INVESTIGATION OF GENOTOXICITY IN INTESTINAL EPITHELIAL CELLS AND LYMPHOCYTES OF CELIAC PATIENTS

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ABSTRACT

Objective: *To investigate the presence of genotoxicity in intestinal epithelial cells and lymphocytes in celiac patients and to assess DNA repair capacity.*

Material and Method: *The study comprised 15 newly diagnosed celiac patients, 14 celiac patients receiving gluten-free diet, and 9 non-celiac patients who had undergone endoscopy for various reasons (control group). Comet assay was performed to detect DNA injury. DNA injury was measured both after being exposed to stress by hydrogen peroxide and 20 minute regeneration period to measure DNA repair capacity.*

Results: *DNA injury in the intestinal epithelial cells was significantly higher in the newly diagnosed celiac patients than that in the controls (Tail DNA%: 25.9±1.5 and 15.7±0.9, respectively, p<0.001). Although DNA injury (Tail DNA%) in the lymphocytes was higher in the newly diagnosed celiac patients (3.7±0.3) than that in the celiac patients on diet (2.9±0.9; p=0.020), it was not different from the controls (4.3±0.3; p=0.100). DNA injury in the lymphocytes after hydrogen peroxide exposure was higher in the newly diagnosed celiac patients than that in the celiac patients on diet and controls (Tail DNA%: 14.2±0.6, 10.7±0.5 and 12.4±0.6, respectively). The remaining DNA injury after regeneration period was also higher in the newly diagnosed celiac patients compared to those in the celiac patients on diet and controls (Tail DNA%: 8.4±0.5, 6.3±0.3 and 6.4±0.4, respectively).*

Conclusion: *Genotoxicity was detected in the intestinal epithelial cells of the newly diagnosed celiac patients. Lymphocytes of these patients were more susceptible against stress and had low DNA repair capacity. Thus, DNA injury in celiac patients may contribute to the development of malignant diseases.*

Key Words: *Celiac disease, comet assay, cancer*

ÇÖLYAK HASTALARINDA BAĞIRSAK EPİTEL HÜCRELERİNDE VE LENFOSİTLERDE GENOTOKSİSİTENİN ARAŞTIRILMASI

ÖZET

Amaç: Çölyak hastalığı (ÇH) olanlarda bağırsak epitel hücreleri ve lenfositlerde genotoksisitenin varlığını araştırmak ve DNA tamir kapasitesini değerlendirmek.

Materyal ve Metot: Çalışmaya yeni tanı alan 15 çölyak hastası (Yeni-ÇH grubu), glutensiz diyet almakta olan 14 çölyak hastası (Diyette-ÇH grubu) ve ÇH olmayan, farklı nedenlerle endoskopi yapılan 9 hasta (kontrol grubu) dahil edildi. Bağırsak epitel hücreleri ile periferik kan lenfositlerinde DNA hasarını saptamak için Comet testi (singlecell gel electrophoresis) yapıldı. Hücrelerdeki DNA tamir kapasitesini ölçmek için de hem hidrojen peroksit ile strese maruz bıraktıktan sonra hem de 20 dakikalık rejenerasyon sürecinden sonra DNA hasarı ölçüldü.

Bulgular: Yeni-ÇH grubunda bağırsak epitel hücrelerinde saptanan DNA hasarı, kontrol grubuna göre anlamlı yüksek bulundu (sırayla 25,9±1,5 ve 15,7±0,9 tail DNA%, p<0,001). Lenfositlerdeki DNA hasarı ise (tail DNA%), Yeni-ÇH grubunda (3,7±0,3) Diyette-ÇH grubuna (2,9±0,9) kıyasla fazla olmasına rağmen (p=0,020) kontrol grubuna göre (4,3±0,3) farklı değildi (p=0,100). Hidrojen peroksit ile muameleden sonra lenfositlerde gelişen DNA hasarı Yeni-ÇH grubunda, Diyette-ÇH ve kontrol gruplarından daha fazla idi (sırasıyla 14,2±0,6; 10,7±0,5 ve 12,4±0,6 tail DNA%). Rejenerasyon süreci sonrasında Yeni-ÇH grubunda kalan DNA hasarının Diyette-ÇH ve kontrol gruplarından fazla olduğu belirlendi (sırasıyla 8,4±0,5; 6,3±0,3 ve 6,4±0,4 tail DNA%).

Sonuç: Yeni-ÇH grubunda bağırsak epitel hücrelerinde genotoksisite saptanmıştır. Bu hastalara ait lenfositlerin strese karşı daha duyarlı oldukları ve DNA tamir kapasitelerinin düşük olduğu belirlenmiştir. Bu nedenle çölyak hastalarındaki DNA hasarının malign hastalıkların gelişmesine katkı sağlayabileceği düşünülmüştür.

Anahtar Kelimeler: Çölyak hastalığı, comet testi, kanser

INTRODUCTION

Celiac disease (CD) is a multifactorial enteropathy, in which gluten sensitivity and predisposing genes (mainly major histocompatibility complex class II genes encoding HLA-DQ2/8 molecules) play a role in etiology, and is characterized by villus atrophy, crypt hyperplasia, and intraepithelial lymphocyte increment in the small intestine.CD is usually a benign disease that can be treated by gluten-free diet. Nevertheless, the risk for malignant diseases has been reported to be higher in CD patients as compared to the healthy individuals of population.[1] It has been demonstrated that the risk for the development of non-Hodgkin lymphoma, small intestinal adenocarcinoma, esophageal cancer, and melanoma is higher in CD patients than general population.[2-6]

It is assumed that DNA injury and impaired DNA repair mechanisms in celiac patients lead to microsatellite instability, which forms a basis for the development of malignant disease.[7-9] The fact that exposure to gliadin reduces cell viability and induces morphological damage has been experimentally demonstrated in the cell culture studies and it has been reported that gliadin-related cell damage is similar to enzyme-mediated cell damage.[10] It has also been reported that these alteration are similar to those observed in the duodenal biopsy samples of untreated CD patients.[10]

The aim of the present study was to identify DNA injury in the intestinal epithelial cells and peripheral blood lymphocytes and to assess DNA repair capacity in CD patients.

MATERIAL and METHOD

Patients:
The present study was approved by the Ethics Committee of Ondokuz Mayıs University Medical Faculty and informed consents of the families were obtained prior to the study. Patients were assigned into three different groups. New-CD group consisted of the patients who were newly diagnosed histopathologically in addition to clinical and serological findings (antigliadin antibody and antiendomysial antibody positivity). On diet-CD group consisted of the previously diagnosed celiac patients who were receiving gluten-free diet for a mean of 27.5±18.3 months (range 9-52 months). Control group consisted of the patients who had no systemic disease, negative antiendomysial antibody, and underwent upper gastrointestinal system endoscopy for dyspeptic complaints.

Sample Collection:
Four mucosa biopsies were taken from the second part of the duodenum of the patients in the new-CD and on diet-CD groups. Two biopsy samples underwent histopathological examination after being stained with hematoxylin and eosin and other two biopsy samples underwent Comet assay. Biopsy samples were exposed to ethylenediaminetetraacetic acid/ethylene glycol tetraacetic acid and intestinal epithelial cells were isolated by means of chelation method. A mean of 100 cells were isolated from each patient. Biopsy samples were also taken from the small intestines of the controls during endoscopic examination.

Tablo 1. General characteristics of the patients

	New-CD Group (n=15)	On diet-CD Group (n=14)	Control Group (n=9)
Age (year)	12.5±4.4	11.5±4.2	14.8±1.4
Gender (F/M)	7/8	10/4	2/7
BMI (kg/m2)	18.4±3.0	17.8±2.9	17.9±1.5
Symptom duration (month)	24.6±33.8	44.4±38.9	-

Values are demonstrated as mean±standard deviation or number (n), where appropriate

For all patients in each group, a blood sample of 4 mL was taken into the tubes containing ethylenediaminetetraacetic acid and centrifuged with 4 mL histopaque-1077 (sigma) to isolate the lymphocytes of peripheral blood.

DNA Injury Analysis (Comet Assay):
DNA injury in the intestinal epithelial cells and lymphocytes was quantitatively detected by Comet assay (percent of DNA in tail).[11,12] A poly-l-lysine-covered slide was prepared and agarose gel and cells were spread over the slide for the cells to undergo lysis. Alkali (pH=13.1) unwinding (separation of DNA chains from each other), alkali (pH=13.1) electrophoresis, neutralization, DNA staining with ethidium bromide, visualization via fluorescent microscope, and Comet scoring via "CASP" image analysis program were performed.

In order to assess DNA repair capacity in the lymphocytes (H_2O_2 exposure-Challenge test), 3 slides were prepared and DNA injury was assessed in three steps; I) Initially existing DNA injury, II) DNA injury after exposing to peroxide (DNA injury assessment via Comet assay by incubating with 75 µM H_2O_2 for 5 minutes at 4°C), III) DNA injury after 20-minute repair period at 37°C in the cells that have been exposed to peroxide.

Statistical Analysis
Comparison between two groups was performed by Student t-test and comparison between more than two groups was performed by One-Way and Two-Way Analysis of Variance. Correlations between the data were performed by Spearman's correlation analysis. Values of $p<0.05$ were considered significant. →

Table2. DNA injury in the lymphocytes (tail DNA%)

	New-CD Group (n=15)	On diet-CD Group (n=14)	Control Group (n=9)	p1	p2	p3
Initial	3.7±0.3	2.9±0.9	4.3±0.3	0.020	0.100	<0.001
Peroxide exposure	14.2±0.6	10.7±0.5	12.4±0.6	<0.001	0.030	0.039
Regeneration	8.4±0.5	6.3±0.3	6.4±0.4	0.001	<0.001	0.890

Values are demonstrated as mean±standard deviation.
p1: New-CD group versus on diet-CD group **p2:** New-CD group versus control group **p3:** On diet-CD group versus control group

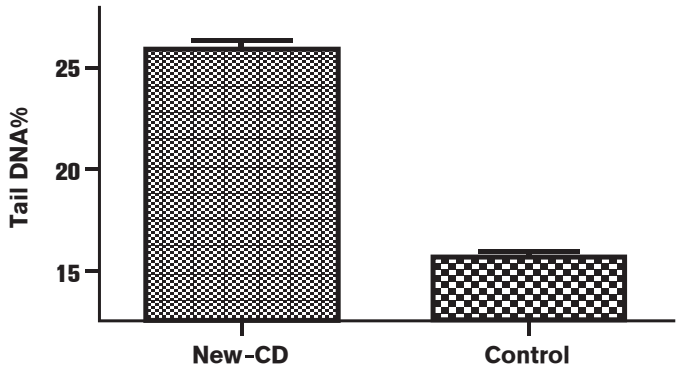

Figure 1. DNA injury in the intestinal epithelial cells of the new-celiac disease group and the control group

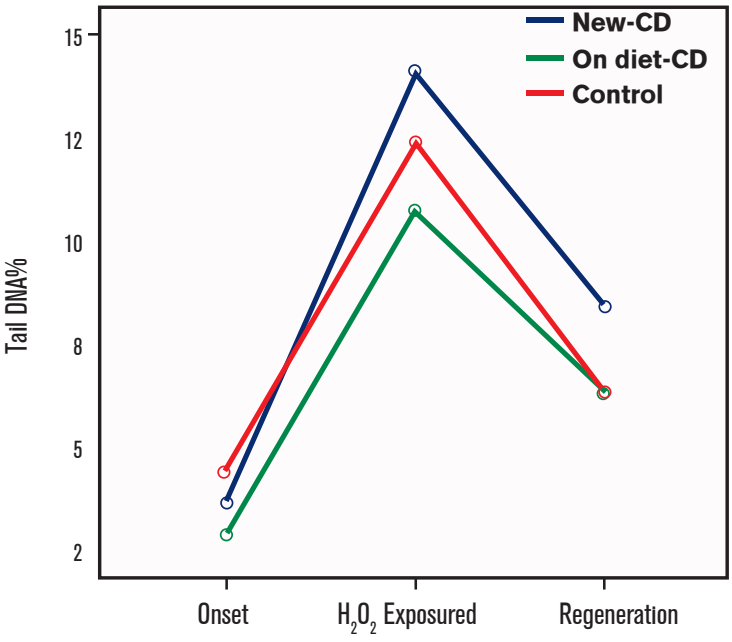

Figure 2. Assessment of DNA repair capacity in lymphocytes

RESULTS

There were no differences between the study groups in terms of age, gender distribution, and body mass index. General characteristics of the patients are presented in Table 1.

Histopathological examination of intestinal biopsy samples revealed that 3 patients had type 1, 4 patients had type 2, and 8 patients had type 3 findings according to Marsh classification in the new-CD group. Histopathological examination of on diet-CD group prior to diet revealed that 1 patient had type 1, 5 patients had type 2, and 4 patients had type 3 findings, whereas 4 patients could not be classified. In these patients, clinical and biochemical signs were improved after gluten-free diet and antigliadin antibody and antiendomysium antibody became negative. Antiendeomysium antibody was negative in all of the patients in the control group and histopathological examination of biopsy sample taken from small intestine showed no villous atrophy or crypt hyperplasia.

DNA injury in the intestinal epithelial cells was significantly higher in the new-CD group than that in the control group (25.9±1.5 vs 15.7±0.9 tail DNA%, p<0.001) (Figure 1).

DNA injury in the lymphocytes was higher in the new-CD group than the on diet-CD group. DNA injury after peroxide exposure and repair period was also higher in the new-CD group as compared to the other two groups. Assessment of DNA injury and DNA repair capacity is demonstrated in Table 2 and Figure 2.

In the new-CD group, no significant correlation was determined between DNA injury in the intestinal epithelial cells and intestinal damage detected by histopathological examination performed after staining with hematoxylin and eosin (according to Marsh classification) (r=-0.057, p>0.05). There was also no significant correlation between DNA injury in the lymphocytes and histopathological intestinal damage (Marsh classification) (r=0.079, p>0.05). Evaluation for the effect of duration of gluten exposure on DNA injury showed negative significant correlation between patient age and DNA injury in the intestinal epithelial cells (r=-0.281, p<0.001). No gender effect was determined on DNA injury in the intestinal epithelial cells and repair capacity of lymphocytes (p=0.100 and p=0.440, respectively).

DISCUSSION

Celiac disease leads to chronic inflammation and atrophy in the intestinal mucosa.[13] It is known that certain malignancies are more prevalent in celiac patients than general population; the risk for intestinal lymphoma, hepatobiliar carcinoma, pancreatic carcinoma, oropharyngeal carcinoma, and esophageal carcinoma is increased. Malignancy occurs generally in 8-13% of celiac patients and is the most common cause of mortality. Factors that lead to such an increment have been the subject of investigations. These factors include gluten-related mucosal injury due to the lack of specific mucosal peptidases →

leading to chronic inflammation by stimulating immunological response, proinflammatory cytokine release, increased permeability for environmental carcinogenesis, and nutritional deficiency.[4,14] Early diagnosis is important since the risks are developed before diagnosis.[15] After 5-year gluten-free diet, the risk is similar to that in the general population.[16] Kolacek et al. compared untreated celiac patients and non-celiac enteropathies (chronic diarrhea, postenteritis syndrome, cow milk protein intolerance) with the control group and found the chromosomal aberrations (instability and fragility) to be significantly higher in both patient groups as compared to the control group.[13] Consequently, they reported that genomic instability was increased not only in celiac disease but also in the other non-celiac enteropathies and children were similarly involved with the adults. However, the above-mentioned study was performed only with peripheral blood sample and it was stated that genomic instability was needed to be studied in the intestinal cells. In the present study, genotoxicity was assessed both in the peripheral blood and intestinal cells.

Comet assay, one of the genotoxicity tests, is an easily applicable method for detection of DNA stand breaks at cellular level.[11,12] As compared to the other tests, advantages of Comet assay may include proven sensitivity for detecting low levels of DNA damage, requirement for small numbers of cells per sample, şexibility, low costs, ease of application, possibility of conducting studies using relatively small amounts of a test substance, and the relatively short time period (a few days) needed to complete an experiment.[12] In the present study, Comet assay was used to detect DNA injury.

Genotoxicity is the damage of genetic material. Genomic instability presents itself in two forms. The first one is microsatellite instability, which results from the defects in DNA repair mechanisms and the second one is chromosomal instability, which is characterized by chromosomal anomalies. Some diseases that display chromosomal instability such as ataxia-telangiectasia, Bloom syndrome, and xerodermapigmentosum have been found to be associated with certain cancers.[17] Cytogenetic studies suggest that genomic instability might be associated with cancer development and might have likely effects on resistance to therapy.[18-20] Genetic instability has an effect on enzymes that replicate DNA (DNA polymerases) or repair DNA (mismatch repair enzymes, nucleotide-excision repair enzymes) as well as on proteins that affect chromosomal stability (chromatin structure and condensation proteins, kinetochore proteins, spindle proteins) or control apoptosis and cell cycle regulation in response to DNA damage (p53 and pRb). Mutations in each of these pathways have been related to the pathogenesis of cancer, either in humans or in animals.[18] Fundia et al. investigated peripheral blood lymphocytes and intestinal samples in 20 adult CD patients and found genomic instability in 35%.[21] High microsatellite transformation was reported particularly in TP53 locus. Likewise, TP53 has been detected in the duodenal mucosa samples of celiac patients and found to have been altered in GIS cancers and NHL. Whilst chronic inflammation in CD patients enhances the risk for cancer by triggering the pathway that leads to instability, genomic alteration and chromosomal instability are reduced with diet. Potter et al. reported that while mismatch repair status decreased in CD patients, microsatellite instability was increased.[8] Martin-Arruti et al. assessed genomic instability, t(14;18), and t(11,14) among children with CD, children with CD but receiving gluten-free diet, and control children and found no difference among the children.[22] In the present study, genotoxicity was detected in the intestinal epithelial cells of CD patients and DNA injury was considerably higher as compared to that in the control group. Moreover, although DNA injury in the lymphocytes (Tail-DNA%) was significantly higher in the new-CD group than that in the on diet-CD group, it was not significantly different from that in the control group.

In the present study, the control group consisted of the patients with dyspeptic complaints. Helicobacter pylori infection was positive in 4 of 9 patients in this group. H. pylori infection enhances the risk for gastric carcinoma and gastric lymphoma. Some studies have reported that H. pylori leads to DNA injury in the mucosal cells of gastric antrum and in the lymphocytes.[20,23] Eradication therapy for H. pylori reduces existing oxidative stress and DNA injury.[24] Under the light of these findings, higher DNA injury in the lymphocytes of the control group might be associated with H. pylori infection and other causes of dyspepsia. If a similar study were made without H. Pylori infection in a greater number of patients and the control group, more meaningful results could be obtained.

DNA repair mechanisms are mandatory for the protection of genome. DNA repair capacity has been demonstrated to be decreased in cancer patients.[25,26] Kolacek et al. reported chromosome aberrations in peripheral blood lymphocytes of CD patients and a significant reduction in the frequency of chromosome aberrations with gluten-free diet.[17] Based on these findings, they concluded that genomic instability was probably a secondary phenomenon to chronic intestinal inflammation. Potter et al. compared CD patients having intestinal adenocarcinoma with controls and detected high rates of defective mismatch repair in →

CD patients.[8] Thus, they concluded that defective mismatch repair might have been effective in the development of adenocarcinoma in CD patients. DNA polymerase has the ability of proofreading during replication. Mismatch excision repair is the mechanism that repairs mismatches remained even after proofreading. DNA mismatch repair is a system for recognizing and repairing erroneous insertion, deletion, and misincorporation of bases that can arise during DNA replication and recombination, as well as for repairing some forms of DNA damage.[27,28] In the present study, while DNA injury in the lymphocytes after hydrogen peroxide exposure was higher in the new-CD as compared to the on diet-CD and control groups, the remaining DNA injury after regeneration period was also higher in the new-CD group. These results seem to corroborate previous studies.

In conclusion, in the present study conducted on CD patients, genotoxicity was detected in the intestinal epithelial cells of CD patients and found the DNA injury to be higher than the control group. DNA injury was higher in the lymphocytes of the new-CD group than that of the on diet-CD group. This could be considered to result from systemic exposure. Lymphocytes of the new-CD patients were more susceptible against peroxide and had less ability to repair. This suggested that DNA repair capacity was insufficient in CD patients. Thus, DNA injury in CD patients was suggested to contribute to the development of malignant diseases.

* The authors declare that there are no conflicts of interest.

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