Alterations of Prolyl Endopeptidase, Oxytocin and Vasopressin Activity in the Plasma of Autistic Children

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ABSTRACT

Introduction: Prolyl Endopeptidase (PEP), oxytocin and vasopressin activity alteration is associated with various psychiatric disorders such as schizophrenia, mania and depression. Autistic Spectrum Disorders (ASD) is neuropsychiatry and behavioral syndromes affecting social behaviors and communication development.

Aim of the Study: Is to assess plasma levels of PEP, oxytocin and vasopressin in autistic children and to correlate the degree of autism and age of the affected children to plasma levels of those neuro-peptides. The diagnosis of autistic children is in accordance with DSM-IV TR criteria.

Subjects and Methods: They were subjected to childhood autism rating scale, Wechsler Intelligence Scale for children and Vineland Adaptive Behavior Scale. Fluorometric assay was used to measure PEP activity in EDTA plasma in children with ASD (n=15) (mean 7.95 years; SD, 2.51). Oxytocin and vasopressin were measured by commercially available ELISA Kits.

Results: These results were then compared to PEP, oxytocin and vasopressin concentration in a control group of non-ASD children (n=11) (mean 7.65 years; SD, 2.5). An alteration in PEP activity was found in children with ASD compared to control group. There was much greater variation of PEP activity in the group of ASD children when compared to the controls (SD=38.9 and SD 9.6, respectively). This variation was significant ($p<0.0005$), although the mean level of PEP activity in the group of ASD children was slightly higher than in the control group (143±38.9 and 134±9.6, respectively). Oxytocin plasma concentration was significantly lower in the autistic group (123±6.2), as compared to the control group (134±9.6, $p<0.05$). Furthermore, vasopressin plasma level was significantly lower in autistic children (119±5.8, $p<0.05$) as compared to the control group (134±9.6). There were no significant correlations between the degree of autism and levels of oxytocin (123±6.2) or vasopressin (119±5.8). Similarly, there were no significant correlations between the age of the affected children and plasma levels of PEP (143±38.9) Oxytocin (123±6.2) or vasopressin (119±5.8). Labor was induced in 40% of autistic children as compared to 27.3% in the control group.

Conclusion: Our preliminary finding suggests a role for PEP enzyme, oxytocin and vasopressin hormones in the patho-physiology of autism. Higher rates of oxytocin (syntocinon) induction were found among the autistic group. This supports an association between exogenous exposure to oxytocin and neurodevelopment abnormalities.

Key words: Behaviors, health risk, students.

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INTRODUCTION

Prolyl Endopeptidase (PEP, EC 3.4.21.26) is a cytosolic endopeptidase. PEP cleaves peptide bonds on the carboxyl side of proline residues in low molecular weight proteins containing the recognition sequence X-Pro-Y, where X is a peptide or protected amino acid and Y is an amide, a peptide, an amino acid, an aromatic amine or an alcohol[1]. PEP can only hydrolyze small peptides and is thought to be involved in the metabolism of hormones and neuropeptides. However, PEP also degrades many active hormones and neuropeptides, e.g. oxytocin, arginine vasopressin (AVP), substance P, neurotensin, luteinizing hormone-releasing hormone (LH-RH) and thyrotropin-releasing hormone (TRH)[2]. These low molecular weight proteins, particularly oxytocin, AVP, TRH, neurotensin and substance P, profoundly affect social behavior, emotions, stress level, responsivity, reward-seeking and positive reinforcement behavior[3]. Altered PEP

activity has been observed in psychiatric disorders such as depression, mania and schizophrenia[4]. Autism is a neuro-developmental syndrome with markedly high heritability affecting social and communicative development[5]. They were classified as developmental disorders in DSM-IV[6]. Severe communication deficits and social and behavioral abnormalities often appear during the first three years of life but the diagnosis is often made later, due to a lack of resources. The etiology of AD is not yet known. There is no single biological or clinical marker for autism[7]. Symptoms of AD are related to the abnormal functioning of certain centers within the brain: In particular the cerebellum, brain stem and limbic region[8]. AD is also associated with several specific dysfunctions including increased levels of opioid[9,10] and high levels of arginine-vasopressin (AVP). Low plasma levels of the neuropeptide hormone oxytocin have also been found in a group of children with AD when compared to the normal age-matched controls [11].

Altered levels of the neuropeptide hormones oxytocin, arginine vasopressin and other related hormones/peptides may be a result of proteolytic enzyme activity such as PEP, which is involved in the formation and degradation of various neuropeptides[12]. The neuropeptides oxytocine and vasopressin are strong candidates for dysregulation in autism[13]. The aim of the current study is to assess plasma levels of PEP, oxytocin and vasopressin in autistic children and to correlate the degree of autism and age of the affected children to plasma levels of those neuropeptides.

SUBJECTS AND METHODS

The current study was conducted in Psychiatry, Neurology and Clinical Pathology Departments of Zagazig University Hospitals between December 2007 and November 2008. 15 children with AD and a control group of 11 non-AD children participated in this study. The original diagnosis of AD was made in accordance with DSM IV–TR[6]. The severity of autism was assessed by using childhood autism rating scale (CARS)[14]. It is a15-item structured interview and observation instrument. Its total scores range between 15 and 60. A higher score means a more severe degree of autism. Wechsler Intelligence Scale for Children (WISC) was used to estimate the children's functional abilities. The Arabic version of Vineland Adaptive Behavioral scale was used to measure personal and social efficiency of autistic children[15]. Neurological examination was done to asses the motor functions, coordination, Reflexes, cranial nerve domains and examination for the presence of neurological soft signs. The AD group consisted of 11 boys and 4 girls ranging from 3.9 to 12 years (mean 7.95 years;SD, 2.51). None of the children in the control group had any mental disabilities. The control group (non-AD) consisted of nine boys and two girls ranging from 3.5-11.8years (mean 7.65 years; SD, 2.5).

Venous blood from AD children and a control group of non-AD children was collected in evacuated 4 mL EDTA tubes, containing 0.084 ml of 0.34 M K3-EDTA solution. Plasma from EDTA-containing blood was produced immediately after collection by centrifugation at 1300 g for 10 min at 4°C. 30 µL of cocktail inhibitors per 1 mL plasma was then added to the produced plasma sample. The inhibitor cocktail stock solution used was Tris 2.0 M, Na-EDTA 0.9 M, Benzamidin 0.2 M, E-64,10 µM and Pepstatin 48 µM. The PEP activity of the samples was analysed immediately after the production of plasma. The remaining samples were stored at -80°C. The method used to assay the PEP using the hydrolysis of the fluorogenic substrate (Z-Gly-Pro-4-methylcoumarinyl-7-amide) has previously been described by Momeni et al.[16]. This study showed that different factors such as temperature, freeze-thawing cycles, substrate concentration, the organic solvent used to dissolve the substrate and the time of incubation of enzyme-substrate mixture influenced the final fluorescence intensity. 20 µL of plasma was incubated with 200 µL of buffer (100 mM phosphate buffer, pH 7.5, with 1 mM EDTA, 1 mM DTT and 1 mM sodium azide) for 10 min at 37°C to reach thermal equilibrium. 5 µL of the substrate solution containing 18.4 mM Z-Gly-Pro-4-methylcoumariny l-7-amide was then added and the mixture incubated at 37°C for 120 min. The reaction was then terminated by the addition of 1000 µL of 1.5 M acetic acid and the release of 7-amino-4-methylcoumarin measured in a fluorimeter (λex:370nm;λem: 440 nm; slit width: 2.5). The substrate solution was prepared by dissolving Z-Gly-Pro-4-methylcoumariny l-7-amide in 100% 1,4-dioxane and then diluting to 50% (v/v) with incubation buffer. Double assays of each sample were carried out. Any variation in results was insignificant, which confirmed for each of the two results was used for the calculations. Oxytocin and vasopressin were measured by commercially available ELISA Kits.

Statistical analysis:
Results were expressed as mean±sd. Statistical analysis for differences among the groups was assessed by Student's t-test. A p value of ≤ 0.05 was considered significant. The Spearman test for correlation was used. (SPSS for Windows, 2001).

RESULTS

The studied sample comprised 15 children, their mean age was 7.59±2.51 while control group their mean age were 7.65±6.25. Male children comprised most of the studied sample (73.3%), while (26.7%) were females. Most of the parents were university graduates, 80% and 74% for fathers and mothers ,respectively. 40% of the sampled children were from high socioeconomic level, 46.7%. were from middle socioeconomic level, while 13.3% were from low level (Table 1).

Table 1: Demographic data of patients and control.

	Patients No. 15	Control No. 11
Age(y)(mean±SD)	7.95±2.51	7.65±6.25
Sex:	no. (%)	no. (%)
Male	11 (73.3)	9 (81.8)
Female	4 (26.7)	2 (18.2)

Fourty six. sixty six of autistic children had scores less than 37% indicating mild to moderate autistic symptoms while 53.34% scored more than 37% indicating severe autistic symptoms. The total CARS mean score was 39.87±6.78. 60% of the sampled children were with moderate mental retardation while 20% were mild mental retardation. The total mean score was 51.3±23.12. All the autistic children presented with socially immature adaptive behavior with a mean total score 42.61±8.93. They were categorized as mild social impairment 33.36%, moderate impairment 53.34% and severe impairment 13.3% (Table 2).

Table 2: Psychometric measures of the autistic children group.

	No	%
1. Severity of autistic symptoms*		
30-≤37 (mild to moderate)	7	46.66
≥37 (severe)	8	53.34
	X(sd)39.87±6.78	
2. IQ level**		
Average	2	13.3
Mild mental retardation	3	20.0
Moderate mental retardation	9	60.0
Severe mental retardation	1	6.7
	X(sd)51.30±23.12	
3. Social adaptive behavior scale***		
Mild impairment	5	33.36
Moderate impairment	8	53.34
Severe impairment	2	13.30
	X(sd)42.61±8.93	

* According to CARS scores
** According to Wechsler Intelligence scale for children
*** According to Vineland adaptive behavior scale

Neurological soft signs were present in 60% of the autistic children. The commonest signs reported were the over flow movement 60%, poor comprehension of instructions 73% and whole body clumsiness 35% (Table 3).

Table 3: Neurological soft signs and dysmorphic soft signs.

	No	%
*** Neurological soft signs**		
Present	9	60.0
Absent	6	40.0
*** Type**		
Nystagmus	3	20.0
Asymmeteric facial movements	4	26.6
Whole body clumsiness	8	53.3
Over flow movements	9	60.0
Poor performing fine or gross motor tasks	11	73.3
Hypo or hypertonia	5	33.3
Dysarthria	4	26.6
Motor impersistance	6	40.0
*** Dysmorphioc soft signs**		
Present	4	26.6
Absent	11	73.4
*** Type**		
Malformed ears	1	6.6
Micro or macrostomia	1	6.6
High arched palate	2	13.3

Basal plasma PEP activities in the control group (n=11) were between 144.8% and 123.2% fluorescence intensity units (134±9.6). The activity of PEP in the 15 A.D children ranged from 97.6% to 188.4% fluorescence intensity units (143±38.9) (Table 4).

Table 4: Plasma PEP, Oxytocin, and Vasopressin concentration (fluorescence intensity unit) in patients and control .

Group	Plasma Concentration (fluorescence intensity unit) (x=sd)
Control	134±9.6
Patients	143±38.9
PEP	123±6.2
Oxytocin	119±5.8
Vasopressin	

Control vs patients (PEP) p<0.0005
Control vs patients (Oxytocin) p<0.001
Control vs patients (Vasopressin) p<0.001
Very highly significant statistically

The mean level of PEP activity in children with AD was only slightly higher than that in the controls but the variation of PEP activity was much larger in AD children than in the controls (SD=38.9 and 9.6, respectively). The difference was significant (p<0.0005). Oxytocin plasma concentration was significantly lower in the autistic group (123±6.2), as compared to the control group (134±9.6, P<0.05). Further more, vasopressin plasma level was significantly lower in autistic children (119±5.8, P<0.05) as compared to

the control group (134±9.6). There were no significant correlations between the degree of autism and levels of oxytocin (123±6.2) or vasopressin (119±5.8). Similarly, there were no significant correlations between the age of the affected children and plasma levels of PEP (143±38.9) Oxytocin (123±6.2) or vasopressin (119±5.8). Type of labor and mode of delivery in the autistic and control groups are shown in (Table 3). Labor was induced in 6 (40%) of autistic children as compared to 3 (27.3%) in the control group, which is a significant difference ($p<0.05$). Alternatively, mode of delivery was approximately similar in both the autistic and control group.

Table 5: The type of labor and the mode of delivery in autistic children and control.

Variable	Autistic No.=15(%)	Control No.=11(%)
Labor		
Spontaneous	9 (60)	8(72.7)
Induced (oxytocin)	6 (40)	3(27.3)
Delivery		
Forceps/vacuum	2(13.3)	2(18.2)
Cesarian section	5(33.3)	4(36.4)
Normal	8(53.3)	5(45.5)

DISCUSSION

Proline endopeptidase, a cytosolic enzyme isolated from human tissues, cleaves different low-molecular-weight neuropeptide hormones such as oxytocin, AVP, TRH, neurotensin, bradykinin and substance P. The neuropeptide hormones, which contain a proline in the carboxyl side of their sequences, act as a substrate for PEP. It has been reported that PEP activity is altered in individuals with depression, mania and schizophrenia[4]. High PEP serum activity has also been reported in patients with PTSD (post-traumatic stress disorder)[17]. The result of this study showed a significantly higher level of the PEP activity in the group of AD children. It also showed that both oxytocin and vasopressin were significantly lower in autistic children as compared to the control group. High PEP serum activity and the levels of both oxytocin and vasopressin were not related to the degree of the affected autism or to the age of the affected child. Our results are consistent with that of[11]. The male to female ratio was 2.7 to 1, less than the known international ratio of 4 to 1 without any significance. This is probably due to small number of our cases participating in the study. On the other hand, the male to female ratio was 11.8 to 1 in the study of Laila Y[18]. who attributed to more males enrolled in the study than females. Furthermore, there were higher rates of induced labor among AD children as compared to controls.

Both cellular and molecular studies have begun to reveal the mechanisms by which PEP, oxytocin and vasopressin neural pathways are related, leading to a preliminary understanding of how these hormones act within the brain to influence complex social behaviors, communication and rituals. Some researchers have suggested that Oxytocin may play a role in the symptoms of autistic disorder; they reported low plasma Oxytocin levels in autistic compared to age matched normal subjects[11].

Whether abnormalities in oxytocin or vasopressin neurotransmission account for several features of autism need further investigations. These 2 hormones have been implicated in the regulation of behavior in animals, but have not yet been examined in depth in autistic children. As autism appears to be a genetic disorder, mutations in peptide receptors or linkage-specific developmental genes could lead to altered oxytocin or vasopressin neurotransmission[19,20]. A profound impairment in social recognition in vasopressin receptors knockout mice has been shown, indicating an important role played by this peptide in social and affective disorders including autism and anxiety disorders[21]. Brain biochemistry seems to be altered, as demonstrated by Young et al.[22]. In a study of 30 controls and 29 autistic children, it was shown that their blood contained on average significantly less oxytocin. The animals behaved normally, except they could not learn to recognize other mice or recognize their mother's smell, though their sense of smell was normal. A single dose of oxytocin into the brain, however, cured the mice. With regards to this Young quoted "That gives you hope that if autism is related to Oxytocin, it's not permanent[23].

The alteration of PEP activity may support hypothesis[23] that PEP might be involved in the etiology of AD. However, AD can be caused or influenced by external events in early childhood, possibly as result of a genetic predisposition. As a result there may be an inappropriate release of the cytosolic proteolytic enzyme PEP into the circulating blood stream and the cerebrospinal fluid (CSF). PEP cleaves different neuropeptides or their precursor molecules leading to an alteration of the concentration of neuropeptides and this may have a negative effect upon proper brain function. Our results are consistent with the previous hypothesis.

The main deficits of children with AD include early difficulties with social contact, such as eye contact and social smile[24,25]. Attention[26], affects[27,28], reciprocity[29,24] turn-taking, timing and answering parents' signals[27]. Many different problems can arise with respect to co-ordination and motor planning[24], body tonus deficits[25] and problems with mobility. Children with AD have a tendency to ignore other people or may even prefer to be alone[25]. They also have difficulties in signaling for attention and they communicate without meeting the gaze of another person[24]. This could be explained by the effects PEP might have on the neuropeptide hormones when cleaving them and interfering with their proper functions in the processes of early brain development.

Oxytocine and vasopressin contribute to a wide variety of social behaviors, including social recognition,, communications parental care, territorial social bonding. The effects of these two neuropeptides are species –specific receptor distribution in the brain[30]. In three children with AD, the PEP activity was lower than the mean activity of the control group. Twelve AD children had higher PEP activity than average and the remaining three had PEP activity equal

to that of the control group. This variation may be related to different psychiatric disorder from which the patients were suffering, such as depression or mania. There was no significant gender difference in enzyme activity in the control group, neither was there any great variation but in the AD group there was a significant variation, randomly distributed between the sexes. A significant result was observed in the current study as regards sex variation in AD group.

Research is also limited regarding PEP activity associated with other psychiatric disorders. Altered prolyl endopeptidase activity in plasma has been associated with major depressed patients (low levels) and with manic and schizophrenic patients (high levels)[4]. This preliminary finding may indicate an association between altered PEP activity and neuropsychiatric disorders such as ADs.

There is an argument that oxytocin may cause some cases of autism as so many mothers of autistic children had oxytocin (syntocinon) to induce labor. It has been proposed that exposure to high levels of exogenous oxytocin at birth, via oxytocin induction of delivery, might increase susceptibility to autism by causing a down regulation of oxytocin receptors in the developing brain in genetically susceptible children. Gale et al.[23]. Examined the rates of labor induction using oxytocin in children with autism and matched controls with either typical development or mental retardation. Birth histories of 41 boys meeting the criteria for autistic disorder were compared to 25 age- and IQ-matched boys without autism (15 typically developing and 10 with mental retardation). There were no differences in Oxytocin induction rates as a function of either diagnostic group (autism versus control) or IQ level (average versus sub average range), failing to support an association between exogenous exposure to oxytocin and neurodevelopmental abnormalities. Others have suggested that the association was more likely caused by the mother/child unit having sulfation problems, which made it difficult for the mother's oxytocin to be produced in sufficient quantity to move labor along, necessitating a jump-start with exogenous oxytocin (syntocinon). The theory is that mothers with sulfation problems would have a higher likelihood for delayed labor.

Resu1ts from the current study demonstrated significant lower levels of oxytocin and vasopressin in autistic as compared to normal children, which might be related to abnormal social behavior in autistic children. Further more, results showed a higher incidence of Oxytocin induced labor as compared to the normal group. The data supports an association between exogenous exposure to oxytocin and neurodevelopmental abnormalities. Pointing out that it is either that Oxytocin induction might induce down regulation of oxytocin receptors in the child's developing brain, or that the mother has the tendency to secrete less oxytocin, requiring some assistant through Oxytocin induction. The child inherits this tendency for low oxytocin levels in the body, as reflected most obviously in his/her behavior. Two studies have demonstrated improvement in AD symptomatology following administration of intranasaly oxytocin[31]. Another study from the same group showed an improvement in recognition of affective prosody[32]. Further clinical studies are recommended to explore the possible therapeutic effects of oxytocin and vasopressin in autism.

CONCLUSION

Our preliminary finding suggests a role for PEP enzyme, Oxytocin and vasopressin hormones in the pathophysiology of autism but further research should be conducted to establish their role in the etiology of psychiatric and neurological disorders, including autism and related spectrum disorders. However, further clinical studies are recommended to explore the possible therapeutic effects of oxytocin and vasopressin in autism.

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