Progress in Nutrition 2016; Vol. 18, N. 2: 118-124

Original article

The effect of gluten free diet on markers of celiac disease and association with behavioral symptoms in children diagnosed with Autism Spectrum Disorders

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Summary. *Purpose:* The objectives of this study were to measure markers of celiac disease before and after implementing a gluten free diet and also evaluating its association with autism severity. *Methods: T*he present randomized clinical trial was accomplished over 80 subjects diagnosed with Autism Spectrum Disorders (ASD) by the Autism Diagnostic Interview-Revised (ADI-R). Participants were randomly divided into Gluten Free Diet (GFD) and regular diet groups for 6 weeks. Immunological markers consisting of IgA, tTGIgA, tTGIgG and EMAIgG were measured. *Findings:* In this study, 6 patients were tTGIgA positive. In the GFD group, tTGIgA decreased insignificantly (from 3.62 ± 4.25 to 3.41 ± 3.51 U/ml; P<0.05) while it increased significantly (from 2.80 ± 3.54 to 3.96 ± 4.10 U/ml; P<0.05) in the regular diet group. *Conclusion:* These results suggest an underlying immune reactivity to gluten in a subset of children with ASD; however, celiac disease (CD) was not detected in any of the cases. Further investigations are necessary to clarify the possible relationship between ASD and celiac disease. Iranian Registry of Clinical Trials (IRCT201404212017N20), http://www.irct.ir

Keywords: autism spectrum disorders, gluten, celiac disease, immunoglobulin A, tissue transglutaminase II

Introduction

According to centers for disease control and prevention's autism and developmental disabilities monitoring network, the prevalence of ASD has increased from about 1 in 88 children in 2012 up to 1 in 68 children in 2014 (1). "Autistic spectrum disorders (ASD) (autistic disorders; pervasive developmental disorders, not otherwise specified; and asperger disorders) are severe neurodevelopmental disorders with diagnostic features that include qualitative impairment in social interactions (e.g., lack of social reciprocity, marked impairment in eye-to eye gaze, lack of joint attention), qualitative impairments in communication (e.g., lack of language development, echolalia, stereotyped, and repetitive use of language), and restricted repetitive and stereotyped patterns of behavior, interests, and activities" (2). ASD is a complex, multi factorial and clinically heterogeneous disorders with a spectrum of symptoms involving the brain and the body (3, 4). The exact etiology of ASD is still unknown. However, both genetic and environmental factors including diet, toxic chemicals and infections along side with immunological and neurological factors play an important role in the etiology of ASD (5, 6, 7).

For several decades, the association between celiac disease and central nervous system dysfunction has been discussed (8, 9). "Celiac disease (CD), also known as

gluten-sensitive enteropathy is a genetically linked, an autoimmune disorder in which eating certain types of grain-based products trigger an immune response that causes damage to the small intestine" (10). Gluten free diet (GFD) has been a popular treatment in controlling autoimmune abnormalities and neurodevelopmental disorders such as schizophrenia and ASD in a sub group of patients, yet it's effectiveness is still ambiguous (11). The similarities in mechanism of action between CD and ASD led to the hypothesis that a link might exist between these conditions, some of these similarities are discussed below. (a) Neurological and psychiatric comorbidity and conditions such as depression, dementia and epilepsy have been reported in cases of CD. Moreover, attention-deficit hyperactivity disorders (ADHD) has also been observed in both ASD and CD (12). (b) Changes in gut microbial population and similar bacterial species have been detected in both conditions. Also, gastrointestinal abnormalities particularly, constipation, diarrhea, bloating and stomachache alongside with nutritional deficiencies predominantly iron deficiency are common in both conditions (12, 13, 14). (c) The Human Leukocyte Antigen (HLA) DQ2 and DQ8 genotypes are found in 95% patients with celiac disease (31). Similarly, numerous HLA haplotypes, particularly HLA-DR4, have been observed in ASD patients as well (3). Furthermore, the association between ASD and low level antibody responses to tissue transglutaminase, appears to be linked to the HLA-DR3/DQ2 and DR7/DQ2 haplotypes (15).

Presently, there is no recognized cure for celiac disease; however, a gluten-free diet can effectively decline symptoms in a significant number of patients (16). Few articles have also declared an improvement on a number of autistic behaviors after implementing a GFD (17, 18). Goodwin et al. (19) concluded that there is a correlation between autism and malabsorption and also between gluten sensitivity and cognitive impairment. Inconsistently, other studies found no association between autism and celiac disease (20, 21, 22). As can be seen, results of studies examining the association between ASD and CD are conflicting. Considering the high prevalence of celiac disease in Iran (1 in 166) and wheat as a major component of the Iranian diet (>150 kg per person per year) (23), it seemed necessary to conduct a study in order to evaluate the effect of GFD on markers of celiac disease and its association with autistic behaviors in children with ASD.

Methods

Study design

The present randomized controlled clinical trial was carried out on 80 patients (ages 4-16 years) with ASD. Subjects were selected from Iranian special education organization for children with pervasive developmental disorders in Tabriz, Iran, after simple random sampling. The diagnosis was confirmed by the autism diagnostic interview-revised (ADI-R) by a psychologist. Study inclusion criteria were patients being diagnosed with ASD by a psychiatrist and a psychologist, aged 4-16 years, without a special restriction diet based on parent report. Exclusion criteria were patients with intellectual disabilities that were not diagnosed as ASD with ADI-R by a psychologist, individuals with feeding difficulties based on parent report or inpatient and children with additional illnesses or abnormalities diagnosed by a physician. The study population was randomly divided into two groups of 40, matched for age and sex. The first group received a GFD and the other group continued their regular diet for 6 weeks. The duration of the diet was based on the minimum period for achieving a relatively acceptable result since it was the first time that a restricted diet was implied in Iran for ASD patients (24). Two of the children in the gluten free diet did not complete the study; this was largely due to the fact that the parents had difficulty keeping their children on the prescribed diet. Therefore, two children in the regular diet group were also put aside based on their physiologic condition that was similar to the excluded patients in the GFD group. In conclusion there were 2 groups of 38. The GFD consisted of gluten free pasta and biscuits (Aysuda food industrial group) and gluten free breads (Institute of Iranian celiac association, registration number 2895). The products were given weekly according to age requirement. Participants were categorized into 3 groups according to age, group 1 (4-8years), group 2 (9-12 years), group3 (13-16 years). At the beginning of the week, the first group received 1 package of biscuits, 1 package pasta and 4

loafs of bread. The second group received 2 packages of biscuits, 1 package pasta and 7 loafs of bread. The third group received 2 packages of biscuits, 1 package pasta and 9 loafs of bread. In the Iranian special education organization for children with pervasive developmental disorders, there is a special room for parents to stay while there children are training, a dietitian visited them every day and monitored children's diet. Also, we called parents frequently to ask if there were any problems regarding the diet and consulted them. Additionally, a brochure containing a list of gluten free and gluten containing foodstuff and also recipes for preparing gluten free meals based on Iranian cuisine were presented to parents. Thus, parents were familiar with the gluten containing foodstuffs and they were highly encouraged to keep them away from children.

At the beginning and end of the gluten free diet, fasting blood samples were obtained. The Gilliam autism rating scale 2 (GARS-2) questionnaire was administered by a psychiatrist for assessing autistic behaviors and demographic information was gathered by interviewing children's parents. Additionally, written informed consent was obtained from parents. The study protocol was approved by the ethics committee of Tabriz university of medical sciences and was registered in the Iranian registry of clinical trials website (IRCT201404212017N20).

Serological measures

Fasting blood samples were gathered at baseline and 6 weeks follow up for assessing markers of gluten sensitivity consisting of Immunoglobulin A (IgA), IgA anti-tissue transglutaminase antibody (tTGIgA), IgG anti-tissue transglutaminase antibody (tTGIgG) and IgG antiendomysial antibodies (EMAIgG). The enzyme tissue transglutaminase was recently identified as the major autoantigen in CD (24). Systematic review of the available studies revealed that the IgA tTG antibody test has greater than 90% sensitivity and specificity for gluten sensitivity (24, 25). In contrast, the sensitivity and specificity of IgA anti-tTG antibody were 38% and 98% in Iran (24). The sensitivity and specificity of IgG anti-tissue transglutaminase antibody is 98.7% and 98.6% respectively. IgG-EmA, as a highly specific marker for celiac disease alongside IgG-tTG could be a sensitive criteria for diagnosing celiac disease in patients with IgA deficiency (26). In our study, IgA was measured using an IgA kit obtained from Binding slite (United Kingdom). The method was based on Nephrometry using the Mini Neph apparatus. Also tTGIgA (>12 U/ml) was, tTGIgG (>20 U/ml) and EMAIgG (>20 U/ml) were determined by Chemiluminescence immunoassay system with human recombinant tTGA and tTGG as antigen, using a commercial kit (Dia Sorin, Italy).

Autism Diagnostic Interview, Revised (ADI-R)

The ADI-R is preformed as a semi-structured interview with children's parents and caregivers and manages to detect children's early and current development features (27). ADI-R consists of 93 items arranged in three functional domains: language/communication, reciprocal social interactions and restricted, repetitive, and stereotyped behaviors and interests (28). The Persian version of this scale was standardized in 2005 with a reported Cronbach's alpha above 0.80 (29).

Gilliam Autism Rating Scale 2 (GARS-2)

GARS-2 is based on DSM-IV diagnostic criteria for Autism. This tool is a behavioral checklist developed for use with children and youth aged 3-22 years. "GARS-2 consists of 42 items (0-126 scores) grouped into three subscales: Stereotyped Behaviors, Communication and Social Interaction". The Persian version was reviewed for language lucidity and appropriateness for use in Iran. This attempt was in cooperation with the Iranian Special Education Organization (ISEO). The tool was then pilot tested with 15 Iranian families from different socioeconomic backgrounds with a child who had screened positive for ASD. The Cronbach's alpha was above 0.70 (30).

Statistical analysis

The Statistical Package for Social Science (SPSS) version 16 was used for the statistical analysis. The normality of variables was tested by the Kolmogorov–Smirnov test. Categorical variables were tested using Chi-square test and continuous variables were tested using Independent t-Test, Paired-t-Test and Wilcoxon test to compare the differences within a group and between groups. Additionally, Pearson correlation test

was used to assess correlation between autism severity and biochemical measures. All statistical tests were two-sided with a P-value <0.05 considered statistically significant.

Results

Demographic and health characteristics are presented in Table 1. There were no statistically significant differences in any demographic variables including age and gender at the beginning of the intervention.

The mean IgA was 101.38 ± 43.25 mg/dl in the gluten free diet group and 103.26 ± 52.36 mg/dl in the regular diet group. According to the reference range of IgA kit, 9 patients had IgA deficiency.

Table 2 summarizes the immunological measures of patients diagnosed with ASD in the GFD and Regular diet groups. For individuals with IgA in the normal range, tTGIgA was measured and for patients with IgA deficiency, tTGIgG and EMAIgG were analyzed. There were no tTGIgG and EMA IgG positive among patients diagnosed with IgA deficiency.

According to Table 3, 6 children were tTGIgA positive (>12 U/ml) and 2 of the children were in borderline (8-12 U/ml). tTGIgG and EMAIgG were also measured for these children and patients with IgA deficiency, but the results were all negative.

According to the findings of this research, tTGIgA decreased in the Gluten free diet group and the results were statistically significant (p<0.001). In the Regular diet group, tTGIgA significantly increased

Table 1. Demographic and health characteristics of study groups at baseline (n=76).

Variable	Total n=76	Gluten free diet n=38	Regular diet n=38	P-value
Age (yr)(mean; SD)	7.92 (3.37)	7.84 (3.55)	8 (3.22)	0.84
Sex (n;%)				
Male	56 (73.7)	28 (73.7)	28 (73. 7)	1.00
Female	20 (26.3)	10 (26.3)	10 (26.3)	1.00

n: number; SD: standard deviation; yr: year. Categorical variables were analyzed by a two-sided Chi-square test and continuous variables by Independent-t-Test test.

Table 2. Comparison of gastrointestinal symptoms between groups at baseline and follow-up

Serological measures	Gluten free diet (n=38)		P-value	Regular diet (n=38)		P-value
	Baseline	6 weeks		Baseline	6 weeks	
tTGIgA(mean;SD)	6.39(3.62)	5.02(2.81)	<0.001	6.41 (2.82)	6.80 (3.16)	0.044
tTGIgG(mean;SD)						
IgA deficient*	1.83(0.96)	1.79(0.83)	0.893	1.50 (0.95)	1.91 (1.06)	0.100
tTGIgA positive**	2.12(1.04)	1.72(1.01)	0.109	0.80(0.47)	0.85 (0.52)	0.109
EMAIgG(mean;SD)						
IgA deficient	4.22(1.73)	2.46(0.73)	0.205	4.24 (2.20)	7.26 (4.68)	0.120
tTGIgA positive	5.55(2.43)	3.57(2.28)	0.050	3.26 (1.56)	2.80 (0.77)	0.828

SD: standard deviation. Categorical variables were analyzed by Wilcoxon test and Paired t-Test
Serological measures are presented as U/ml
**children with IgA deficiency*
***children with tTGIgA positive*

(p=0.044). In the IgA deficiency subgroup, tTGIgG (p=0.893) and EMAIgG (p=0.205) declined; however, results were statistically insignificant.

According to the results from Pearson correlation test, there was no correlation between a subset of children with tTGIgA positive and autism severity based on GARS-2 questionnaire (p=0.527). Results indicate that there is no correlation between markers of CD and ASD. These results suggest an underlying immune reactivity to gluten in a subset of children with ASD. Therefore, although immunological markers of CD declined in some cases, our study indicates no connection between ASD and celiac disease and raises the possibility of immunological dysfunction.

Discussion

In the GFD group, tTGIgA decreased insignificantly, while it increased significantly in the regular diet group. In a study among 150 children with ASD, 3 patients were diagnosed with selective IgA deficiency. Moreover, there were no evidence of markers of CD (antibodies to gluten or tissue transglutamic acid) in autism patients (31). Also, in another study, increased serum IgG2 and IgG4 concentrations were present in children with autism and were associated with certain behavioral outcomes (32). A study by Elder et al. (24), demonstrated that 87% of the children with autism had high titer IgG antibodies to gliadin. On the contrary, in one study, immunological markers for CD (gliadin IgA, gliadin IgG, tTG, and EMA) were within normal range for all patients with autism (33). In our study there was no correlation between tTGIgA and autism severity. Therefore, this study does not support the relationship between autism and celiac disease. Similarly, in a study by Lau et al. (34), the level of specific markers of celiac disease did not differ between patients and controls. This study believes that the mechanisms of action between ASD and CD are different.

It has been hypothesized that immune aberrations in autistic children have a link with wheat protein "gluten" (35). Disruption in the mucosal lining of the gut best known as intestinal dysbiosis and abnormal carbohydrate digestive enzyme activity causes malabsorption of large proteins such as gluten, gliadin (36). The incomplete digest of these proteins produces gluteomorphin peptides which have opioid activity and pass through an abnormally permeable intestinal membrane and enter the central nervous system and are believed to act like neuropeptides and alter neurologic function. Additionally, they can stimulate T-cells, induce peptide-specific T-cell responses and abnormal cytokine which could cause inflammation, autoimmune reaction and disrupt neuroimmune correlation (37, 38).

Another condition which has been discussed in the last two decades is the term non celiac disease gluten sensitivity (NCGS). NCGS is a non-autoimmune and non-allergic condition without the celiac disease diagnostic criteria anti-TTG and anti-EMA positive. NCGS can be expected in patients with gluten intolerance that is not diagnosed as CD or wheat allergy, the common aspect is that they all get better with a gluten free diet. Although NCGS is the most com-

Table 3. Results of serological tests (tTGIgA, tTGIgG, EMAIgG) in cases with tTGIgA positive

Groups	Sex	Age	tTGIgA (U/ml)		tTGIgG (U/ml)		EMAIgG (U/ml)	
			Baseline	6weeks	Baseline	6weeks	Baseline	6weeks
Gluten free diet (n=38)	female	8	14.78	12.23	2.50	1.53	6.95	5.46
	male	5	16.96	13.82	2.54	1.78	7.97	6.49
	male	6	14.91	5.50	2.89	2.71	6.65	1.89
Regular diet (n=38)	male	6	13.36	17.20	0.46	0.78	2.15	3.35
	male	4	12.82	13.53	0.61	0.64	4.37	2.25
	male	5	12.97	14.75	1.35	1.45	3.67	6.40

mon syndrome of gluten intolerance, the only known antibodies in these patients are IgG antigliadin antibodies (IgG-AGA), which are detected in only half of the patients and have low sensitivity (39, 40). Future studies should be given further attention to the relationship between ASD and NCGS and specific diagnostic measures.

Our study has the following limitations; firstly, the relatively small number of subjects enrolled in this study and heterogeneity of the age of subject's clearly present the most significant limitations to this study. Also, the short duration of the intervention is another limit to this study. Secondly, even though parents tried to hide prohibited foods and were careful about food sneaking, they were not always successful. Ideally, it would have been superior performing the study in a more supervised setting and lowering the possibility of diet disruption. Thirdly, although CD is usually detected by serologic testing of specific antibodies, particularly tTG and EMA, duodenal biopsy and DQ2 or DQ8 encoding genes which are necessary to confirm the diagnoses were not implied in our study (41).

Increased intestinal permeability resulting from damage to the intestinal epithelial barrier in those with ASD may be responsible for increased exposure of the immune system to partially digested gluten fragments, resulting in the detected increase in antibody response (34, 42, 18). Evidence suggests that manipulation of the immune system and restricting potential pathogens could improve core features of ASD (11). Therefore, better understanding of the mechanisms involved in the gluten sensitivity observed in ASD is a helpful strategy for investigating suitable therapies. Searching for ASD subpopulations that will best respond to the gluten free diet is another aspect that must be taken into account.

Acknowledgement

The authors are grateful for the financial support of the Nutrition Research Center, Tabriz University of Medical Sciences. The authors also are deeply indebted to all patients who participated in this study and their caregivers.

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