Evaluation of plasma soluble fatty acid synthase levels among Saudi autistic children

Relation to disease severity

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ABSTRACT

الأهداف: قياس مستوى مخلق الحامض الدهني السائل في أطفال التوحد في المملكة العربية السعودية وعلاقته بشدة المرض.

الطريقة: أجريت هذه الدراسة خلال الفترة من مايو2011م حتى أبريل 2012م في قسم علم وظائف الأعضاء ومركز علاج وأبحاث التوحد، كلية الطب ومستشفى الملك خالد الجامعي، جامعة الملك سعود، الرياض، المملكة العربية السعودية. وقد اشتملت الدراسة على 60 طفلاً، 20 من الأصحاء و20 من أطفال التوحد متوسط الدرجة و20 من أطفال التوحد شديد الدرجة وتم قياس مستوى مخلق الحامض الدهني السائل في بلازما الدم.

النتائج: أظهرت الدراسة ارتفاع مستوى مخلق الحامض الدهني السائل في بلازما الدم ارتفاعاً احصائياً في أطفال التوحد بالمقارنة بالأطفال الأصحاء وكذلك كان هناك زيادة إحصائية في أطفال التوحد شديد الدرجة بالمقارنة مع أطفال التوحد متوسط الدرجة وعلاقة طردية بين مستوى مخلق الحامض الدهني السائل في بلازما الدم وشدة مرض التوحد.

خاتمة: تظهر نتائج الدراسة ارتفاع مستوى مخلق الحامض الدهني السائل في بلازما الدم في أطفال التوحد الشديد الدرجة في المملكة العربية السعودية مما يؤدى إلى اعتباره مؤشر لدرجة التوحد وهذا يشير إلى عامل علاجي أو تشخيصي جديد لمرض التوحد.

Objective: To investigate the role of an apoptotic marker soluble fatty acid synthase (s-Fas) antigen in children with autism and its correlation to disease severity.

Methods: The study was conducted between May 2011 and April 2012 at the Department of Physiology and Autism Research and Treatment Center (ARTC) at King Khalid University Hospital and King Saud University (KSU) in Riyadh, Kingdom of Saudi Arabia. Sixty children were enrolled, 20 as controls, 20 mild, and 20 with severe autism. Plasma samples were analyzed for s-Fas.

Results: The levels of s-Fas were significantly higher in autistic children compared with control children ($p<0.05$). Furthermore, this increase was significantly more pronounced in children with severe autism as compared with mild autism, and there was a positive correlation between s-Fas levels and severe autism ($p<0.05$).

Conclusion: The s-Fas level is high in Saudi children with severe autism, and can be considered an indicator of disease severity. These findings may offer a new therapeutic or diagnostic tool for children suffering from severe autism.

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Autism is a neurodevelopmental disorder manifesting pervasive abnormalities of social interaction and communication, repetitive behaviors, restricted interests, language and speech.[1] The disorder affects approximately 4 times more boys than girls. The diagnosis is generally made between 18-30 months after birth, but the autistic characteristics can be found in children as young as one-year-old.[1] Although the etiology and pathogenesis of autism remain largely

unknown, several factors have been implicated: genetic, immune, viral, metabolic, and neurochemical.[1] Moreover, the abnormal brain blood flow and endothelial dysfunction could also contribute to the pathogenesis of the disorder.[2] In addition, emerging evidence points to inflammatory and apoptotic mechanisms being responsible for certain neuropsychiatric disorders including autism.[2] In recent years, certain growth factors; for example, vascular endothelial growth factor, platelet derived growth factor, and programmed cell death (apoptosis) factors (for example soluble-fatty acid synthase ligand [s-Fas-L]) have been linked to neurodegenerative and neuropsychiatric diseases.[3] These factors may have a pivotal role in the survival and growth of the brain neuronal axons, inflammation, and autoimmune pathology. Recent studies suggest that apoptotic mechanisms may partially contribute to the pathogenesis of autistic disorders. Sheikh et al[4] stated that increased cathepsin D in the cerebellum of autistic subjects suggested that altered regulation of apoptosis, and altered activities of cathepsin D in the autistic brain, may play an important role in the pathogenesis of autism. Fatty acid synthase-mediated apoptosis has been described in other CNS diseases, such as traumatic brain injury, multiple sclerosis, and ischemic stroke,[5,6] although its role in autism has not been yet defined. Based on the aforementioned considerations, and the idea that autism is a disorder of the developing nervous system, the objective of this study was to measure concentrations of s-Fas as an antiapoptotic marker in the plasma of autistic patients in an attempt to understand the role and relationship of this biochemical parameter in the etiology of autism and its commonly related psychiatric conditions.

Methods. ***Study design.*** The study was conducted between May 2011 and April 2012 in the Department of Physiology, and the Autism Research and Treatment Center (ARTC) at King Khalid University Hospital and King Saud University (KSU) in Riyadh, Kingdom of Saudi Arabia. A total of 60 male children (40 autistic children and 20 healthy children) up to the age of 10 years were recruited for the study. Healthy children (n=20) serving as the normal controls (Group I) were recruited from the pediatrics department. Autistic children were divided into 2 groups: 20 children with a mild form of autism (Group II) and 20 children with a severe form of autism (Group III). The diagnosis of autism was performed by a licensed psychologist, psychiatrist, or neurologist according to criteria described in the fifth edition of the Diagnosis and Statistical Manual of Mental Disorders (DSM-5).[7] The severity of the disease was determined according to the Childhood Autism Rating Scale (CARS) diagnostic criteria for autistic spectrum disorders (ASD). According to this scale, a score of 30-36 indicated a mild form of autism, whereas a score of 37-60 indicated a severe form of autism.[8] The study was approved by the Ethics Committee of King Khalid University Hospital and all procedures were performed according to guidelines and declaration of the Institutional Review Board (IRB) according to the principles of the Helsinki Declaration. Written consent was obtained from parents of children prior to the start of the study, and the parents were asked to fill out a questionnaire regarding the child's medical and behavioral history.

Children were excluded from the study if they had organic aciduria, dysmorphic features, or a diagnosis of Fragile x gene or other serious neurological (for example, seizures), psychiatric (for example, bipolar disorder or depression), or known medical conditions. All participants were screened for current and past physical illness, children with known endocrine, cardiovascular, pulmonary, liver, kidney, or other medical diseases were excluded from the study.

Laboratory parameters analysis. Blood samples were collected in EDTA tubes. After thorough mixing, the tubes were centrifuged at 1,500 xg for 10 minutes to separate plasma, which was collected and stored at -70°C. Plasma samples were analyzed for s-FAS by enzyme immunoassays (EIA). Samples were assayed in duplicate in a single large batch. The EIA kits for these proteins were purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France).

ELISA method for assaying s-Fas.[9] This assay method was carried out according to the instructions of the s-Fas-ELISA Kit manufacturer. Briefly, 100 µl of diluent RD1W was added per well of a microtiter plate. Then 100 µl per well of s-Fas standard, control, or plasma sample was pipetted. The plate was incubated for 2 hours at room temperature. Then, the contents were aspirated and the plate was washed 3 times with washing buffer (20 ml of wash buffer concentrate available with the kit diluted into deionized or distilled water to yield 400 ml of 1x wash buffer). Then, 200 µl per well of s-Fas conjugate (biotinylated anti-human Fas) was added, followed by a 2-hour incubation at room temperature. The contents were aspirated, and microwells were washed 3 times. Then 200 µl per well of substrate solution (TMB substrate) was added, protected from light, and incubated for 25 minutes at room temperature. A stop solution (sulfuric acid) was added as 50 ul/well to stop the enzyme reaction and a microplate reader (ELX 800-BioTek, Highland

Park, Winooski, Vermont, USA) was used to read the microplates within 30 minutes at 450 nm, and lastly, the concentration of s-Fas was calculated according to kit directions.

Statistical analysis. The data were analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA). All data were reported as means ± standard deviation (SD). Unpaired T test was used to compare study groups, and the difference was considered significant when the *p*-value was ≤0.05, and confidence interval of 95%. Spearman's correlation coefficient was applied to assess the strength of correlation between continuous quantitative variables, and the difference was considered significant when the *p*-value was ≤0.05.

Results. Table 1 summarizes the demographic, educational, and psychiatric hospitalization data for children with autism versus control children. The mean age at admission to the Autism Center at King Khalid University Hospital, Riyadh, Saudi Arabia was approximately 9.18 in Group I, 9.53 in Group II, and 9.22 years old in Group III. All groups comprised Saudi boys. Children did not differ significantly in educational data ($p>0.05$) and prior psychiatric hospitalization ($p>0.05$). School attendance in the children's respective school settings was virtually identical, and no significant change was observed between all groups ($p>0.05$) (**Table 1**). Concerning co-occurring psychiatric diagnoses such as attention deficit/hyperactivity (ADHD), oppositional defiant disorder (ODD), depressive, and bipolar disorder, disruptive behavioral disorder (DBD), adjustment disorder, anxiety disorders, or obsessive compulsive disorder (OCD), and conduct disorder all showed insignificant change between the mild and severe autism groups (**Table 2**). As summarized in **Table 3**, the plasma levels of s-Fas proteins were significantly higher in severely autistic children (Group III) compared with normal (Group I), and mildly autistic children (Group II), whereas the children with the mild form of autism (Group II) showed no significant change of s-Fas compared with normal children (Group I). Correlations between different variables (**Table 4**) showed that the correlation between s-Fas and mild autism was not significant (r=0.317) ($p=0.174$), but it was significant and positive in severe autism (r=0.499) ($p=0.002$).

Discussion. Children with autism have impairments in 4 core domains: socialization, communication, restricted interests, and repetitive behaviors.[1] Protection of the brain from injury during the fetal, neonatal, and postnatal periods is of major importance owing to the significant number of infants who now survive early brain injury but develop neurodevelopmental and motor disabilities. Although autism is generally considered to

Table 1 - Socia-demographic data in the controls, mild autism, and severe autism groups.

Demographic data	Controls (n=20)	Children with mild autism (n=20)	Children with severe autism (n=20)	*P*-value
Gender (% male)	100%	100%	100%	>0.05
Nationality	Saudi	Saudi	Saudi	-
Age at time of study (years)	9.18	9.53	9.22	>0.05
Family income ($/year)	10,012	10,251	10,231	>0.05
Social problems	-	-	-	-
Population density (resident county)				
Rural				
Densely settled rural				
Semi-urban	20%	10%	11%	>0.05
Urban	80%	90%	89%	
Prior psychiatric hospitalization	-	-	-	-
Last academic performance				
Failing or below average	2%	4.5%	5.5%	
Average	89%	69.3%	76.9%	>0.05
Above average	9%	26.2%	17.7%	
Last school attendance				
Regular (90-100%)	97.5%	92.6%	95.4%	>0.05

Percentages for variables were calculated without missing cases in the denominator. Difference between mild and severe autism groups was non significant at $p\geq0.05$

Table 2 - Co-occurring psychiatric diagnosis in controls, mild autism, and severe autism groups.

Psychiatric diagnosis	Controls (n=20)	Children with mild autism (n=20)	Children with severe autism (n=20)	*P*-value
Thought problems	-	-	-	-
Aggressive/delinquent behavior	-	1.9%	1.8%	>0.05
Attention deficit hyperactivity disorder (ADHD)	-	3.7%	4.1%	>0.05
Depressive disorders (major depressive disorder, dysthymic disorder)	-	0.5%	0.7%	>0.05
Disruptive behavioral disorder NOS	-	-	-	-
Adjustment disorder	-	0.1%	0.2%	>0.05
Mental retardation	-	-	3.1%	-
Anxiety disorders (generalized anxiety disorder, panic disorder, social phobia, anxiety disorder NOS)	-	2.8%	-	>0.05
Obsessive compulsive disorder	-	-	-	-
Conduct disorder	-	2.1%	2.4%	>0.05

Percentages for variables were calculated without missing cases in the denominator. Difference between mild and severe autism groups was non significant at $p \geq 0.05$, NOS - not otherwise specified

Table 3 - Plasma levels of soluble fatty acid synthase (s-Fas) in controls, mild autism, and severe autism groups.

Biomarker	Controls (n=20)	Children with mild autism (n=20)	Children with severe autism (n=20)	*P*-value
Plasma s-Fas (pg/ml)	809.95±101.75	847.99±153.20	897.17±138.08*†	0.013*, 0.04†

Results are expressed as mean ± SD
*Significant changes in the severe autism group in comparison with controls ($p<0.05$)
†Significant changes in the severe autism group in comparison with the mild autism group ($p<0.05$)

Table 4 - Correlation between plasma level of soluble fatty acid synthase (s-Fas) and autistic severity as assessed by CARS.

Biomarker	Correlation coefficient	*P*-value*
Mild autism and s-Fas	0.317	0.174
Severe autism and s-Fas	0.499	0.002

CARS - Childhood autism rating scale, *significant correlation

be a multifactorial disorder, the pathogenesis of the disorder remains poorly understood. Environmental, genetic, and immune factors are commonly linked to the disorder.[1-3] Recently, neurochemical factors like brain-derived neurotrophic factor (BDNF) and oxidative stress (OS) have also been suggested to contribute to this disorder.[9,10]

Apoptosis or programmed cell death is well known to play a pathogenic role in neurodegenerative diseases, but its role in autism is not known. Emerging evidence points to inflammatory and apoptotic mechanisms being responsible for certain neuropsychiatric disorders including autism.[11,12] Localized inflammation of the CNS may contribute to the pathogenesis of autism, and that elevation of plasma cytokines such as TNFα and IL6 involved in tissue inflammation and necrosis could be an early event followed by infiltration of macrophages, cytokines, and proapoptotic factors across the blood brain barrier. Also, a previous report by Nilufer Yonguc et al[13] demonstrated a link between inflammatory marker TNFα and the major effectors of its apoptotic signal; namely, Caspase 1 and 3. They identified the downstream effectors of TNFα apoptotic signaling, and showed a positive correlation of TNFα with Caspase 3. A pathogenic role of apoptosis in autism is also supported by recent studies of Krey et al[14] who suggested a mechanism relating Ca2+-signaling induced apoptosis in brain cells of autistic patients, and showed altered expression of 2 other apoptotic markers; namely, p53 and Bcl-2. The level of p53 was increased whereas the level of Bcl-2 was decreased in the brain of autistic children.

All the aforementioned studies confirm the role of neuroinflammation and apoptosis in the pathogenesis of autism. Soluble-Fas is the soluble form of a cell-surface receptor molecule known as Fas (CD95), and it is well known as a regulator of apoptosis or programmed cell death.[15] This factor (s-Fas) has been shown to be involved in the pathophysiology of neurodegenerative diseases,[16,17] autoimmune diseases,[18,19] and diabetic retinopathy or neuropathy.[20,21] Soluble-Fas inhibits Fas-mediated apoptosis by neutralizing Fas-L, or the anti-Fas antibody. Therefore, the observed rise of s-Fas in severe autism in the present study may be compensatory to induce an antiapoptotic effect and to secure outgrowth and neuronal survival.[22] Also these findings suggest that neuronal cells or the peripheral blood lymphocytes or cells concerned with immune activation upregulate or stimulate s-Fas production to protect neuronal cells from Fas-mediated apoptosis.[22] This indicates the importance of s-Fas in inhibiting apoptosis in autistic brains.

The results of the present study are in line with the reports of Siniscalco et al,[23] who suggested a possible role of the capsase pathway involved in apoptosis in the clinical outcome of autism, and the use of caspase as potential diagnostic and/or therapeutic tools in autistic disorder management. Also, recent works[24] suggested that down-regulation of the brain derived neurotrophic factor (BDNF)-Akt-Bcl2 antiapoptotic signaling pathway in the autistic brain could be one of the underlying mechanisms responsible for the pathogenesis of autism. As described in the present study, the significant increase of the s-Fas biomarker in the plasma of severe autistic children, and the positive correlation between s-Fas level and the severity of autism, suggests a compensatory protective role for this biochemical factor in the pathophysiology of the disorder and suggests a possible role of s-Fas as an antiapoptotic factor in clinical outcome of autism and the potential use of s-Fas as a diagnostic tool in autistic disorders.

Brain autoimmunity has been found to be involved in the pathogenesis of autism.[25] Soluble-Fas concentrations rise in the blood of patients with autoimmune diseases.[26] This in accordance with the findings of the present study, which described the increase of s-Fas in severely autistic children and supports the notion that s-Fas, not only as antiapoptosis marker, may play a role in the pathogenesis of autism, and may also be related to the autoimmune pathogenesis of autism.

Study limitations. One limitation of this study was that a number of the children were irritable and scared, some refused blood collection by venipuncture because of distress of needles, and were therefore excluded from the study.

In conclusion, the findings of the present study reveal that the s-Fas level is high in Saudi children with severe autism, and that it can be considered an indicator of disease severity. Soluble-Fas may have a compensatory protective role against neuronal apoptosis. While additional research is necessary, the findings of this study may offer s-Fas as a new therapeutic target or a diagnostic tool for children suffering from autism.

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