children, suggesting a pathological role of oxidative stress and SHH in autism spectrum disorders.

Keywords Autism spectrum disorders · Sonic hedgehog · BDNF · OFR

Introduction

Autism spectrum disorders (ASD) refer to a broad spectrum of neurodevelopmental disorders known as pervasive developmental disorders (PDD), including autism, Asperger’s syndrome, Rett’s syndrome, and childhood disintegrative disorder. By definition, ASD are characterized by impairments in verbal and nonverbal communication and social interaction [1], with onset usually occurring around the first 36 months of childhood. Repetitive, stereotyped, purposeless behaviors as well as attention and sensory dysfunctions are common findings in patients with ASD. In recent years, the prevalence of ASD has increased dramatically, and this increase, cannot be attributed completely to improved diagnostic techniques and increased awareness only [2]. Latest reports estimate that ASD affects approximately one in every hundred children, with a male-to-female ratio of four to one (4:1) [2]. Despite that the fact that there is an increase in autism research worldwide, exact etiology of autism and ASD remains largely unknown.

It is well known that oxidative stress resulting from excess generation of reactive-oxygen species (ROS) is a major contributing factor in neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease and ASD [3].

Sonic hedgehog (SHH) protein is a signaling protein from the Hedgehog family. When SHH binds to its receptor Patched-1 (PTCH1), PTCH1 cannot interact with the transmembrane protein Smoothened (SMO), resulting in activation of transcription factor GLI. The activated GLI regulates expression of many target genes that control cell growth, survival, and differentiation in a wide variety of cells, including neurons [4]. SHH signaling is vital during embryonic development. Previous studies have demonstrated that SHH signaling is activated in adult organism after injury and is involved in tissue repair mechanisms [4, 5].

Activation of SHH pathway up-regulates two neurotrophic factors, namely vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). As a result, activation of SHH signaling protects cortical...
neurons against oxidative stress that also led to the proposal of a potential role of SHH for the clinical treatments of brain ischemia and neurodegenerative disorders [6]. Thus the aim of the present study was to explore the relationship of SHH pathway, oxidative stress and BDNF in children with ASD.

Materials and Methods

Patients and Subjects

Forty-four children with ASD were recruited in the study (41 males and 3 females) ranging in age from 3 to 9 years and 40 age- and sex- matched healthy children (38 males and 2 females) served as the control group. The diagnosis of autism was made by child neurophysiologist, and pediatrician based on the criteria of autistic disorder as defined in the DSM-IV, CARS. Complete diagnostic work-ups including medical, neurological, psychiatric, and psychological evaluations were done for all of the subjects. All were in good physical health and were not taking any medications or nutrient supplements. Written consent was obtained from the parents of each subject, according to the guidelines of the ethical committee of King Khalid Hospital, King Saud University, Riyadh, Saudi Arabia.

Blood Samples

After an overnight fasting, blood samples (10 ml) were collected from children in both groups in plain test tubes. Blood samples were centrifuged at 3,000 rpm to collect serum samples, which were stored frozen in a freezer at −80°C until the time of analytical assays.

SHH Assay

Serum level of SHH was measured using a commercially available sandwich enzyme immunoassay (ELISA) kit from CUSABIO BIOTECH CO. Ltd (Wuhan, China).

BDNF Assay

Serum level of BDNF was measured using a commercially available sandwich enzyme immunoassay (ELISA) kit from Emax Immunoassay System (Promega Corp., Madison, Wisconsin).

Blood PMNL Isolation

Polymorphonuclear leukocytes (PMNL) were separated by using PMNL isolation medium (Robbins Scientific Corporation, Sunnyvale, CA). Five to seven milliliter of heparinized blood was layered over 4 ml of PMNL-Isolation Medium in a 15 ml tube and then centrifuged at 400×g for 30 min at room temperature. The leukocyte-rich plasma was carefully removed with a Pasteur pipette and transferred to a 15 ml conical centrifuge tube, filled with phosphate buffered saline (PBS) and centrifuged at 350×g for 10 min (Jouan centrifuge Model B4i, France). Two milliliter of lysing buffer (0.87% NH4Cl) were added to lyse residual erythrocytes and the cell suspension was, centrifuged at 250×g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 5% fetal calf serum (FCS). A cell count taken that was then to a desired final concentration [7, 8]. Cell viability was determined by trypan blue (0.2%) exclusion method [7, 8].

Chemiluminescence Assay and Oxygen Free Radicals Measurement: Luminol-Enhanced Chemiluminescence

A Berthold (AutoLumatPlus LB 953) luminometer with a constant temperature (37°C) controller (Berthold Technologies GmbH & Co. KG, Calmbacher Straße 22, D-75323 Bad Wildbad-Germany) connected to a computer was used. The reaction mixture consisted of 100 μl of whole blood or PMNLs suspension and 900 μl medium containing 10−5M luminol (5-amino-2,3-dihydro,1,4-phthalazinedione Sigma Chemical Co., St. Louis, MO, USA), 2 ng/ml phorbol myristate acetate (PMA) Sigma Chemical Co., St. Louis, MO, USA, 1.25 mg/ml opsonized zymosan (OPZ) Sigma Chemical Co., St. Louis, MO, USA and phosphate buffered saline (PBS). Whole blood or PMNLs suspension, triggered by adding prepared PMA or OPZ solution. Light emission was recorded in millivolts (mV) and the readings were recorded at 1 min intervals for 30 min. CL emission was quantified as the peak height in mV [7, 8].

Statistical Analysis

Metabolic data are presented as means ± standard deviation (SD). Statistical differences were ascertained by using the Student’s t test with significance set at a P value of 0.05 or lower.

Results

The results of the respiratory burst of whole blood and PMNLs from autistic and control subjects are shown in Tables 1, 2, 3 and 4. The respiratory burst of whole blood stimulated with PMA and OPZ, autistic (males and females) were significantly higher (P < 0.05) compared to control children (Tables 1 and 2). This increase was not related to the severity of the disorder. Similar findings were
also found in the respiratory burst of PMNLs from autistic and control children (Tables 3 and 4) and the increase was not related to the severity of the disorder.

Furthermore, BDNF serum level were significantly higher in mild autistic children compared to age and sex matched control subjects (442 ± 20 (pg/ml), 290 ± 90 (pg/ml), \( P < 0.05 \)) respectively. On the other hand, serum level of BDNF, was not statistically significant in severe autism as compared to age and sex matched control/subjects (323 ± 114 (pg/ml), 290 ± 90 (pg/ml), \( P > 0.05 \)) respectively, Fig. 1. In addition, Sonic hedgehog serum level were very highly statistically significant in mild (10.4 ± 3 (pg/ml)) and severe autism (24.2 ± 6 (pg/ml)) compared to control (2.6 ± 2 (pg/ml)) \( P < 0.0001 \), Fig. 2. In addition, the level of SHH were positively correlated with the severity of autism (r = 0.69) Fig. 3.

### Table 1
Respiratory burst of whole blood stimulated by PMA, as measured by chemiluminescence (CL), in control and children with ASD

| Subjects participated (n = no. of subjects) | CL measurement (mV) |  |
|--------------------------------------------|---------------------|---|
|                                            | Basal               | Maximum peak (mV) | Time to peak (min) |
| Control                                   |                      |                  |                  |
| Male (n = 38)                             | 0.90 ± 0.6          | 10 ± 4           | 1,800 ± 53       |
| Female (n = 2)                            | 0.92 ± 0.7          | 12 ± 6           | 1,721 ± 65       |
| Autistic                                  |                      |                  |                  |
| Male (n = 41)                             | 1.81 ± 0.5          | 24.13 ± 2*       | 1,960 ± 70       |
| Female (n = 3)                            | 1.5 ± 0.3           | 23.7 ± 6*        | 1,720 ± 21       |

PMA concentration = 2 µg/cuvete; Luminol concentration = 2 M\(^4\)/cuvete; Values are expressed as mean ± SD
* \( P < 0.05 \) (as compared to control group)

### Table 2
Respiratory burst of whole blood stimulated by OPZ, as measured by chemiluminescence (CL), in control and children with ASD

| Subjects participated (n = no. of subjects) | CL measurement (mV) |  |
|--------------------------------------------|---------------------|---|
|                                            | Basal               | Maximum peak (mV) | Time to peak (min) |
| Control                                   |                      |                  |                  |
| Male (n = 38)                             | 0.90 ± 0.6          | 10 ± 4           | 2,216 ± 65       |
| Female (n = 2)                            | 0.92 ± 0.7          | 12 ± 6           | 2,310 ± 54       |
| Autistic                                  |                      |                  |                  |
| Male (n = 41)                             | 1.81 ± 0.5          | 24.13 ± 2*       | 3,258 ± 76       |
| Female (n = 3)                            | 1.5 ± 0.3           | 23.7 ± 6*        | 3,174 ± 62       |

OPZ concentration = 200 µg/ml; Luminol concentration = 2 M\(^4\)/cuvete; Values are expressed as mean ± SD
* \( P < 0.05 \) (as compared to control group)

### Table 3
Respiratory burst of polymorphoneuclear leukocytes (PMNLs), stimulated by PMA, as measured by chemiluminescence (CL) in control and children with ASD

| Subjects participated (n = no. of subjects) | CL measurement (mV) |  |
|--------------------------------------------|---------------------|---|
|                                            | Basal               | Maximum peak (mV) | Time to peak (min) |
| Control                                   |                      |                  |                  |
| Male (n = 38)                             | 17.32 ± 6           | 303 ± 34         | 728 ± 35         |
| Female (n = 2)                            | 18.43 ± 1.5         | 298 ± 26         | 875 ± 42         |
| Autistic                                  |                      |                  |                  |
| Male (n = 41)                             | 31.8 ± 3            | 378 ± 51*        | 593 ± 54         |
| Female (n = 3)                            | 29.7 ± 4            | 412 ± 41*        | 643 ± 75         |

PMA concentration = 2 µg/cuvete; Luminol concentration = 2 M\(^4\)/cuvete; Values are expressed as mean ± SD
* \( P < 0.05 \) (as compared to control group)
The etiology of ASD is not well understood, though it likely involves genetic, immunologic, and environmental factors [9]. The dramatic increase in reported prevalence has encouraged an intense effort to identify early biological markers [10]. Such markers could allow for earlier identification and therapeutic intervention, contributing to improved prognosis [11]. In the current study, we speculated an important connection between SHH, BDNF pathways and oxidative stress. In our study, we demonstrated statistically significant increase in free radicals production (superoxide anion (O2) hydrogen peroxide (H2O2), and hydroxyl radicals (OH)), from whole blood and isolated human PMNLs in autistic children when compared to age and sex matched control. This increase was not related to the degree of autism, or to the age of affected child.

### Table 4 Respiratory burst of polymorphonuclear leukocytes (PMNLs), stimulated by OPZ, as measured by chemiluminescence (CL) in control and children with ASD

| Subjects participated (n = no. of subjects) | CL measurement (mV) |  |
|---------------------------------------------|---------------------|---|
|                                             | Basal               | Maximum peak (mV) | Time to peak (min) |
| Control                                     |                     |                   |                   |
| Male (28)                                   | 17.32 ± 6           | 303 ± 34          | 2,206 ± 45        |
| Female (18)                                 | 18.43 ± 1.5         | 298 ± 26          | 2,234 ± 54        |
| Autistic                                    |                     |                   |                   |
| Male (35)                                   | 31.8 ± 3            | 378 ± 51*         | 1,713 ± 64        |
| Female (5)                                  | 29.7 ± 4            | 412 ± 41*         | 1,905 ± 54        |

OPZ concentration = 200 µg/ml; Luminol concentration = 2 M4/cuvete; Values are expressed as mean ± SD

* P < 0.05 (as compared to control group)

![Fig. 1](image1.png)  
**Fig. 1** Serum levels of BDNF in control and autistic children. BDNF in mild and severe ASD, compared to control subject. BDNF serum level were significantly high in mild but not severe autistic children compared to age and sex matched subjects 442 ± 20 (pg/ml), 290 ± 90 (pg/ml), P < 0.05, respectively.

![Fig. 2](image2.png)  
**Fig. 2** Serum levels of Sonic hedgehog protein in control and autistic children. Highly statistically significant Sonic hedgehog serum level in mild and severe autism

![Fig. 3](image3.png)  
**Fig. 3** Relationship between SHH serum level and CARS score. Serum Level of SHH protein was positively correlated with the severity of autism (r = 0.69)

### Discussion

The etiology of ASD is not well understood, though it likely involves genetic, immunologic, and environmental factors [9]. The dramatic increase in reported prevalence has encouraged an intense effort to identify early biological markers [10]. Such markers could allow for earlier identification and therapeutic intervention, contributing to improved prognosis [11]. In the current study, we speculated an important connection between SHH, BDNF pathways and oxidative stress. In our study, we demonstrated statistically significant increase in free radicals production (superoxide anion (O2) hydrogen peroxide (H2O2), and hydroxyl radicals (OH)), from whole blood and isolated human PMNLs in autistic children when compared to age and sex matched control. This increase was not related to the degree of autism, or to the age of affected child.
addition, we demonstrated higher serum level of SHH concentration, which was positively correlated with the degree of autism. Furthermore, we found a statistically significant higher level of BDNF in mild but not in severe autism.

Oxidative stress is a process caused by exposure to reactive oxygen intermediates, such as superoxide anion \((O_2^-)\) hydrogen peroxide \((H_2O_2)\), hydroxyl radicals \((OH)\) and nitric oxide \((NO)\) which can damage proteins, nucleic acids and cell membranes. The ROS within the cells are neutralized by antioxidant defense mechanisms. SOD, catalase and glutathione peroxidase \((GPx)\) are the primary enzymes involved in direct elimination of ROS, whereas glutathione reductase and glucose-6-phosphate dehydrogenase are secondary antioxidant enzymes, which help in maintaining a steady concentration of glutathione and NADPH necessary for optimal functioning of the primary antioxidant enzymes [12–15]. Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species \((ROS)\) and the antioxidant capacity of the cell [16, 17]. In pathological conditions, over production of OFR or less effective antioxidant enzymatic system, takes place, resulting in OFR overflow which leads to tissue damage. Oxidative stress is an important mechanism involved in brain damages, as consequences of exposure to reactive oxygen species \((ROS)\) [15, 17].

Brain-derived neurotrophic factor \((BDNF)\) is a small protein found throughout the central nervous system \((CNS)\) and peripheral blood. BDNF is the most widely distributed neurotrophin in the CNS. BDNF plays a critical role in axonal and dendritic growth and guidance. In addition, BDNF participates in neurotransmitter release [18]. BDNF is involved in the survival and differentiation of dopaminergic neurons in the developing brain [18, 19] and plays an important role in the formation and plasticity of synaptic connections [20].

Within the nervous system, SHH protein is associated with development and patterning of the central nervous system \((CNS)\) and peripheral blood. BDNF is the most widely distributed neurotrophin in the CNS. BDNF plays a critical role in axonal and dendritic growth and guidance. In addition, BDNF participates in neurotransmitter release [18]. BDNF is involved in the survival and differentiation of dopaminergic neurons in the developing brain [18, 19] and plays an important role in the formation and plasticity of synaptic connections [20].

An increase in SHH protein was initially reported in the gray matter from multiple sclerosis brains lesions [31] or in animal models of this pathology including Experimental Autoimmune Encephalomyelitis and cuprizone-induced demyelination [32, 33]. The clinical improvement and reduced demyelination driven by either interferon-b or triiodothyronine in several rodent models of demyelinating diseases are likely linked to an enhanced expression of SHH [32, 33]. It points out towards a possible protective
effect of exerted by SHH and it is with agreement to our findings in autism. The most likely explanation for higher level of SHH in autistic examined in this study is as a result of increased oxygen free radicals production as a protective mechanism secondary to increase oxidative stress inside the autistic. The roles of the SHH signaling pathway in the CNS are gaining some interest lately, due to a multifunctional properties of SHH, ranging from the regulation of new cells production to the modulation of neuronal electrophysiological activity. The higher levels of OFR and consequently SHH, demonstrated in the current study, in mild ASD, resulted in an increase BDNF production as a protective mechanism. In severe ASD the further increase in OFR and SHH, produced a negative feed back response on the production of BDNF, as demonstrated by lower level of BDNF in severe but not in mild ASD. Existing data provide support for considering SHH signaling as an important mechanism in tissue-repair process in brain diseases, and as a target for novel therapeutic approaches for the treatment of brain disorders and in particular ASD.

Conclusion

The present study demonstrated the possible critical protective role played by SHH and BDNF in ASD, particularly in relation to oxidative stress. Larger studies are strongly recommended, to investigate the significant role played by BDNF in ASD phenotypes. Explanation of the underlying molecular mechanism may help develop a novel therapeutic intervention for neuronal protection in ASD.

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