International Immunopharmacology 47 (2017) 1–8

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The effects of modified *versus* unmodified wheat gluten administration in patients with celiac disease

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ARTICLE INFO

Article history:
Received 6 February 2017
Received in revised form 9 March 2017
Accepted 9 March 2017
Available online 23 March 2017

Keywords:
Celiac disease
Alternative celiac disease treatment
Enzyme-modified wheat flour
Gluten-free diet
Microbial transglutaminase

ABSTRACT

Celiac disease (CD) treatment requires a gluten-free diet (GFD), although alternative approaches have been proposed. Modification of gliadin peptides using microbial transglutaminase (mTG) inhibits their ability to induce immune response *in vitro*. Our aim was to evaluate the safety of mTG-modified wheat flour ingestion in CD patients. Twenty-one CD patients in remission were randomized to receive mTG-modified (n = 11) or unmodified (n = 10) wheat flour rusks, in double-blind fashion. Monthly, patients completed a symptom questionnaire. Serum anti-tTG, EMA and creatinine levels were monitored. At baseline and after 90 days, serum anti-actin antibodies (AAA) were measured and upper endoscopy was performed. Data were analyzed by non-parametric tests. 7/11 patients eating modified rusks and 7/10 patients receiving unmodified rusks completed the study. At baseline, all patients showed negative serum anti-tTG and EMA results. At the end, 2/7 (28.6%) patients ingesting modified and 4/7 (57.1%) patients taking unmodified rusks presented positive serum anti-tTG and EMA results. Creatinine results were unmodified. Moreover, 1/7 (14.3%) patients ingesting modified and 4/7 (57.1%) patients taking unmodified rusks presented villous atrophy. In patients who received unmodified rusks, the AAA levels increased significantly and duodenal anti-tTG levels appeared higher than those measured in patients who ate modified rusks. Abdominal swelling, bloating and nausea were more severe in patients ingesting unmodified rusks than those taking modified rusks. Our results may support larger clinical trials to confirm the enzymatic treatment of wheat flour as an alternative to GFD.
Clinicaltrials.gov registration no: NCT02472119.

1. Introduction

Celiac disease (CD) is a chronic immune-mediated inflammatory disorder of the intestinal mucosa, triggered by ingestion of wheat gluten and related proteins from rye and barley in human leukocyte antigen (HLA)-DQ2 and/or -DQ8 positive individuals [1]. Gluten is essentially composed of two protein fractions - gliadins and glutenins - which are characterized by high levels of glutamine (30–35%) and proline (10–15%). DQ2 has a preference for binding peptides containing negatively charged residues at the P4, P6 or P7 anchor positions and DQ8 prefers motifs containing two acidic residues at the P1 and P9 pockets. In the small intestine of CD patients, after deamidation, gliadin peptides increase their affinity binding for both DQ2 and DQ8 molecules [2,3]. In particular, specific glutamine residues are converted to glutamic acid by tissue transglutaminase (tTG) [4]. Furthermore, the proline residues in gluten, which are resistant to intestinal proteases, ensure the survival of the immunostimulatory epitopes [5]. The gliadin peptides induce alterations in the duodenal mucosa by activating intestinal-specific CD4+ T cells, resulting in intraepithelial lymphocytosis, crypt

Abbreviations: AAA, anti-actin antibodies; anti-tTG, anti-tissue transglutaminase; CD, celiac disease; ELISA, enzyme-linked immunosorbent assay; EMA, endomysium antibodies; GFD, gluten-free diet; HLA, human leukocyte antigen; IECs, intestinal epithelial cells; IELs, intraepithelial lymphocytes; K-C_2H_5, lysine ethyl ester; K-CH_3, lysine methyl ester; mTG, microbial transglutaminase; tTG, tissue transglutaminase; VAS, visual analogue scale.

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http://dx.doi.org/10.1016/j.intimp.2017.03.012
1567-5769/

hyperplasia and villous atrophy [1,2]. The gluten-induced immune reactions also lead to production of specific antibodies against tTG (anti-tTG) and endomysium (EMA), suggesting an autoimmune mechanism [6,7], as well as antibodies against deamidated gliadin peptides [8]. The treatment of CD is currently based on a lifelong gluten-free diet (GFD) to normalize the antibody serum levels and to recover the intestinal mucosal integrity. However, GFD compliance is very difficult and over the years, alternative approaches have been proposed in order to improve the quality of life of CD patients [9]. Some of the strategies considered include detoxification of dietary gluten, reduction of the immunogenicity of gliadin peptides, modulation of intestinal permeability, inhibition of antigen presentation (using anti-tTG or HLA-blockers) and modulation of inflammation (by anti-IFN-γ, anti-IL-15 or anti-TNF-α) [10,11]. Recent studies have reported an alternative therapeutic approach that inhibits the immunotoxicity of gliadin peptides by enzymatic transamidation using food-grade microbial transglutaminase (mTG), a transamidase of the *endo*-γ-glutamine:ε-lysine transferase type [12]. The mTG was shown to exhibit a site specificity similar to tTG on synthetic peptides, but lacks deamidase activity [13]. In detail, the gliadin transamidation following treatment of wheat flour with mTG and lysine methyl ester (K-CH_3) or lysine ethyl ester (K-C_2H_5) inhibits the ability of gluten to induce the CD-specific immune response in *in vitro* human and murine experimental models [13,14]. In the food industry, the covalent attachment of amino acids is also accepted to improve the nutritional quality and functional properties of food proteins. Moreover, the presence of ε-(γ-glutamyl)-lysine in gliadins is safe for humans and the final catabolic step of transamidated gluten occurs in the kidneys, where the isopeptide provides a substrate for γ-glutamylamine cyclotransferase [15].

Based on these findings, our aim was to perform a double-blind, gluten-controlled clinical trial to evaluate the safety of a protracted ingestion of mTG-modified wheat flour in CD patients. The primary outcome was to evaluate the tolerance of CD patients to treated flour by analyzing serum anti-tTG and EMA levels. The secondary outcomes included the evaluation of changes in serum anti-actin antibodies (AAA), in the Marsh-Oberhuber degree of intestinal biopsy samples, in anti-tTG antibodies in the organ culture system, as well as the appearance of clinical symptoms. In addition, we measured serum creatinine levels to monitor the integrity of renal function.

2. Materials and methods

2.1. Patients

The study was a randomized, controlled clinical pilot trial. The randomization was performed by a researcher who did not participate in data collection using a computer-generated number sequence (www.randomization.com).

Twenty-two CD patients in remission (7 male/15 female, mean age 39.4, range 19–65 years), who were referred to our Gastroenterology Unit and agreed to undergo the trial, were enrolled in this study. All patients had been diagnosed in agreement with the British Society of Gastroenterology diagnostic guidelines [16,17]. In particular, they presented a histological pattern corresponding to type III based on the Marsh-Oberhuber classification, positive anti-tTG and EMA serological results and complained of gastrointestinal and/or extra-intestinal symptoms at the time of diagnosis. Moreover, one patient presented dermatitis herpetiformis. At the beginning of the trial, all patients were on a GFD for at least 18 months and presented negative anti-tTG and EMA serum antibody results for at least one year. Patients with inflammatory bowel disease, tumors and liver diseases were excluded. All patients presented normal renal function.

One out of 22 patients declined participation after elucidation of the protocol study; the remaining 21 patients were randomized to receive 100 g/day of rusks manufactured with mTG-modified (n = 11 patients) or unmodified (n = 10 patients) wheat flour, in a double blind fashion. During treatment, other 7 patients (4 from the modified and 3 from the unmodified group) discontinued their participation because of the low palatability of the product and/or the occurrence of clinical symptoms. Finally, only 14 CD patients concluded the 90 day trial. Patients were monitored every month for the appearance of the main gastrointestinal and/or extra-intestinal symptoms present at diagnosis using a modified questionnaire. The perceived severity of symptoms was quantitatively evaluated by means of a visual analogue scale (VAS): a 10 cm straight line with two ends corresponding to “no symptoms” and “maximum possible” (or the maximum patient has experienced). The VAS is a one-dimensional tool that quantifies what the patient subjectively perceives. Both the questionnaire and VAS were elaborated according to the Salerno Experts' Criteria in the diagnosis of non-celiac gluten sensitivity [18]. Serum anti-tTG and EMA, as well as creatinine concentration were also monitored. At baseline and after 90 days, patients were subjected to upper endoscopy with biopsy to evaluate histological alterations.

All procedures followed in this study were in accordance with the ethical standards of the institutional committee responsible for human experimentation. Furthermore, an informed consent was obtained from each participant being studied.

(Local Ethics Committee code: Prot. 672/10 - Rif. 1907/22.07.2010; ClinicalTrials.gov identifier: NCT02472119).

2.2. Durum wheat flour transamidation and rusk preparation

A pilot-scale transamidation of commercial durum wheat flour was conducted using food-grade mTG (8 U/g flour; ACTIVA®WM; 81–135 U/g, Ajinomoto Foods, Hamburg, Germany) and 20 mM K-C_2H_5 (NutraBio.com, Middlesex, NJ) for 2 h at 30 °C, followed by centrifugation of the flour suspension and a second step for 3 h at 30 °C with fresh enzyme and K-C_2H_5. The recovered dough was used to manufacture the double-baked bread slices under gluten-free conditions by adopting a classical baking procedure; the control bread was similarly treated but without the addition of enzyme. A R5-sandwich enzyme-linked immunosorbent assay (ELISA) of the manufactured bread was performed by a certified independent Institute (Istituto Ricerche Agrindustria, Modena, Italy) and the detectable gluten level was found to be 80 ppm.

2.3. Purification of gliadins

A sample of 20 mL semolina suspension from transamidated flour was centrifuged at 3000*g* for 10 min. The residual gliadin fraction was extracted from the protein pellet using a modified Osborne procedure [19]. The protein content was assessed by Bradford analysis [20].

2.4. In vitro immunological assessment

Transgenic mice expressing the HLA-DQ8 molecule in the absence of endogenous mouse class II genes [21] were reared in gluten-free and pathogen-free conditions at our animal facility (accreditation n. 164/99-A). All procedures met the guidelines of the Italian Ministry of Health. Six-week-old mice (n = 6) were immunized by intraperitoneal injection with gliadins (100 μg) emulsified in Freund's adjuvant (Sigma, Milan, Italy) on days 0, 7 and 14. Mice were sacrificed on day 21 to recover their spleens. Spleens were passed through a stainless steel wire mesh to dissociate cells. Erythrocytes were removed by treating the cell suspensions with a Tris-buffered ammonium chloride solution. 5×10^6 mouse spleen cells were incubated in 1 mL culture medium in 24-well flat bottom plates at 37 °C for 72 h in the presence of antigens (200 μg/mL). The culture supernatants were then collected and analyzed for IFN-γ by in-house sandwich ELISA.