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THE CORRELATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN OXYTOCIN AND OXYTOCIN RECEPTOR GENES AND THE SERUM OXYTOCIN LEVEL IN THE IRAQI MALE CHILDREN WITH AUTISM

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ABSTRACT

The aim of the present study was to test the possible correlation between oxytocin (rs2770378) and oxytocin receptor (rs53576) polymorphisms with the level of oxytocin in association with the severity of social and cognitive dysfunctions in children with autism. The study was done on 60 males with autism who were recruited from the Pediatric Department at Al-Sader General hospital, Baghdad-Iraq between November 2014 and April 2015. The levels of oxytocin (OXT) were measured in the sera of 60 Iraqi autistic male patients, categorized as mild, moderate and severe (20 patients each) and compared with 26 age- and gender-matched control subjects. The genetic polymorphism OXT/rs2770378 and OXTR/rs53576 was detected by polymerase chain reaction Restriction fragment length polymorphism (PCR-RFLP) method. From the genetic work it was concluded that OXT/rs2770378 polymorphism showed to have an effect on the level of oxytocin in the autistic children that in turn may involve in the pathogenesis of autism. Whereas, OXTR/rs53576 polymorphism showed a non-significant association with autism in the studied children and no effect of this polymorphism was demonstrated on the level of oxytocin.

KEYWORDS: **Autism; Autism Spectrum Disorder (ASD); oxytocin; oxytocin receptor; rs2770378; rs53576.**

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INTRODUCTION

Autism is a neurological developmental disorder that characterized by deficits in social interaction, behavioral patterns, repetitive and restricted interests, and social communication. Symptoms of autism manifested in children usually by the age of 3.[1] It also characterized by impaired language and social skills, and restricted areas of interest. Autistic features may also include poor eye contact, repetitive pattern of behavior, sensory modulatory dysfunction, and varying levels of cognition and motor disturbances.[2] Yet, specific genetic mechanisms underlying this heritability have not yet been discovered. In addition, a substantial number of environmental factors are known, at least in their extremes, to lead to autism-like symptoms. [3, 4] An important challenge in the determination of biomarkers in autism is that these biomarkers may reflect genetic and neurobiological changes or epigenetic processes which may be active only during particular periods of time and do not define the disorder, only the process that led to it. [1] In addition, the research done for autism treatment should ideally include biomarkers that are useful to be used to predict improvements in clinical symptoms from clinical interventions [5] which are important to predict if an intervention is affecting or targeting an active biomedical process that relates to response in the autistic patients at that time. [1] Many hormones and immunological mediators may be involved in the severity and pathogenesis of autism. Oxytocin is a nine-amino-acid peptide, which is synthesized in the paraventricular and supraoptic nucleus of the hypothalamus and released into the bloodstream by the posterior pituitary. It is also widely distributed in the central nervous system and has been shown to play a role in social recognition, memory, and attachment, as well as in stereotyped behaviors such as exaggerated grooming. [6] Oxytocin and its related pathways had been of particular interest in ASD, because of its unique role in influencing social behaviors and its potential for generating animal models with behavioral deficits that may be relevant to ASD. [6] There were also specific hypotheses (supported by varying degrees of evidence) linking oxytocin and ASD, specifically, that autistic children tend to be characterized by lower levels of plasma oxytocin [7], and that infusion of oxytocin was related to the reduction of repetitive behaviors in patients with ASD. For example, it has been hypothesized that administration of oxytocin during labor can generate excess oxytocin in the fetal brain. [8] Such excess might cause down regulation of oxytocin receptors and, subsequently, to imbalance of the oxytocin system and unavailability of oxytocin for further signal transduction cascades. [8] Also of note is that maternal oxytocin has been related to switching GABA signaling in the fetal brain during delivery. Because GABA signaling is seen as one of the disrupted process in ASD, it is possible that oxytocin is indirectly associated with this deficiency.[8] Physiologically oxytocin (OXT) plays a major role in the establishment of affiliative bonds. [9] Some clinical studies report that oxytocin may be a promising drug for psychiatric diseases such as depression, anxiety disorders, schizophrenia, and autism. [10, 12] It also suggests that the brain oxytocin and arginine vasopressin systems are promising pharmaco- therapeutic targets to improve social behavior and to reverse social deficits.[11, 12] Not surprisingly, initial randomized clinical trials of intranasal oxytocin were yielding promising results both on autism core symptoms [6, 13] and on parent–child play interactions when administered to ASD children and to their parents, respectively. [14, 15] On the other hand, studies conducted on animal models suggested a role for oxytocin (OXT, located at 20p13) and oxytocin receptor (OXTR, OXTR, located at 3p26) genes in social-emotional behaviors, and several studies indicate that the oxytocin/oxytocin receptor system is altered in individuals with autism. [16-18] In another study conducted on human, a link was found between the autism phenotype and polymorphism in these genes. [8, 19] Several previous studies demonstrated that many single nucleotide polymorphisms (SNPs) in oxytocin and OXTR genes may associate with the incidence of autism. [18, 21] Genetic studies identified a nominal correlation between the single nucleotide polymorphism (SNP) Rs2770378 in oxytocin gene [21] and Rs53576 in OXTR with autistic traits. [18, 20] These two SNPs were studied in the present work to illustrate their correlation with autism in Iraqi children. A goal of this research in autism is to identify the different abnormal genetic and epigenetic processes that underlie the phenotype of the disorder. This might allow individuals with autism to be classified into subgroups with certain biomarker profiles that may respond more favorably to specific treatments. It also has the potential to clarify the physiologically abnormal aspects that lead to autism, which could improve the understanding of the disorder and lead to earlier diagnosis and more specific and targeted treatments.

MATERIALS AND METHODS

This study was done for 60 male patients who had autism recruited from the Pediatric Department at Al-Sader General hospital, Baghdad, Iraq between November 2014 and April 2015.Their ages were ranged between 3 and 13 years (mean± SD 7.28 ± 2.89 years). The control group comprised of 26 age -matched apparently healthy male children with mean± SD age 6.92 ± 2.59 years. The patients met the diagnostic criteria of autism according to the Diagnostic and Statistical Manual of Mental Disorders (5th Ed.). [22] Patients were sub-grouped into mild (n=20), moderate (n=20) and severe autism (n=20). The control children were normally developing, healthy, unrelated to the autistic children and without any of the exclusion criteria which includes: Any disorder that may be related to the incidence of ASD (e.g. Rett syndrome, focal epilepsy), neurologic problems that involving pathology above the brain stem, except uncomplicated non-focal epilepsy, inherited medical conditions involving the central nervous system (CNS), clinically significant defects in vision or hearing, severe nutritional or psychological deprivation, metabolic disorders (eg. Phenylketonuria), active administration therapy, history of upper respiratory tract diseases, previous inflammatory diseases, allergic history and infectious diseases or neuropsychiatric disorders. The Institutional Review Board at the College of Medicine, University of Al-Nahrain, Baghdad, Iraq, had approved this study. An informed written consent for participation in the study

was signed by the parents or the legal guardians of the investigated subjects according to the Helsinki principles.

Sample collection

Five milliliters of blood samples were collected from both autistic children and control groups then about three milliliters of blood were put into SST and separated sera were kept at −20°C for measurement of serum oxytocin and about two ml of the blood samples was put into an EDTA tube for DNA extraction and subsequent analysis of polymorphism using PCR-RFLP.

Biochemical assays

Oxytocin measurement

The enzyme-linked immunosorbent assay kit (Cusabio, China) was used for the *in vitro* quantitative determination of human oxytocin concentrations in serum. Standards or samples were added to the pre-coated wells with a horseradish peroxidase (HRP) - conjugated antibody and substrate solutions were then added to each well. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution and the color change was measured spectrophotometrically at 450 nm. The oxytocin concentration in the samples was then determined by comparing the optical density of the samples to the standard curve generated for oxytocin.

Genotyping method

The genomic DNA was isolated from fresh sample of whole blood using genomic DNA Mini Kit (Geneid, Taiwan). Genotyping was determined using polymerase chain reaction Restriction fragment length polymorphism (PCR-RFLP) method and the primers' sequences for the detection of OXT/rs2770378 and OXTR/ rs53576 gene) polymorphism [23,24] are shown in table 1 .Subsequently, the PCR product for OXT/rs2770378 gene polymorphism was digested with the restriction enzyme FauI (New England Biolabs, UK).For OXTR/rs53576 polymorphism, the PCR product was digested with the restriction enzyme Sau 96I.

Table 1

Sequence of primer used for PCR amplification of oxytocin (OXT) rs2770378 and OXTR/ rs53576 gene polymorphism

		Sequence (5'->3')	Template strand	Length	Tm	GC%
OXT /rs27703 78	Forward primer	ACTCCACCTCTTCCTCCAGAC	Sense	21	53.4	57.1
	Reverse primer	TTTATTGCACAGGCAGGAGTAG	Antisense	22	53.6	45.5
	Product length	799				
OXTR/ rs53576	Forward primer	GCTGGACTCAGGAGGAATAGGGAC	Sense	24	60.2	58.3
	Reverse primer	GCCCACCATGCTCTCCACATC	Antisense	21	61	61.9
	Product length	340				

PCR conditions used in the detection of OXT/ Rs2770378 and OXTR/53576 polymorphism that give best results were summarized in table (2).

Table 2

The PCR reaction program for the detection of OXT/ rs2770378 and OXTR/ rs53576 gene polymorphisms.

SNP	Type of Cycle	Temperature	Time	No. of Cycle
OXT /rs2770378	Initial Denaturation	95°C	5 min	1 cycle
	Denaturation	95°C	30 sec	35 cycle
	Annealing	55°C	30 sec	
	Extension	68°C	45 sec	
	Final Extension	68°C	10min	1 cycle
OXTR/ rs53576	Initial Denaturation	95°C	5 min	1 cycle
	Denaturation	95°C	30 sec	35 cycle
	Annealing	60°C	30 sec	
	Extension	72°C	45 sec	
	Final Extension	72°C	10min	1 cycle

RESULTS

The FauI restriction enzyme digestion pattern of PCR products of OXT gene is revealed in figure (1). Accordingly, the genotypes of the studied subject were divided into the three following groups based on the presence or absence of A and G alleles in the rs2770378 of the OXT gene polymorphism.

1-G/G homozygous, demonstrated a 76 bp fragment and a18 bp fragment (not seen in figure 1).

2-A/A homozygous state presents the expected 94 bp fragment

3-G/A heterozygous state exhibited 94 and 76 bp fragments.

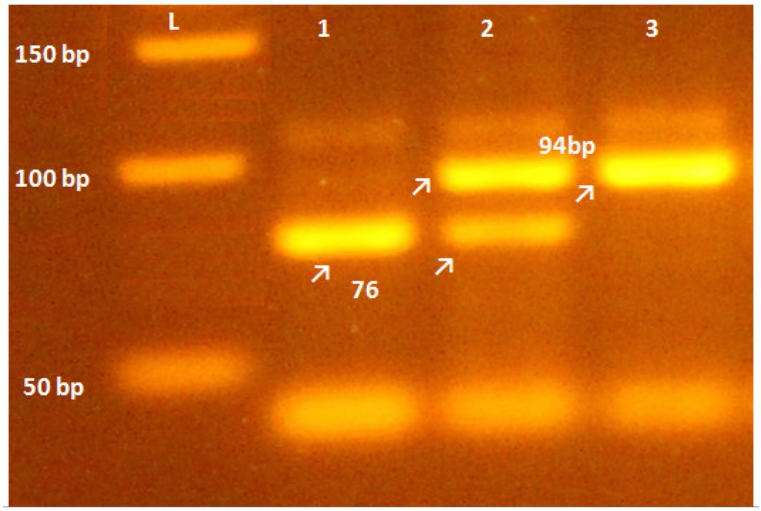

Figure 1
An electrophoreticograph of restriction enzyme (Faul) digestion of PCR products in detection of the OXT rs2770378 Gene polymorphism.

Lane L: 50 b.p DNA Ladder
Lane 1 : shows band of 76 b.p. ; G/G Genotype (homozygote state)
Lane 2: shows band of 76 and 94 b.p. ; G/A Genotype (heterozygote state)
Lane 3 : shown band of 94 b.p. ; A/A Genotype (homozygote state)

On the other hand, the digestion of PCR products of an OXTR gene by the restriction enzyme Sau96I (figure 2) revealed G/G homozygous state (one 219 bp fragment) , A/A homozygous state (one 280 bp fragment) and G/A heterozygous state presented as 280 and 219 bp fragments.

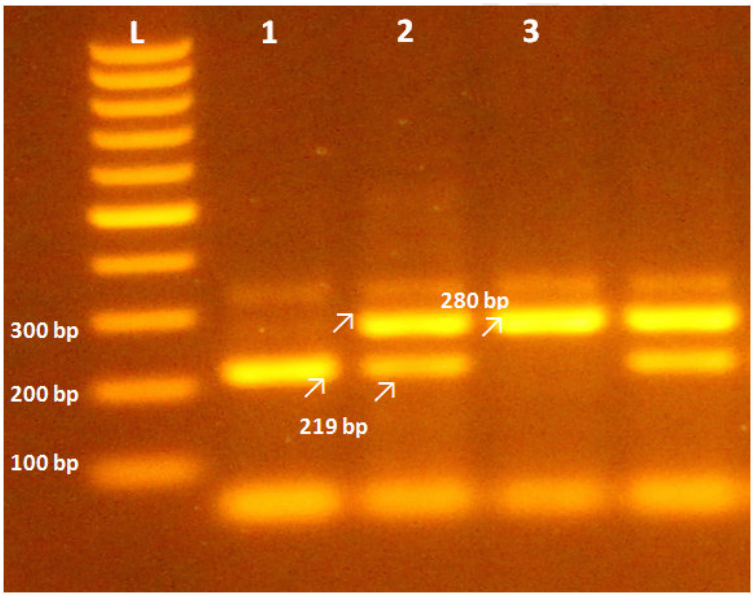

Figure 2
An electrophoreticograph of restriction enzyme (Sau96I) digestion of PCR products of the OXTR rs53576 Gene polymorphism using 2% agarose, 70V, and for 4 hours (7 µl of DNA loaded in each well).

Lane L: 100 b.p DNA Ladder
Lane 1: shows band of 219 b.p. ; G/G Genotype (homozygote state)
Lane 2: shows bands of 280 and 219 b.p. ; A/G Genotype (heterozygote state)

Genotypes and Allele frequencies for OXT rs2770378 and OXTR rs53576 polymorphism

As shown in table (3), for OXT/rs2770378 the highest genotype percentages in the control group was G/G (50%) followed by A\A genotype (30.77%) and 19.23 % for A\G genotype. In whole autistic patients, the highest genotype was the G/G (63.33%) followed by A\A (20%) and A/G genotypes (16.67%). In patients with mild autism, it was revealed that the highest genotype was G/G (60%) followed by both A\A and A/G genotypes (20% for each). In moderate autistic patients, the highest genotype was the G/G (50%) followed by A\A (35%) and A\G genotypes (15%) while in patients with severe autism, the highest genotype was G/G (80%) followed by A/G genotype (15%) and 5 % for A/A genotype (table 3). By using Chi square test, it was also

demonstrated that there were non-significant differences in OXT gene A, G polymorphism between autistic group patients and control groups.While for OXTR/rs53576 the highest genotype in the control group was A/G (50%) followed by G\G genotype (42.31%) and 7.69 % for A\A genotype. In whole autistic patients, the highest genotype was the G/G (50%) followed by A\G (36.67%) and A\A genotypes (13.33%). In patients with mild autism, it was demonstrated that the highest genotype was A/G (50%) followed by G/G genotype (40%) and 10 % for A\A genotype. In moderate autistic patients, the highest genotype was the G/G (65%) followed by A\G (35%) and A\A genotypes (10%), and in patients with severe autism, it was demonstrated that the highest genotype was G/G (45%) followed by A\G genotype (35%) and 20 % for A\A genotype as showed in table (1). By using Chi square test, it was also demonstrated that there were non-significant differences in OXTR gene A, G polymorphism between autistic group patients and control groups.

Table 3

OXT rs2770378 and OXTR rs53576 Genotypes and allele frequencies among autistic patients and control groups.

SNP	Group	Genotype, n (%)			Allele frequency		Pearson's chi-square	
		A/A	G/G	A/G	A	G	x^2	P
OXT/rs27 70378	Control (n=26)	8 (30.77)	13 (50)	5 (19.23)	40.38	59.62		
	Autistic Patients (n=60)	12(20)	38(63.33)	10(16.67)	28.33	71.67	1.52	0.47
	Mild Autism (n=20)	4(20)	12(60)	4(20)	30	70	0.71	0.7
	Moderate Autism (n=20)	7(35)	10(50)	3(15)	42.5	57.5	0.18	0.91
	Severe Autism (n=20)	1(5)	16 (80)	3(15)	12.5	87.5	4.54	0.1
OXTR/rs5 3576	Control (n=26)	2 (7.69)	11 (42.31)	13 (50)	32.69	67.31		
	Autistic Patients (n=60)	8 (13.33)	30 (50)	22 (36.67)	31.67	68.33	1.51	0.47
	Mild Autism (n=20)	2 (10)	8(40)	10(50)	35	65	0.08	0.96
	Moderate Autism (n=20)	2(10)	13(65)	7(35)	27.5	82.5	2.99	0.22
	Severe Autism (n=20)	4(20)	9(45)	7(35)	27.5	72.5	1.92	0.38*

Odd ratios were calculated by comparison of control individuals and autistic groups and the following results were obtained as showed in table (2)

Table 2

The distribution of the A, and G genotypes of OXT/rs2770378 and OXTR/rs53576 polymorphism in autistic groups.

SNP	Group	Genotype	OR	95% CI	P
OXT/rs2770378	Autistic Patients (n=60)	G/G Vs. A/A	1.98	1.01-3.89	0.046*
		A/GVs. A/A	1.39	0.59-3.29	0.46
		G/G Vs. A/G	1.41	0.66- 2.99	0.37
	Mild Autism (n=20)	G/G Vs. A/A	1.86	0.95-3.66	0.072
		A/GVs. A/A	1.63	0.70-3.79	0.25
		G/G Vs. A/G	1.4	0.55-2.37	0.73
	Moderate Autism (n=20)	A/A Vs. G/G	1.13	0.61-2.10	0.7
		A/AVs. A/G	1.43	0.62-3.29	0.4
		G/G Vs. A/G	1.27	0.58-2.77	0.55
	Severe Autism (n=20)	G/G Vs. A/A	9.892	3.62-27.2	<0.001*
		A/GVs. A/A	4.9	1.53-15.65	0.007*
		G/G Vs. A/G	2.03	0.94-4.35	0.07
OXTR/rs53576	Autistic Patients (n=60)	A/A Vs. G/G	1.37	0.52-3.61	0.53
		A/AVs. A/G	2.2	0.83-5.84	0.11
		G/G Vs. A/G	1.61	0.89-2.9	0.11
	Mild Autism (n=20)	A/A Vs. G/G	1.31	0.470-3.66	0.6
		A/AVs. A/G	1.25	0.46-3.43	0.66
		A/G Vs. G/G	1.05	0.59-1.88	0.87
	Moderate Autism (n=20)	G/G Vs. A/A	1.24	0.45-3.39	0.68
		A/AVs. A/G	1.79	0.64-4.98	0.27
		G/G Vs. A/G	2.21	1.234-3.95	0.007*
	Severe Autism (n=20)	A/A Vs. G/G	2.33	0.93-5.86	0.07
		A/AVs. A/G	3.57	1.41-9.02	0.007*
		G/G Vs. A/G	1.53	0.84-2.8	0.17

OR: Odd Ratio, CI: confidence interval.

THE EFFECT of the OXT A, G AND OXTR/ A, G POLYMORPHISM on the Levels of Oxytocin.

As shown in table (3), in the OXT A, G polymorphism, there was a significant decrease in the level of oxytocin in whole autistic patients with G/G genotype when compared with autistic patients with A/A genotype (p= 0.014). It was also demonstrated that the level of oxytocin showed a significant difference (p=0.047) among whole autistic patients with a different genotypes (A/A, G/G and A/G).

Table 5
The effect of the A, and G genotypes of the OXT and OXTR gene polymorphism on the levels of Oxytocin (µIU/ml) in autistic and control groups.

SNP	Group	Genotype (mean± SD)			P^a	P^b	P^c	P^d
		A/A	G/G	A/G				
OXT/rs2770378	Control	45.30 ±18.08	93.29 ±28.83	99.46 ±32.16	0.61	0.8	0.91	0.88
	Autistic patients	63.92 ±23.41	36.7 ±16.63	52.14 ±27.38	0.014*	0.62	0.09	0.047 *
	Mild Autism	101.83 ±41.38	43.77 ±13.28	62.97 ±26.77	0.043*	0.53	0.34	0.16
	Moderate Autism	46.48 ±22.66	91.61 ±15.32	65.34 ±21.22	0.672	0.38	0.16	0.39
	Severe Autism	31.85± 0.00	28.32 ±11.07	24.5 ±6.95	0.76	0.46	0.58	0.79
OXTR/rs53576	Control	79.57 ±33.75	111.44 ±46.23	97.67 ±39.55	0.74	0.82	0.77	0.91
	Autistic patients	48.73 ±29.8	43.65 ±22.67	44.71 ±24.21	0.67	0.84	0.91	0.94
	Mild Autism	98.38 ±35.44	48.32 ±16.93	60.11 ±26.86	0.12	0.49	0.63	0.51
	Moderate Autism	37.5 ±15.44	51.91 ±16.93	38.05 ±14.19	0.5	0.98	0.33	53
	Severe Autism	24.53 ±14.29	27.56 ±9.24	27.47 ±1.64	0.77	0.98	0.79	0.95

P^a value between A/A and G/G genotypes in autistic patients
P^b value between A/A and A/G genotypes in autistic patients
P^c value between A/G and G/G genotypes in autistic patients
P^d value among A/A,G/G and A/G genotypes in autistic patients.

In mild autistic patients, the level of oxytocin showed a significant decrease in patients with G/G genotype when compared with autistic patients with A/A genotype (p= 0.043).On the other hand, in the OXTR/ A, G polymorphism, there was non-significant difference in the levels of Oxytocin among patients and control subjects with either A/A, G/G or A/G genotypes.

DISCUSSION

Many biomarkers might be involved in the etiology, pathogenesis, diagnosis and prognosis of autism. Most of them have an effect on the behavior, cognition and other neurological problems. [10] The present study depends on the information obtained from measuring serum oxytocin and determination of the polymorphism in the oxytocin gene and oxytocin receptor gene which may be related to the autism. [8, 19, 21] Previous studies suggested that the social impairments exhibited by autistic children were associated with changes in plasma oxytocin levels. [2, 7, 16, 17] It was also demonstrated previously that the effects of oxytocin known to be mediated through its specific receptors, and numerous studies had implicated oxytocin receptors in the regulation of social cognition and behavior. [18, 25] For oxytocin gene, many SNPs was studied and linked to autism, such as OXT/rs2770378 that considered as one of the important SNPs that may relate to the incidence of autism among children. [26] Additionally, several SNPs thought to affect the responsiveness of the oxytocin receptors and OXTR/rs53576 was one of these SNPs that studied previously to illustrate its correlation to autism. [25] In the current study, non-significant differences of genotype distribution and allele frequencies of the OXT/rs2770378 between patients with autism and healthy controls were obtained (table 1) which is in agreement with Yrigollen et al., 2008 [8] who reported that there was no association between OXT/rs2770378 and autism and a more recent study which reported that there was an association between the OXT rs2770378 and autism-like traits among 1774 Swedish twin female, but not male subjects. [21] On the other hand, table (2) revealed that the autistic children carried G/G genotype showed to have a significant (p=0.046) higher risk of autism when compared with autistic children carried the A/A genotype. Children with severe autism carrying the G/G genotype also showed a highly significant (p<0.001) increase in the risk of autism in a comparison with a severe autistic children who carried the A/A genotype these finding were also in agreement with study which revealed that autistic patients with A-allele at rs2770378 showed greater improvement in the social withdrawal than G-carriers [27] when treated with risperidone. These significant differences in the risk of autism between G/G and A/A genotypes also accompanied by a significant (p=0. 014) decrease in the level of oxytocin in autistic patients who carried the G/G genotypes when compared with autistic patients harboring the A/A genotypes as demonstrated in the table 3.On the contrary, Goldani et al.(2014) reported that autistic patients carrying A-allele showed a higher level of oxytocin than G-carrier autistic children and respond better to risperidone because oxytocin affects social affiliation and social communication deficits. [1] In this study, non-significant differences in genotype distribution and allele frequencies of the OXTR/rs53576 between patients with autism and control subjects were obtained (table 1) which is in agreement with some studies [20, 28] and these results were inconsistent with other studies. [18, 29] Previous work showed that individuals with one or two copies of the A allele (A/A or A/G genotypes) have an increased likelihood of an autism when compared with patient with G/G genotype. [30] These findings match the results obtained in this study, especially those of patients with severe autism who showed an increase in the prevalence of A/A genotype (20%) in comparison with

control subjects (7.69%). Moreover, the OXTR/rs53576 polymorphism had no effect on the levels of oxytocin and carriers of the A/A genotype showed a non-significantly higher risk of autism than other genotypes studied in autistic children. Collectively, all these factors may affect the autistic child's behavior, sleep pattern and response to treatments and may be used in the future for the diagnosis and prognosis of children vulnerable to autism. The major limitations of this case-control study include limited number of children examined that affect the statistical power of the study, and the second limitation is that the patients were recruited from single hospital which does not reflect the actual problem among Baghdad inhabitants. So, further work is needed on a larger scale from different areas of Iraq and on female gender to address the actual scope of this clinical problem. In conclusion, OXT/rs2770378 polymorphism showed to have an effect on the level of oxytocin in the autistic children subjected to this study that in turn might be involved in the pathogenesis of autism.

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