Study of dual angiogenic/neurogenic growth factors among Saudi autistic children and their correlation with the severity of this disorder

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ABSTRACT

الأهداف: دراسة مستوى عامل نمو الإندوثيليام المبطن للأوعية الدموية، وعامل النمو الناتج من الصفائح الدموية في الأطفال السعوديين المصابين بالتوحد.

الطريقة: أُجريت هذه الدراسة في قسم علم وظائف الأعضاء، كلية الطب، جامعة الملك سعود، وفي مركز أبحاث وعلاج التوحد، مستشفى الملك خالد الجامعي، جامعة الملك سعود، الرياض، المملكة العربية السعودية وذلك خلال الفترة من مايو 2010م إلى أبريل 2011م. شملت الدراسة 60 طفلاً منهم 20 أصحاء، و40 مصاباً بالتوحد. لقد تم تحليل بلازما الدم لمعرفة مستوى عامل نمو الإندوثيليام المبطن للأوعية الدموية وعامل النمو الناتج من الصفائح الدموية.

النتائج: أظهرت النتائج تغير غير ملحوظ في مستوى عامل نمو الإندوثيليام المبطن للأوعية الدموية في الأطفال المصابين بالتوحد بالمقارنة مع الأطفال الأصحاء (p=0.065)، بينما كان هناك زيادة ملحوظة في مستوى عامل النمو الناتج من الصفائح الدموية في الأطفال المصابين بالتوحد بالمقارنة مع الأطفال الأصحاء (p=0.01)، وهذه الزيادة كانت ملحوظة في الأطفال المصابين بالتوحد من الدرجة العالية عنها في الأطفال المصابين بالتوحد من الدرجة المتوسطة (p=0.001)، وهذه الزيادة غير مرتبطة إحصائياً بدرجة المرض.

خاتمة: أظهرت نتائج هذا البحث ارتفاع مستوى عامل النمو الناتج من الصفائح الدموية ذو الدور المحفز لنمو الأوعية الدموية والمنظم لنمو الخلايا العصبية في الأطفال السعوديين المصابين بالتوحد وهذا يؤكد أنه ربما يكون له دور في هذا المرض. و ربما يكون لوحده أو بالتعاون مع عوامل أخرى تنظم نمو الخلايا العصبية لتؤدي إلى نمو أوعية دموية جديدة تغذي وتحمي الخلايا العصبية في مخ الأطفال المصابين بالتوحد. ويفسر عدم وجود تغير ملحوظ في مستوى عامل نمو الإندوثيليام المبطن للأوعية الدموية في الأطفال المصابين بالتوحد أن ليس له دور مسبب أو تعويضي في الإصابة بهذا المرض وخاصة في هذه الدراسة.

Objectives: To investigate the role of 2 angiogenic/neurogenic growth factors, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) in Saudi children with autism.

Methods: The study included a total of 60 children that included 20 controls and 40 patients with a confirmed diagnosis of autism. The study was conducted in the Department of Physiology, Faculty of Medicine, King Saud University, and in the Autism Research and Treatment Center, King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia between May 2010 and April 2011. Collected blood plasma samples were analyzed for VEGF and PDGF.

Results: The levels of VEGF showed a non-significant change in autistic children compared with the control children (p=0.065). The levels of PDGF were significantly higher in autistic children compared with the control children (p=0.01). Furthermore, this increase was significantly more pronounced in children with severe autism as compared with children with mild autism (p=0.001), and it was not correlated to the severity of the disorder.

Conclusion: A rise in PDGF may contribute to the pathophysiology of the disorder, either alone or in synergy with other neurotrophic factors to induce an angiogenic-neuroprotective effect. Plasma VEGF has no causative or compensatory contribution to the pathology of this disorder.

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Autism is a neuro-developmental disorder manifesting pervasive abnormalities of social interaction and communication, repetitive behaviors, restricted interests, language, and speech. The disorder affects around 4 times more boys than girls.[1] 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 factors, immune factors, viral factors, metabolic factors, and neuro-chemical factors.[2-4] Moreover, abnormal brain blood flow and endothelial dysfunction could also contribute to the pathogenesis of the disorder.[5] Certain growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have been linked to neurodegenerative and neuropsychiatric diseases in recent years.[5-8] These factors have also been associated with angiogenesis, cell proliferation and migration, neuronal survival, axonal outgrowth, inflammation, and autoimmune pathology.[9,10]

The VEGF enhances angiogenesis and promotes blood brain barrier leakage in the ischemic brain.[11] It has dual roles as a potent angiogenesis factor and neural regulator. It also has a role in regulation of early forebrain development.[12] The modulatory effect of a single bolus of VEGF and PDGF in combination on astrocytes and microglia to acute cerebral injury was examined. A combination of VEGF and PDGF injected into the striatum of adult male Sprague-Dawley rats delayed the inflammatory response to traumatic injury, which initiated an increase in astrocyte and microglial infiltration as part of an inflammatory response to injury and lead to local progressive cell death.[13] During development, the brain neuronal wiring and vasculature follow the same routes, so they share some of the same angiogenic/neurogenic growth factors for guidance.[14] In human primary brain tumors, the expression of dual angiogenic/neurogenic growth factors VEGF and also PDGF and their receptors were differentially regulated, either increased or decreased, in relation to the type and grade of the tumor.[14] One important factor that has been identified for its potential dual role in the vascular and nervous systems is VEGF, vascular endothelial growth factor receptor 2 (VEGFR2) is expressed throughout the CNS vasculature.[15] The VEGFR2 is also present in the forebrain neuro-epithelium.[15] While VEGF has been shown to be required for vascular development, it also plays a direct role in nervous system development.[15] The CNS expression of VEGFR2 promotes neuronal survival and axon outgrowth in cultured peripheral neurons in vitro, and cortical neurons in vitro and in vivo.[16]

Based on these considerations and the idea that autism is a disorder of the developing nervous system, the primary aim of the present study was to evaluate plasma levels of VEGF and PDGF in Saudi autistic children, as angiogenic/neurogenic factors play a role in brain development, and to correlate their levels to the severity of the disorder.

Disclosure. This research was conducted under the Grant of Deanship of Scientific Research, King Saud University, Riyadh, Kingdom of Saudi Arabia. The authors declare no conflicting interests.

Methods. The study was conducted between May 2010 and April 2011. The site of the study was the Department of Physiology, Faculty of Medicine, King Saud University, and the Autism Research and Treatment Center (ARTC), King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia. 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). The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki. Written consent was obtained from the parents of children prior to the start of the study according to the guidelines of the Ethics Committee of King Khalid University Hospital, King Saud University.

Study design. 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) served as the normal controls (group I). Autistic children were divided into 2 groups: 20 children with the mild form of autism (group II), and 20 children with the 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 4th edition of the Diagnosis and Statistical Manual of Mental Disorders (DSM-IV).[17] The severity of the disease was determined according to the Childhood Autism Rating Scale (CARS) diagnostic criteria for autistic spectrum disorders (ASD).[18] 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.[18]

Children were excluded from the study if they had organic aciduria, dysmorphic features, or 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 1500 r/m for 10 minutes to separate plasma, which was collected and stored at –70°C. Plasma samples were analyzed for VEGF and PDGF by enzyme-linked immunosorbent assays (ELISA). The kits were purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France). Samples were assayed in duplicate in a single large batch.

ELISA method for assaying VEGF.[19] This assay method was carried out according to instructions in the VEGF-ELISA Kit. Briefly, 100 µl of diluent was added per well of a micro titer plate. Then 100 µl per well of VEGF 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 wash buffer (20 ml of wash buffer concentrate available with the kit was diluted into deionized or distilled water to yield 400 ml of 1 x wash buffer) then 200 µl per well of VEGF conjugate (polyclonal antibody against VEGF conjugated to horseradish peroxidase) was added, followed by a 2 hour incubation at room temperature. The contents were aspirated, and micro wells were washed 3 times. Then 200 µl per well of substrate solution (Color Reagents A and B mixed together in equal volumes within 15 minutes of use) was added, protected from light, and incubated for 25 minutes at room temperature. The enzyme reaction was stopped by adding 50 µl per well of stop solution (sulfuric acid) and within 30 minutes the micro plate was read at 450 nm using a micro plate reader (ELX 800-BioTek-USA). The concentration of VEGF was calculated as instructed in the kit.

ELISA method for assaying PDGF.[20] This assay method was carried out according to the instructions in the PDGF-ELISA Kit. Briefly, 100 µl of diluent RD1W was added per well of a microtiter plate. Then 100 µl per well of PDGF 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 1 x wash buffer). Then 200 µl per well of PDGF conjugate (antihuman PDGF-BB) was added, followed by a 2 hour incubation at room temperature. The contents were aspirated, and the microwells were washed 3 times. Then, 200 µl per well of substrate solution was added, protected from light, and incubated for 25 minutes at room temperature. The enzyme reaction was stopped by adding 50 µl per well of stop solution (sulfuric acid) and within 30 minutes the microplate was read at 450 nm using a microplate reader (ELX 800-BioTek-USA). The concentration of PDGF was calculated as directed in the kit.

Statistical analysis. The data were analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) computer program. All data were reported as mean ± standard deviation (SD). Paired T test was used to compare study groups, and the difference was considered significant when the *p*-value was ≤0.05. 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. As summarized in **Table 1**, the plasma levels of VEGF were not significant in all autistic children compared with normal children (p=0.065), but the PDGF plasma levels were significantly higher in autistic children compared with normal children (p=0.01). The data in **Table 2** show that the plasma levels of VEGF were not significant in the mild (group II) and severe (group III) autistic children compared with normal children (group I) (p=0.063 and p=0.061), also this was not significant in mild autistic children compared with severe autism children (p=0.075). Children with severe (group III) and mild (group II) forms of autism showed

Table 1 - Plasma levels of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) levels in control and autistic groups.

Biomarker	Group I Controls (n=20)	Group II Autistics (n=40)	*T*-value	*P*-value*
Plasma VEGF (pg/ml)	304.81±52.07	363.22±109.59	2.01	0.065
Plasma PDGF (pg/ml)	331.53±172.68	1200.53±445.84	10.8	0.01

Results are expressed as mean ± S.D. *group II compared with group I

Table 2 - Plasma levels of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) levels in the controls, and the mild autistic and severe autistic groups.

Biomarker	Group I Controls (n=20)	Group II Mild autism (n=20)	Group III Severe autism (n=20)	*P*-value
Plasma VEGF (pg/ml)	304.81±52.07	312.60± 40.365	340.62±69.23	0.061[a] 0.063[b] 0.075[c]
Plasma PDGF (pg/ml)	331.53±172.68	498.94±245.86	701.59±199.98	0.032[a] 0.000[b] 0.028[c]

Results are expressed as mean ± S.D., [a]group III compared with group I, [b]group II compared with group I, [c]group III compared with group II

Table 3 - The correlation between plasma platelet-derived growth factor (PDGF) levels and autism severity as assessed by the Childhood Autism Rating Scale (CARS).[18]

Severity of autism and PDGF levels	Correlation coefficient	*P*-value
Mild autism and PDGF	-0.087	0.717
Severe autism and PDGF	-0.008	0.972

a significant increase of PDGF when compared with the control children (group I) (p=0.032 and p=0.000), also the PDGF was significantly increased in severe autism compared with mild autism children (p=0.028). Regarding correlations between different variables (**Table 3**), the correlation between controls and the mild form of autism was not significant for PDGF and mild autism. Similarly, the correlation between controls and the severe form of autism was not significant for PDGF.

Discussion. Although autism is generally considered to be a multi factorial disorder, the pathogenesis of the disorder remains poorly understood. Environmental factors, genetic factors, and immune factors are commonly linked to the disorder.[21] Recently, plasma levels of neurochemical factors like brain-derived neurotrophic factor (BDNF) and oxidative stress (OS) have also been suggested to contribute to autism.[22,23] The VEGF is a well-known angiogenic/neurogenic inducing factor that also impacts the function of neurons and glial cells of the CNS as it crosses the blood brain barrier to induce growth of brain vasculature.[15,24] It has been shown to be involved in the pathophysiology of neurodegenerative and autoimmune diseases,[25] diabetic retinopathy,[26] and diabetic neuropathy.[27]

The findings of the present study that plasma VEGF showed non-significant changes in Saudi children with mild or severe autism compared to normal controls is in contradiction with recent reports of Emanuele et al.[28] They reported low levels of VEGF in severe autism and explained that as a causative factor in the disorder, as VEGF acts as a key signaling molecule in the CNS, and is involved in neuroprotection, neuronal survival, axonal outgrowth, and neuronal-excitation regulation.[16-24] This variation between the present study and Emanuele et al[28] could be due to different sample size and age of patients. Emanuele et al's study was conducted on 22 adult patients with severe autism, whereas our study was conducted on 40 children with mild and severe autism.

Autism is characterized by excessive excitation of motor neurons, and this explains repetitive motor behavior in these patients. McCloskey et al[24] showed that a stimulus that increases neuronal and motor activity, namely, convulsive seizure, increases VEGF expression in the forebrain motor neurons. They explained this VEGF over-expression on the basis that it has a compensatory protective mechanism to depress this neuronal excitation and motor activity. The findings of the present study suggest that plasma VEGF has no causative or compensatory contribution to the pathology of this disorder. Further research is required to elucidate this point.

As described in the present study, we found an increase of PDGF in the plasma of autistic children, suggesting a role for PDGF biochemical factors in the pathophysiology of the disorder. Our data agree with the report of Kajizuka et al,[29] who indicated that serum levels of PDGF-BB homodimers are increased in male children with autism, and this increase appeared to be particularly related to the severity of the disorder. The significance of this finding is not clear at the moment, but it may be related to PDGF's well-known neuroprotective role as it crosses the blood brain barrier to induce its effect.[30] Autism also involves a myelin

defect,[31] and PDGF is known to promote survival of myelin-producing progenitor cells.[7] It is possible that the PDGF increase observed in this study is a protective compensatory mechanism for the myelin abnormalities found in autism. Autoimmunity to the brain has been found to be involved in the pathogenesis of the autism.[32] Therefore, a rise of PDGF may also play a protective role in brain autoimmune diseases. However, this possibility needs further study.

One limitation of this study was that some of the children refused blood collection by venipuncture because of fear and distress of needles, and so they were excluded from the study.

In conclusion, the findings of the present study elucidate that angiogenic/neurogenic growth factor PDGF is increased in Saudi autistic children, and this may contribute to the pathophysiology of the disorder. The PDGF might work alone or in synergy with other neurotrophic factors to induce an angiogenic-neuroprotective effect in the autistic brains. While additional research is necessary, we believe that the findings of the present study may offer new targets of therapeutic intervention for children suffering from autism. The non-significant change of plasma VEGF in autistic children found in this study suggests that plasma VEGF has no causative or compensatory contribution to the pathology of this disorder. However, further studies should be conducted to shed light on this.

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