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Investigation of the Effects of Glutens on Serum Interleukin-1 Beta and Tumor Necrosis Factor-Alpha Levels and the Immunohistochemical Distribution of CD3 and CD8 Receptors in the Small Intestine in Male Rats

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HIGHLIGHTS

- Gluten is the main storage protein of cereals grains (wheat, barley, corn, etc.).
- Gluten is composed of glutenin polymers and gliadin monomers.
- Gluten consumption leads to severe adverse effects on the intestinal tract.
- Glutens leads to severe adverse effects blood cytokine parameters.

Abstract: While the role of cytokines in celiac disease has been investigated in detail, cytokine release in the event of the exposure of healthy subjects to glutens has only recently been studied. This study was aimed at determining the effects of corn and wheat glutens, incorporated as protein sources into the diet, on serum interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) levels and the immunohistochemical distribution of CD3 and CD8 receptors in the small intestine in male rats. The study material comprised 24

twenty-day-old male Wistar albino rats, which were randomly assigned in equal numbers to three groups (2 rats/replicate and 4 replicates/group). The feed rations provided to all three groups contained high levels of proteins, which were soybean meal, corn gluten and wheat gluten in the control, corn and wheat groups, respectively. The in Control, Corn and Wheat groups serum IL-1 beta and TNF-alpha levels respectively 55.83 - 46.37; 81.65 - 61.95 and 81.65-61.31 was determined but these differences were statistically insignificant. Furthermore, immunohistochemical examination demonstrated a mathematical increase to have occurred in the distribution of the CD3 and CD8 receptors in the duodenum, jejunum and ileum samples of the corn and wheat groups. In result, based on the findings obtained in this study, we suggest that the long-term feeding of rats on high levels of gluten causes systemic adverse effects.

Keywords: cytokine; gluten; immunohistochemical; rat.

INTRODUCTION

As is the case with livestock, experimental animals are also commonly fed with cereals. Gluten is a complex molecule, which is composed of glutenin polymers and gliadin monomers, and is found in various cereals, including among others wheat, rye and barley [1]. While glutenin comprises proteins of high and low molecular weight, gliadin belongs to a large family of proteins comprising the α-, β-, γ- and ω types [2]. Both glutenin and gliadin contain high levels of prolines (20%) and glutamines (40%), which prevent them from being fully broken down in the gastrointestinal tract, and thus, reduce their digestibility [2]. The best characterized gliadin peptides are the 57-89 peptide (33-mer) α-gliadin fragment [3], cytotoxic peptide, gut permeating peptides, and IL-8 releasing peptide [4]. These indigestible peptides have been demonstrated to show various biological activities in the gastrointestinal tract, including increased intestinal permeability and cytotoxic and immunomodulatory effects [5]. Gluten intake has been identified as the cause of celiac disease (CD), which is an autoimmune enteropathy activated in the intestinal lamina propria of individuals with genetic predisposition [6]. Several studies have shown that gluten consumption leads to severe adverse effects, primarily on the intestinal tract, autoimmune system and blood cytokine parameters [7-9].

Polypeptide cytokines are cell-regulating proteins, which are secreted by induced lymphocytes, monocytes and macrophages as well as by some somatic cells, and affect the behavior of target cells. Interleukin-1 (IL-1) and tumor necrosis factor (TNF), known as proinflammatory cytokines, are involved in inflammatory alterations and the generation of the rapid immune response, which eliminates pathogens [10].

Being a T-cell surface marker, CD3 plays an important role in pathogen invasion and the maintenance of body health. Mature T-cells, which act as a major regulator of immune functions in the small intestine, carry CD3 receptors that are involved in T-cell proliferation, and the direct and indirect activation of cytokines [11,12]. CD8 is known as the most effective cytotoxicity marker found on T-cells and shows effect against intracellular pathogens. It is also involved in the secretion of cytokines responsible for the immune response, such as TNF-α and interferon. These cytokines are described as being effective against intracellular pathogens [13].

While the role of cytokines in celiac disease has been investigated in detail, cytokine release in the event of gluten exposure and the onset of associated symptoms has only recently been studied. This study was aimed at determining the effects of high levels of glutens, incorporated as protein sources into rat feed, on blood cytokine levels and intestinal immunohistochemical markers.

MATERIAL AND METHODS

Animals, Study Groups and Feed

This study was approved by the Local Ethics Board for Animal Experiments of Sivas Cumhuriyet University (Decision number: 2017/18). The investigation was conducted at the premises of the Experimental Animals Unit of Sivas Cumhuriyet University, Medical Faculty in Turkey. Since the average weaning age for rats is approximately 3 weeks, animals 20 days-old were used in the study. The study material comprised 24 twenty-day-old weaned male Wistar albino rats (initial body weights averages 40 g), which were supplied from the Experimental Animals Unit. The rats were randomly assigned to 3 groups, each of 8 animals (2 rats/replicate and 4 replicates/group). The feed rations provided to all three groups contained high levels of proteins, which were soybean meal, corn gluten and wheat gluten in the control, corn and wheat groups, respectively. Since the average puberty age for rats is approximately 70 days, the feeding trial was continued for a period of 50 days and the study was terminated when the rats reached 70 days of age. Feed and water

were provided ad libitum. The animals were housed at the comfort temperature (22°C) and were fed on a ration containing 22% of crude protein (CP) and 2598 kcal/kg of metabolic energy (ME) throughout the study period.

Collection of Blood Samples for Biochemical Analyses

On the last day of the study (Day 70), cardiac blood samples were collected from each animal, under anesthesia (The Xylazin HCL 2 mg/kg, Rompun, BAYER, intraperitoneal and Ketamine HCL 13 mg/kg Ketalar, PFIZER intraperitoneal were used as anesthetics), into dry tubes. After being kept at room temperature for coagulation, the blood samples were centrifuged at 4,000 g for 5 min (Hettich 38R, Hettich Zentrifugen, Tuttlingen Germany), and thereby, sera were extracted. The serum samples were stored at -80 °C until being analyzed.

Serum TNF-α (Rat, TNF-alpha ELISA Kit, Invitrogen Co., Carlsbad, CA, USA) and IL-1β (Rat, IL-1 Beta ELISA Kit, Invitrogen Co., Carlsbad, CA, USA) levels were determined using commercial ELISA test kits. Optic density values were measured at 450 nm on a plate reader (Multiskan GO, Thermo Scientific).

Immunohistochemical Examination

At the end of the study, on Day 70, the animals were sacrificed under anesthesia, and intestinal tissue samples were taken for immunohistochemical examination. Accordingly, tissue samples taken from the duodenum, jejunum and ileum, all three which form the small intestine, once fixed in 10% buffered formaldehyde solution for 24 h, were processed by routine histological methods. Sections cut at a thickness of 4 microns, were stained with the ABC method for the determination of CD3 and CD8 activity [14]. Following deparaffinization, the sections were maintained in 3% of H2O2 for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed in 0.01 M citric acid (pH 6.0) for 20 min. Nonspecific staining was blocked by placing the sections in ¼ goat serum (Thermo). Next, the sections were incubated overnight at 4°C with CD3 (Thermo Fisher Scientific, 14-0030-81) (1/100) and CD8 (Thermo Fisher Scientific, 14-008-82) (1/75) primary antibodies. This was followed by staining with biotin (Thermo) for 45 min, streptavidin (Thermo) for 42 min, and AEC (Zymed) for 10 min. Hematoxylin was used for counterstaining. The negative controls were subjected to the same procedures, excluding incubation with primary antibodies. Immunohistochemical evaluation was based on the assessment of the cells that stained red with AEC as (+). The distribution of the cells that displayed (+) reaction was investigated at x20, x40, and x100 magnification. Semi-quantitative scoring was performed as follows: no reaction (-), weak reaction (±,+), moderate reaction (++), strong reaction (+++). Selected areas in the regions displaying (+) reaction were photographed.

Statistical Analysis

For all analyses, SPSS® 22.0 (IBM, New York, ASA) for Windows was used and $P<0.05$ was considered significant [15]. Prior to data analysis, normality and homogeneity of variances to meet assumptions of parametric tests were verified with a Skewness and Kurtosis tests. In the biochemical data obtained were evaluated with one-way analysis of variance (ANOVA), with Duncan test used to locate differences between groups. The nonparametric Kruskal–Wallis test were used to detect the differences for histopathological and immunohistochemical parameters. The results obtained in this study are expressed as mean ± standard deviation (SD).

RESULTS

Biochemical Findings

It was determined that serum IL-1 beta and TNF-alpha levels had mathematically increased in the groups that received corn and wheat glutens in feed ($P>0.05$) (Figure 1).

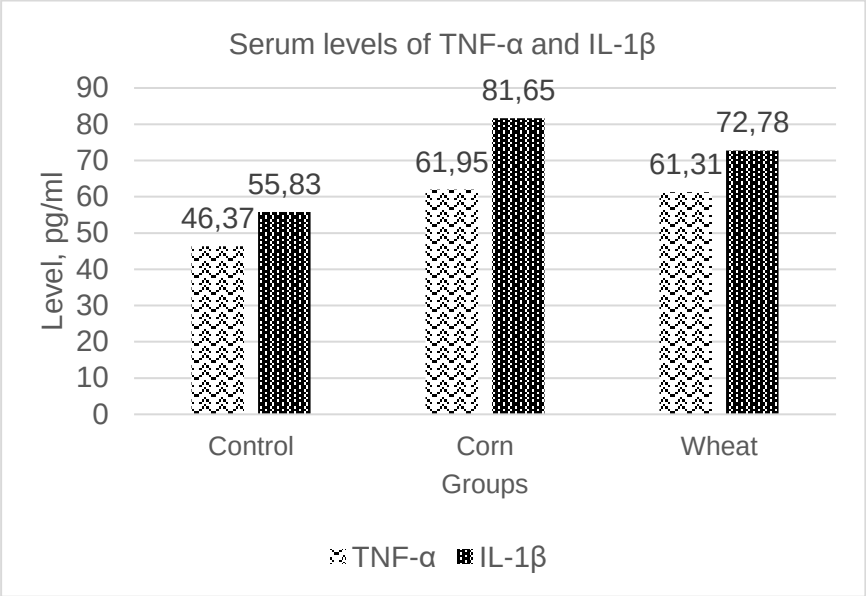

Figure 1. Effects of different protein sources used in ration on TNF-α and IL1-β levels in serum.

Immunohistochemical Findings

Tissue samples taken from the duodenum, jejunum and ileum were immunohistochemically stained for CD3 and CD8. Immunohistochemical reactions for CD3 and CD8 were assessed semi-quantitatively on the basis of the (+) staining of lymphocytes with AEC. Cells, which stained positively with AEC, were observed to be distributed in the lamina epithelialis (intraepithelial localization), lamina propria and submucosa, and the tunica muscularis + tunica serosa. The distribution of the CD3-positive (Table 1 and Table 2), and CD8-positive (Table 3 and Table 4) lymphocytes are shown in Figure 2 and Figure 3, respectively. Statistical analyses demonstrated that, when compared to the control group, CD8-positive reactions had significantly increased only in the lamina propria and mucosa of the ileum in the corn and wheat groups ($P<0.05$). On the other hand, the distribution of the CD3- and CD8-positive reactions was statistically similar in all mucosal layers of the duodenum, jejunum and ileum ($P>0.05$).

Table 1. Immunohistochemically semi-quantitative display of CD 3 (+) lymphocytes in the small intestine tissues of study groups.

Groups	**Lamina epithelialis (IEL)**			**Lamina propria + Submucosa**			**Tunica muscularis + Tunica serosa**		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Control	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(-)
Corn	(-)	(-)	(-)	(++)	(±)	(+)	(-)	(-)	(-)
Wheat	(+)	(-)	(±)	(++)	(-)	(+)	(+)	(-)	(-)

(-): no reaction, (±, +): little reaction, (++): medium, (+++): intense. (n=6).

Table 2. Statistical representation of CD 3 values belonging to small intestine tissues of study groups.

		Control	Corn	Wheat	P value
The CD 3 values of Lamina Epithelialis					
Duodenum	X±SD	0.167±0.408	0.167±0.408	0.667±0.817	0.299
	Median	0.000	0.000	0.500	
Jejunum	X±SD	0.000±0.000	0.167±0.408	0.167±0.408	0.588
	Median	0.000	0.000	0.000	
Ileum	X±SD	0.167±0.408	0.333±0.516	0.500±0.837	0.729
	Median	0.000	0.000	0.000	
The CD 3 values of Lamina propria+submucosa					
Duodenum	X±SD	1.000±0.894	1.833±1.169	1.833±1.169	0.300
	Median	1.000	2.000	2.000	
Jejunum	X±SD	0.167±0.408	0.833±1.329	0.167±0.408	0.588
	Median	0.000	0.000	0.000	
Ileum	X±SD	1.000±1.265	1.167±0.753	1.167±0.753	0.839
	Median	0.500	1.000	1.000	
The CD 3 values of Tunika muscularis+Tunica serosa					
Duodenum	X±SD	0.333±0.516	0.167±0.408	0.667±0.817	0.423
	Median	0.000	0.000	0.500	
Jejunum	X±SD	0.167±0.408	0.000±0.000	0.167±0.408	0.588
	Median	0.000	0.000	0.000	
Ileum	X±SD	0.167±0.408	0.167±0.408	0.167±0.408	1.000
	Median	0.000	0.000	0.000	

All values are given as mean ± standard deviation (SD), (n=6).

Table 3. Immunohistochemically semi-quantitative display of CD 8 (+) lymphocytes in the small intestine tissues of study groups.

Groups	Lamina epithelialis (IEL)			Lamina propria + Submucosa			Tunica muscularis + Tunica serosa		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Control	(-)	(±)	(-)	(++)	(+)	(+)	(-)	(-)	(-)
Corn	(+)	(-)	(+)	(+++)	(++)	(++)	(-)	(-)	(-)
Wheat	(+)	(+)	(-)	(+++)	(++)	(++)	(-)	(-)	(±)

(-): no reaction, (±, +): little reaction, (++): medium, (+++): intense. (n=6).

Table 4. Statistical representation of CD 8 values belonging to small intestine tissues of study groups.

		Control	Corn	Wheat	P value
The CD 8 values of Lamina Epithelialis					
Duodenum	X±SD	0.167±0.408	0.833±0.753	0.500±0.548	0.190
	Median	0.000	1.000	0.500	
Jejunum	X±SD	0.500±0.837	0.167±0.408	0.833±0.408	0.106
	Median	0.000	0.000	1.000	
Ileum	X±SD	0.333±0.817	1.000±0.894	0.167±0.408	0.128
	Median	0.000	1.000	0.000	
The CD 8 values of Lamina propria+submucosa					
Duodenum	X±SD	2.000±1.096	2.833±0.408	2.667±0.516	0.171
	Median	2.000	3.000	3.000	
Jejunum	X±SD	1.333±0.817	1.833±0.408	2.000±0.633	0.243
	Median	1.500	2.000	2.000	
Ileum	X±SD	0.833±0.753[c]	2.500±0.548[a]	2.000±0.633[b]	0.007
	Median	1.000	2.500	2.000	
The CD 8 values of Tunika muscularis+Tunica serosa					
Duodenum	X±SD	0.333±0.516	0.167±0.408	0.000±0.000	0.322
	Median	0.000	0.000	0.000	
Jejunum	X±SD	0.167±0.408	0.167±0.408	0.167±0.408	1.000
	Median	0.000	0.000	0.000	
Ileum	X±SD	0.333±0.816	0.167±0.408	0.500±0.837	0.734
	Median	0.000	0.000	0.000	

All values are given as mean ± standard deviation (SD), (n=6).

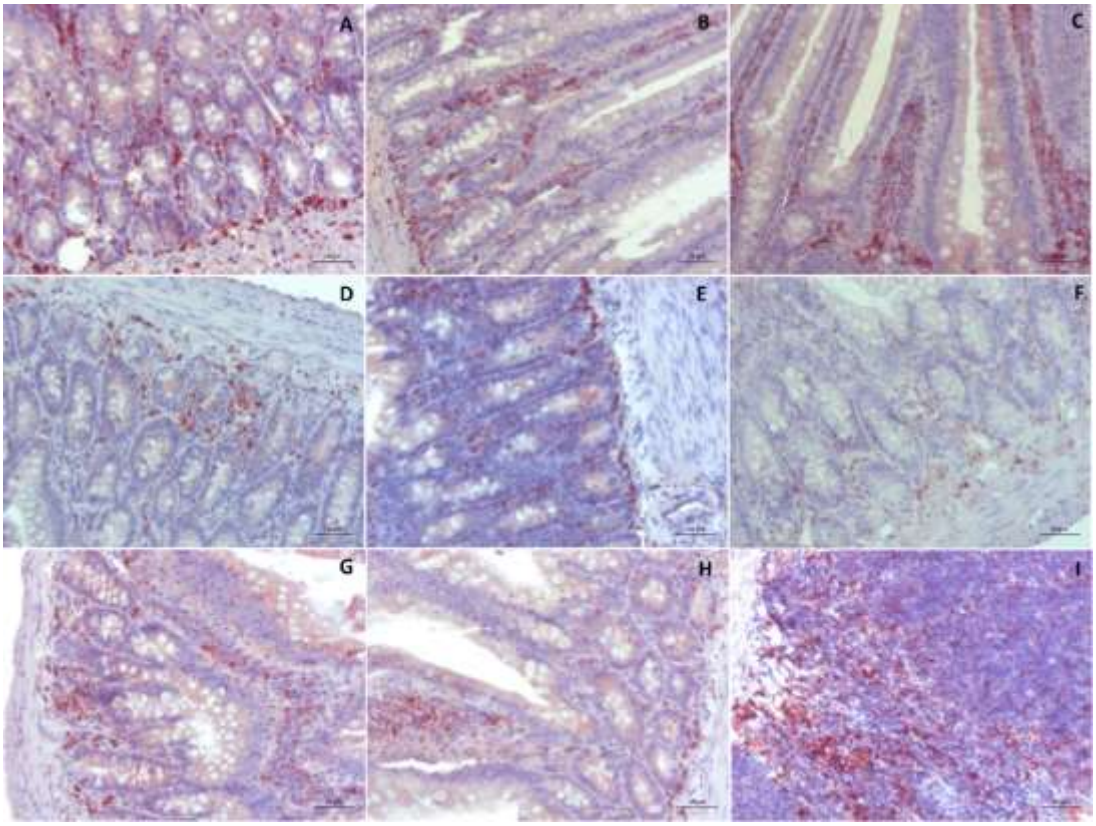

Figure 2. Immunohistochemically CD 3 (+) reactions. **A.** Control group, duodenum. **B.** Corn group, duodenum. **C.** Wheat group, duodenum. **D**. Control group, jejunum. **E.** Corn group, jejunum. **F.** Wheat group, jejunum. **G.** Control group, ileum. **H**. Corn group, ileum. **I**. Wheat group, ileum. A,B,C,D,E,F,G,H,I Barr: 50µ.

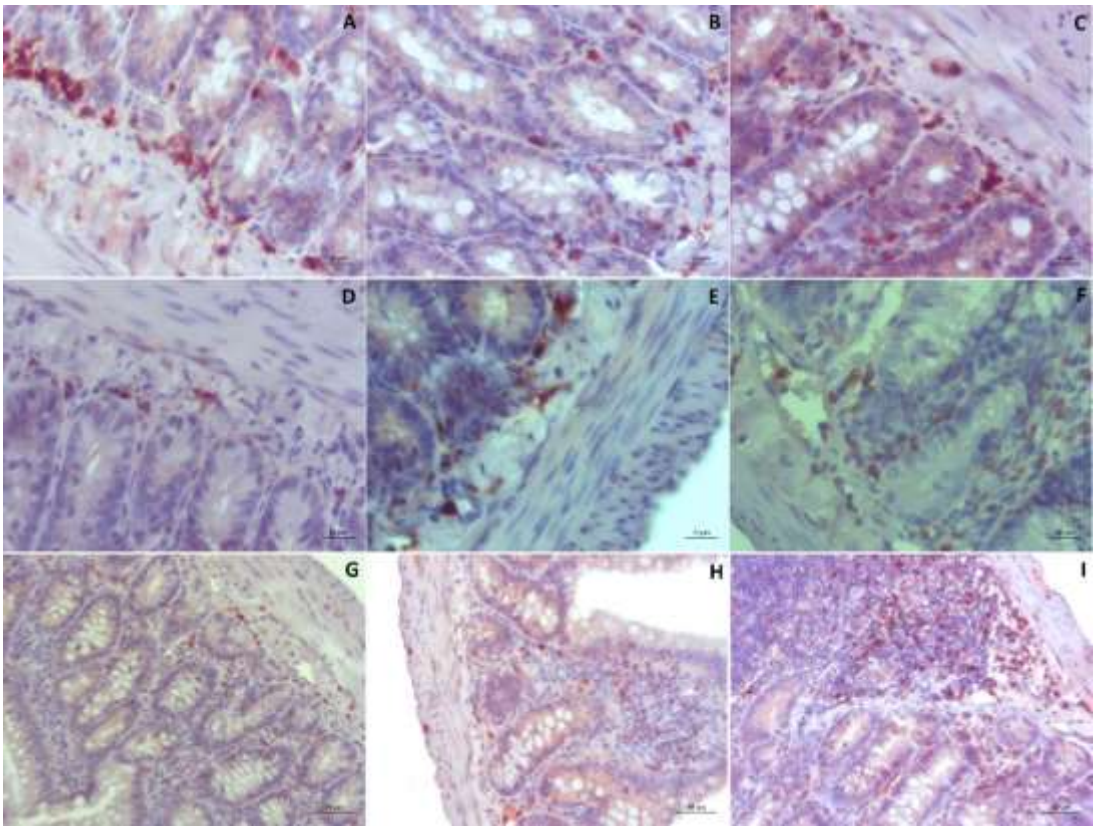

Figure 3. Immunohistochemically CD 8 (+) reactions. **A.** Control group, duodenum. **B.** Corn group, duodenum. **C.** Wheat group, duodenum. **D**. Control group, jejunum. **E.** Corn group, jejunum. **F.** Wheat group, jejunum. **G.** Control group, ileum. **H**. Corn group, ileum. **I**. Wheat group, ileum. A,B,C,D,E,F,G,H,I Barr: 50µ.

DISCUSSION

Gluten is a complex mixture, which is found in various cereals including wheat, barley and rye, and is comprised of 2 proteins, the main constituents of which are glutenin and gliadin, as well as of starch, other proteins, lipids and sugars [16]. While wheat contains 18 amino acids, two-thirds of its total protein content comprises glutamine, proline, cysteine and cystine [17]. Corn gluten is made of 70-80% protein, and is considered a highly digestible excellent protein source [18,19].

Cytokines regulate both immunity- and inflammation-related processes, including cell growth, cell repair and systemic response to injury [20]. Interleukin and TNF-alpha are primarily secreted by activated macrophages, and both cytokines regulate T-cell functions by increasing cytokine production in other cells. Untreated celiac disease patients have been ascertained to display increased TNF-α and IL-6 production in the intestinal mucosa [21,22]. Increasing evidence is being gathered on celiac disease being caused by a T cell-mediated hypersensitivity to gliadin and the most characteristic histopathological lesion of this disease being related to subsequent cytokine secretion [21,23]. It has been suggested that the role played by IL-1β in the mediation of mucosal damage is an integral process associated with inflammation [24]. It has been reported that dietary supplementation with corn gluten meal induced a higher level of expression of the genes encoding proinflammatory cytokines, including IL-1β, IL-8 and TNF- α, in turbot [25]. Similarly, in the present study, it was determined that serum IL-1 beta and TNF-α levels had mathematically increased in the groups that received dietary gluten (Figure 1). This result was attributed to gluten-induced increased cytokine production.

The dietary intake of glutens is followed by the development of clinical and histological findings [26]. In sensitive organisms, gluten ingestion is followed by the development of histological lesions, such as the infiltration of the lamina propria by lymphocytes, macrophages and plasma cells [27,28]. Increasing evidence is available on these defects being caused by T cell-mediated hypersensitivity to gliadin and the characteristic lesion being related to partial subsequent cytokine secretion [29]. It has been reported that gliadin exposure alone may alter the barrier function of intestinal epithelial cells [30]. Also, gluten-sensitive enteropathy is an immune-mediated chronic inflammatory disorder of the small intestine triggered by a combination of environmental and genetic influences (14). In a previous study in turbot, it was determined that the incorporation of corn gluten meal in the diet caused a dose-dependent increase in inflammatory changes in the intestinal tissue, and increased both the width and cell infiltration level of the lamina propria and submucosa [25].

CD3 is a common marker of all T lymphocytes. Of all lymphocytes found in the intestinal mucosa, more than 90% are CD3+ T lymphocytes, whilst 65-80% of the lymphocytes localized to the lamina propria are CD3+ T lymphocytes. Thus, the number and distribution of CD3 (+) T lymphocytes in the mucosa of the small intestine reflects the number and distribution of activated lymphocytes. CD8 is a marker of cytotoxic T lymphocytes. Cytotoxicity is rather effective in intestinal mucosal defense. Alterations in the numbers of both lymphocyte surface receptors, CD3 and CD8, provide input on mucosal immunity status in the small intestine [31,32]. In the present study, it was aimed to investigate CD receptors, which are lymphocyte markers that emerge after enteropathies caused by dietary gluten intake and are readily affected by minor mucosal anomalies, and to demonstrate any potential differences between the segments of the small intestine. Accordingly, it was ascertained that in the duodenal, jejunal and ileal tissue samples of the rats fed on corn gluten and wheat gluten, immunohistochemically, a statistically insignificant mathematical increase had occurred in the T lymphocyte markers CD3 and CD8. In a previous study, it was determined that patients with nonceliac gluten sensitivity (NCGS) show levels of CD3 higher than healthy controls but lower than celiac disease (33).

CONCLUSION

In conclusion, it is suggested that the dietary intake of corn and wheat glutens causes a mathematical increase in serum IL1 beta and TNF-alpha levels. The dietary intake of gluten not only increases the number of but also alters T cells, which are involved in cellular immunity, in the intestines, and thus suppress the immune system. This clearly shows that, although conceptually perceived as being simple factors, dietary changes in fact have significant effects on living organisms.

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