https://doi.org/10.52973/rcfcv-e34500 Revista Científica, FCV-LUZ / Vol. XXXIV, rcfcv-e34500

Effects of experimental wheat and corn gluten on liver tissue in rats: biochemical, histopathological, immunohistochemical and immunofluorescence methods

Efectos del gluten de trigo y maíz experimental sobre el tejido hepático de ratas: métodos bioquímicos, histopatológicos, inmunohistoquímicos y de inmunofluorescencia

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ABSTRACT

This study was aimed at determining the effects of wheat gluten, corn gluten and soybean meal, incorporated into feed as protein sources, on the hepatic tissue of rats, based on the investigation of histopathological parameters (degeneration, inflammation, biliary hyperplasia and fat droplets), immunohistochemical parameters (transglutaminase, gliadin, IgA, IgG, CD4 and CD8), and the serum levels of hepatic enzymes [(aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH)]. Three groups, referred to as Wheat Group, Group Corn and Group Soybean, were established, and were given high levels of wheat gluten, corn gluten and soybean meal as protein sources in the feed, respectively. The study material comprised forty-eight 20-day-old female Sprague Dawley rats. In the group, which received dietary wheat gluten, the hepatic tissue presented with numerically higher histopathological and immunohistochemical parameters on day 45 of the study, and numerically higher histopathological parameters on day 165 of the study (P>0.05). On day 165 of the study, when compared to Groups Corn and Soybean, Group Wheat displayed a significantly different level of sensitivity to immunohistochemical parameters (transglutaminase, gliadin, IgA, IgG, CD4 and CD8). It was determined that gluten significantly reduced the activity of the liver enzymes LDH and AST. In result, it was ascertained that rats, not carrying the genes HLA-DQ2 and DQ8, when fed on a ration incorporated with a high level of wheat gluten, displayed slightly affected hepatic tissue histopathological parameters and significantly increased immunohistochemical parameters (transglutaminase, gliadin, IgA, IgG, CD4 and CD8).

Key words: Rat; gluten; wheat; corn; immunohistochemical

RESUMEN

Este estudio tuvo como objetivo determinar los efectos del gluten de trigo, gluten de maíz y harina de soya, incorporados al alimento como fuentes de proteína, sobre el tejido hepático de ratas, con base en la investigación de parámetros histopatológicos (degeneración, inflamación, hiperplasia biliar y gotas de grasa), parámetros inmunohistoquímicos (transglutaminasa, gliadina, IgA, IgG, CD4 y CD8), y los niveles séricos de enzimas hepáticas [(aspartato aminotransferasa (AST), alanina transaminasa (ALT), fosfatasa alcalina (ALP), lactato deshidrogenasa (LDH)]. Se establecieron tres grupos, denominados Grupo Trigo, Grupo Maíz y Grupo Soja, a los que se les suministró en el alimento altos niveles de gluten de trigo, gluten de maíz y harina de soya como fuentes de proteína, respectivamente. El material de estudio estuvo compuesto por cuarenta y ocho ratas Sprague Dawley hembras de 20 días de edad. En el grupo que recibió gluten de trigo en la dieta, el tejido hepático presentó parámetros histopatológicos e inmunohistoquímicos numéricamente más altos el día 45 del estudio, y parámetros histopatológicos numéricamente más altos el día 165 del estudio (P>0,05). El día 165 del estudio, en comparación con los grupos Maíz y Soja, el Grupo Trigo mostró un nivel significativamente diferente de sensibilidad a los parámetros inmunohistoquímicos (transglutaminasa, gliadina, IgA, IgG, CD4 y CD8). Se determinó que el gluten redujo significativamente la actividad de las enzimas hepáticas LDH y AST. Como resultado, se comprobó que las ratas no portadoras de los genes HLA-DQ2 y DQ8, cuando fueron alimentadas con una ración incorporada con un alto nivel de gluten de trigo, mostraron parámetros histopatológicos del tejido hepático ligeramente afectados y parámetros inmunohistoquímicos significativamente aumentados (transglutaminasa, gliadina, IgA, IgG, CD4 y CD8).

Palabras clave: Rata; gluten; trigo; maíz; inmunohistoquímica

Received: 06/08/2024 Accepted: 28/10/2024 Published: 31/12/2024

INTRODUCTION

Wheat (*Triticum aestivum*) and corn (*Zea mays*) are two main cereal grains, which are most commonly produced and consumed Worldwide [1]. The main structural constituents of these grains are starch, cellulose and gluten. The term gluten refers to a complex mixture of proteins, which is found in grains such as wheat, corn, barley (*Hordeum vulgare*), rye (*Lolium multiflorum*) and oat (*Avena sativa*), and remains after these grains are processed and washed of their starch. Nearly 5.4% of wheat grain [2] and 3–3.55% of corn grain is made of gluten [3].

Normally, gluten contains proteins that are readily digested in the human intestines [4]. On the other hand, individuals not carrying the genes HLA-DQ2 and DQ8 are not capable of digesting these proteins. The consumption of gluten-containing diets by these persons may lead to multiple health problems. These health problems include coeliac disease, gluten intolerance, non-coeliac gluten sensitivity, wheat allergy, and dermatitis herpetiformis [5, 6]. The sensitivity of tissues to gluten is determined in view of histopathological findings and immunohistochemical analyses [7].

In the present study, healthy rats were provided with gluten-rich feed for a long-term period and the sensitivity of their hepatic tissue to gluten was investigated. Histopathological alterations in the hepatic tissue were assessed on the basis of several parameters, including degeneration, inflammation, biliary hyperplasia, and fat droplets, whilst immunohistochemical analyses were performed for the measurement of transglutaminase, gliadin, IgA, IgG, CD4 and CD8 levels. Also, AST, ALT, ALP and LDH levels, which are liver enzymes, were investigated in serum tissue.

MATERIALS AND METHODS

Animal material, experimental groups, and feed

In this study, 48 healthy female Sprague-Dawley rats (*Rattus norvegicus*), 20 days (d) old, were raised until they reached 185 d of age (the trial period lasted 165 d) by being fed experimental diets. The rations given to the study groups were isonitrogenous and isocaloric (TABLE I). Three study groups were established, named Group Wheat, Group Corn, and Group Soybean, each receiving dietary wheat gluten, corn gluten, and soybean meal, respectively. Throughout the study, the animals were fed for 165 d, with feed and water available ad libitum, and were maintained at a comfort temperature of 22°C.

In the present study, healthy rats, confirmed not to carry the genes HLA-DQ2 and DQ8, were fed on rations supplemented with high levels of wheat gluten and corn gluten for a period of 165 d, with to investigate the effects of gluten and the sensitivity displayed by hepatic tissue to gluten. The effects of gluten on hepatic tissue were determined based on the measurement of histopathological and immunohistochemical parameters and enzyme activity in hepatic tissue samples on d 45 and 165 of the study.

Pathological examination

Rats were sacrificed under anesthesia at the average ages of 65 and 185 d, and tissue samples were collected. Liver tissue samples were obtained for histopathological and immunohistochemical analysis, and lesions were scored semi-quantitatively based on the microscopic (Olympus BX51, Japan) examination of 10 different areas at 40× magnification. Histopathologically, Degeneration, Inflammation, Biliary hyperplasia and Fat drop scoring were performed in liver tissues. Immunohistochemically, Transglutaminase, Gliadin, IgA, IgG, CD4 and CD8 expression levels were scored. The scoring was as follows: 0 (negative), +1 (slight), +2 (moderate), +3 (severe), and +4 (very severe) [8].

TABLE I
Ingredients and nutrient composition of rat diet in the study

Ingredients, %	Groups		
	Wheat	Corn	Soybean
Wheat bran	1.8	3.5	3.24
Oat, %11 CP	68	64	62.11
Sunflower meal, % 28 CP	13	13	6
Corn gluten meal, % 62 CP	-	17	-
Wheat gluten meal, % 75 CP	24.85	-	-
Soybean meal, % 51 CP	-	-	24.85
Animal fat	2.2	1.5	2.8
Vitamin-mineral premix*	1	1	1
Nutrient composition			
Crude protein, %	22	22	22
Metabolisable energy, (kcal·kg^{-1})	2,599	2,657	2,598
Ca, %	0.15	0.11	0.14
Methionine + cysteine, %	0.66	0.83	0.68
Lysine, %	1.17	0.63	1.15

*The vitamin-mineral premix provides the following (per kg): vitamin A 6,000,000 IU; vitamin D3 800,000 IU; vitamin E 8,000 mg; vitamin K3 2,000 mg; vitamin B1 1,200 mg; vitamin B2 3,000 mg; vitamin B6 2,000 mg; vitamin B12 8 mg; niacin 10,000 mg; folic acid 400 mg; d-biotin 20 mg; choline chloride 160,000 mg; manganese 32,000 mg; iron 16,000 mg; zinc 24,000 mg; copper 2,000 mg; iodine 800 mg; cobalt 200 mg; selenium 60 mg; Cal-D-Pan. 4,000 mg; antioxidant 4,000 mg. CP: Crude protein

Histopathological examination

For histopathological examination, tissue specimens were initially fixed in 10% buffered formalin for 48–72 hours and then rinsed under running water for 6–8 hours. The specimens were then processed using a routine tissue processing method, which involved passing them through graded alcohols (70°, 80°, 90°, 96°, and 100°) and xylene series. After embedding in paraffin, 4 µm thick sections were cut from the paraffin blocks and mounted on glass slides. The histopathological sections were stained with hematoxylin-eosin (HE) [9, 10] and were examined and imaged using an Olympus BX52-1 light microscope (Japan) with a DP72 camera system, at the Pathology Department Laboratory of Atatürk University, Faculty of Veterinary Medicine.

Immunohistochemical examination

All sections mounted on poly-L-lysine-coated adhesive glass slides for immunoperoxidase analysis were subjected to deparaffinization and dehydration by passing through a series of graded xylene and alcohol, followed by a 5-min wash in distilled water. The slides were then immersed in phosphate buffer solution (PBS, pH 7.2) for 5 min and subsequently treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase activity. Following this, the slides were rinsed in PBS for 5–10 min and incubated with a protein-blocking solution compatible with all primary and secondary antibodies for 5 min to

prevent non-specific background staining. After incubation, excess blocking solution was removed, and without further washing, the slides were exposed to primary antibodies, including CD4 (Catalog No: BS-0647-R), CD8 (Catalog No: BS-0648-R), IgA (Catalog No: BS-0648-R10491-R), IgG (Catalog No: BS-0392-R), gliadin (Catalog No: NB600-54713374-R), and transglutaminase 2/TGM2 (Catalog No: NB600-547). Depending on the specific primary antibody, incubation was conducted either for 1 hour at room temperature or overnight at +4°C. The slides were then washed twice in PBS, each time for 5 min, and incubated with a biotinylated secondary antibody at room temperature for 10-30 min. After another PBS wash, the slides were treated with streptavidin-peroxidase for 10-30 min and washed again with PBS. The sections were then treated with DAB (3,3-diaminobenzidine) as a chromogen for 5-10 min. Finally, background staining was performed using Mayer's hematoxylin for 1-2 min, followed by rinsing with tap water and mounting with a water-based adhesive [1, 11].

Immunofluorescence examination

All sections mounted on poly-L-lysine-coated adhesive glass slides for immunofluorescence analysis underwent deparaffinization and dehydration by passing through a series of graded xylene and alcohol solutions, followed by a 5-min rinse in distilled water. The slides were then immersed in phosphate buffer solution (PBS, pH 7.2) for 5 min before being treated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase activity. Afterward, the slides were rinsed in PBS for 5-10 min and incubated with a protein-blocking solution for 5 min to prevent non-specific background staining. At the conclusion of the incubation, any excess blocking solution was removed from the slides, which were then directly treated with the primary antibodies gliadin (Catalog No: NB600-54713374-R) and transglutaminase 2/TGM2 (Catalog No: NB600-547) without additional washing. Depending on the specific primary antibody, incubation was carried out either for 1 hour at room temperature or overnight at +4°C. The slides were subsequently washed twice in PBS, each time for 5 min, and then incubated with a secondary fluorescein antibody (FITC) in a dark room for 45 min. Finally, the sections were washed with PBS, covered with a coverslip, and examined under a fluorescence microscope (ZEISS AXIO Scope A1)(ZEISS AXIO Scope A1, Germany), with images captured from the necessary areas [12].

Biochemical examination

On the 45th and 165th d of the study, venous blood samples were collected from eight animals per study group into 10 mL glass tubes containing a coagulation accelerator. In the Biochemistry Department Laboratory at Atatürk University, Faculty of Veterinary Medicine, the blood samples were centrifuged at 3,000 G and +4°C for 5 min. The extracted serum was stored at -82°C until analysis. Serum levels of AST, ALT, LDH, and ALP were measured using an automatic analyzer with commercial test kits (Cobas 6000 analyzer, Roche) [13].

Statistical analyses

The statistical analysis of the results from this study was conducted using the Statistical Package for the Social Sciences (SPSS) software [14]. One-way analysis of variance (ANOVA) was employed to analyze serum levels of AST, ALT, LDH, and ALP, while Duncan's test was used to determine the significance of differences between study groups. Histopathological and immunohistochemical changes in the liver were assessed using the Kruskal-Wallis test, a non-parametric method. Data were presented as mean ± standard error of the mean (SEM). Values of $P<0.05$ and $P<0.01$ were considered statistically significant.

RESULTS AND DISCUSSIONS

Findings pertaining to the histopathological, immunohistochemical, and immunofluorescence parameters of the hepatic tissue on day 45 of the study (at 65 d of age) are presented in TABLE II, the graphic representation of these findings is shown in FIGS. 1 and 2, and the results of the statistical analysis of the findings are given in TABLE III. Furthermore, findings pertaining to the histopathological, immunohistochemical, and immunofluorescence parameters of the hepatic tissue on day 165 of the study (at 185 d of age) are presented in TABLE IV, the graphic representation of these findings is shown in FIGS. 3 and 4, and the results of the statistical analysis of the findings are given in TABLE V.

TABLE II
Values obtained with the histopathological, immunohistochemical, and immunofluorescence staining in liver tissues taken from average 65 days old rats

n	Histopathological Parameters				Immunohistochemical Parameters					
	Deg	Inf	Bh	Fd	TG IHC/IF	Gld IHC/IF	IgA	IgG	CD4	CD8
Wheat Group										
1	+2	+2	+2	+2	+2	+2	+3	+2	+1	+2
2	+2	+2	+1	+1	+2	+2	+3	+2	+2	+2
3	+2	+1	0	0	+2	+2	+2	+2	+1	+2
4	+1	+1	0	0	+1	+2	+2	+2	+1	+2
5	+1	+1	0	0	+1	+2	+1	+1	+1	+1
6	+1	+1	0	0	+1	+2	+1	+1	+1	+1
7	0	0	0	0	+1	+1	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
Corn Group										
1	+1	+1	0	0	+1	+1	+2	+2	+1	+1
2	+2	+2	+1	+1	+1	+1	+2	+2	+1	+1
3	+1	+1	0	0	+1	+1	+1	+1	+1	+1
4	+1	+1	0	0	+1	+1	+2	+1	+1	+2
5	+1	0	0	0	+1	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
Soybean Group										
1	+1	+1	+1	+1	+1	+1	+1	+2	+1	+1
2	+1	+1	0	0	+1	+1	+2	+1	+1	+1
3	+1	0	0	0	+1	0	+1	+1	+1	+1
4	+1	0	0	0	+1	0	+1	+1	0	+1
5	+1	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0

Deg: Degeneration, Inf: Inflammation, Bh: Biliary hyperplasia, Fd: Fat drop, TG: Transglutaminase, Gld: Gliadin, IgA: Immunoglobulin A, IgG: Immunoglobulin G

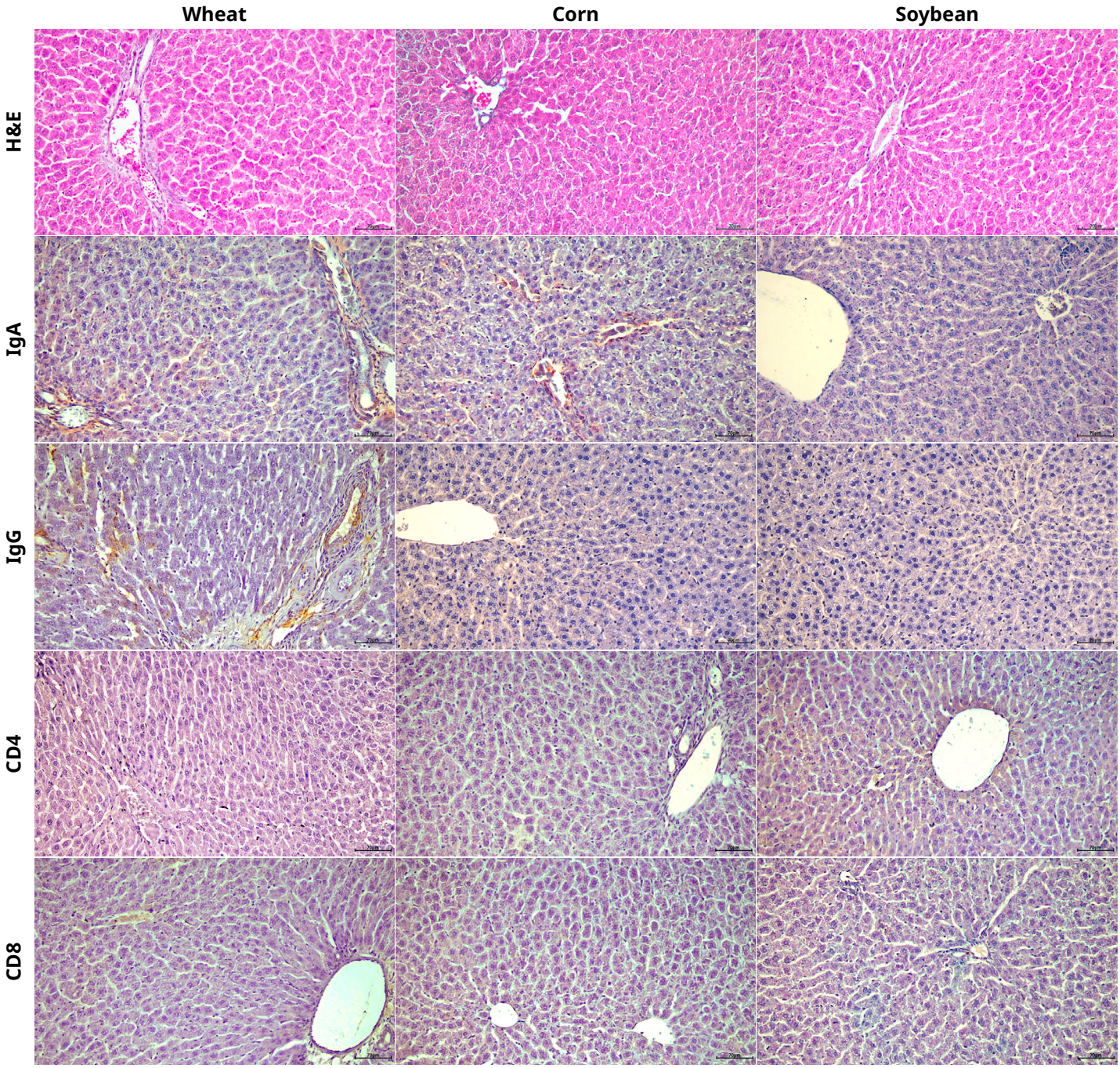

FIGURE 1. Liver tissue, 65–day–old rat, histopathological appearance, H&E, Bar: 70 µm. immunohistochemical staining results, IHC–P, Bar: 70 µm

The histopathological findings detected in the hepatic tissue on d 45 and 165 of the study (degeneration, necrosis, inflammation, biliary hyperplasia, fat droplets) were more severe in Group Wheat, compared to the other groups, yet this numerical difference was statistically insignificant ($P>0.05$) (TABLES III and V). Images of the study are given in FIGS. 1 and 3.

In view of the length of period, during which the animals were fed on the trial rations supplemented with wheat gluten, corn gluten and soybean meal, it was observed that the severity of the histopathological findings in the hepatic tissue samples of all groups had significantly increased over time (165 d of feeding), and the severity was greatest in the group, which was given wheat gluten ($P<0.05$). Furthermore, the hepatic histopathological findings of the group that received dietary soybean meal were more severe, compared to the group fed on corn gluten.

On d 45 of the study, the immunopositivity rates determined for anti–transglutaminase, anti–gliadin, IgA, IgG, anti–CD4 and anti–CD8

TABLE III
Statistical values of the histopathological, immunohistochemical, and immunofluorescence parameters of the liver tissue samples taken from average 65 d old rats

Parameters		Groups			*P*–values
		Wheat	Corn	Soybean	
Histopathological					
Deg	X̄ ± SEM	1.13 ± 0.295	0.75 ± 0.250	0.83 ± 0.166	0.482
	Median	1.00	1.00	1.00	
Inf	X̄ ± SEM	1.00 ± 0.267	0.63 ± 0.263	0.33 ± 0.210	0.156
	Median	1.00	0.50	0.00	
Bh	X̄ ± SEM	0.38 ± 0.263	0.13 ± 0.125	0.17 ± 0.166	0.733
	Median	0.00	0.00	0.00	
Fd	X̄ ± SEM	0.38 ± 0.263	0.13 ± 0.125	0.17 ± 0.166	0.733
	Median	0.00	0.00	0.00	
Immunohistochemical and Immunofluorescence					
TG IHC/IF	X̄ ± SEM	1.25 ± 0.250	0.63 ± 0.183	0.57 ± 0.202	0.066
	Median	1.00	1.00	1.00	
Gld IHC/IF	X̄ ± SEM	1.63 ± 0.263[a]	0.50 ± 0.189[b]	0.29 ± 0.184[b]	0.001
	Median	2.00	0.50	0.00	
IgA	X̄ ± SEM	1.50 ± 0.422	0.88 ± 0.350	0.83 ± 0.307	0.351
	Median	1.50	0.50	1.00	
IgG	X̄ ± SEM	1.25 ± 0.313	0.75 ± 0.313	0.83 ± 0.307	0.390
	Median	1.50	0.50	1.00	
CD4	X̄ ± SEM	0.88 ± 0.226	0.50 ± 0.188	0.50 ± 0.223	0.314
	Median	1.00	0.50	0.50	
CD8	X̄ ± SEM	1.25 ± 0.313	0.63 ± 0.263	0.67 ± 0.210	0.198
	Median	1.50	0.50	1.00	

The values are given as mean ± standard error of the mean (SEM), n=8, [a, b]: Means in the same line with different superscripts differ significantly (*: *P*<0.05), (**: *P*<0.01). Deg: Degeneration, Inf: Inflammation, Bh: Biliary hyperplasia, Fd: Fat drop, TG: Transglutaminase, Gld: Gliadin, IgA: Immunoglobulin A, IgG: Immunoglobulin G

TABLE IV
Values obtained with the histopathological, immunohistochemical, and immunofluorescence staining in liver tissues taken from average 185 days old rats

n	Histopathological Parameters				Immunohistochemical Parameters					
	Deg	Inf	Bh	Fd	TG IHC/IF	Gld IHC/IF	IgA	IgG	CD4	CD8
Wheat Group										
1	+3	+2	+2	+3	+3	+4	+4	+2	+2	+3
2	+3	+2	+2	+3	+3	+4	+4	+2	+2	+3
3	+2	+2	+2	+2	+3	+3	+3	+2	+2	+3
4	+2	+2	+1	+2	+3	+3	+3	+1	+2	+3
5	+2	+1	+1	+2	+2	+3	+3	+1	+1	+2
6	+2	+2	+1	+1	+2	+2	+2	+1	+1	+2
7	+1	+2	+1	+1	+1	+1	+2	+1	+1	+2
8	+1	+1	0	0	+1	+1	+1	+1	+1	+1
Corn Group										
1	+2	+2	+2	+2	+2	+2	+3	+1	+2	+2
2	+2	+1	+2	+2	+2	+2	+3	+1	+2	+2
3	+2	+2	+2	+1	+1	+2	+3	+1	+1	+2
4	+1	+1	+1	0	+1	+1	+2	+1	+1	+1
5	+1	+1	+1	+1	0	+1	+1	+1	+1	+1
6	+1	+1	+1	0	0	+1	+1	0	0	+1
7	+1	0	0	0	0	0	+1	0	0	0
8	+1	0	0	0	0	0	0	0	0	0
Soybean Group										
1	+2	+2	+2	+2	+2	+2	+2	+1	+1	+2
2	+2	+1	+2	+1	+2	+1	+2	+1	+1	+2
3	+2	+2	+2	+2	+1	+1	+1	+1	+1	+2
4	+2	+2	+1	+1	0	+1	+1	+1	+1	+2
5	+1	+1	+1	+1	0	0	+2	0	+1	+1
6	+1	+1	+1	0	0	0	+1	0	0	+1
7	+1	0	0	0	0	0	0	0	0	0

Deg: Degeneration, Inf: Inflammation, Bh: Biliary hyperplasia, Fd: Fat drop, TG: Transglutaminase, Gld: Gliadin, IgA: Immunoglobulin A, IgG: Immunoglobulin G

antibodies, by means of the immunohistochemical examination of the hepatic tissue samples, were found to be higher in the group fed on wheat gluten, compared to the other study groups, yet this difference was except for gliadin statistically insignificant (TABLE III). It was ascertained that the immunopositivity rates for anti–transglutaminase, anti–gliadin, IgA, IgG, anti–CD4 and anti–CD8 antibodies increased over time (highest on day 165 of feeding) in all groups with the highest rates having been determined in the group fed on wheat gluten (*P*<0.05)(TABLE V). The images obtained in this study are presented in FIGS. 3 and 4.

On d 185 of the study, the immunopositivity inase, anti–gliadin, IgA, IgG, anti–CD4, and anti–CD8 antibodies by means of the immunofluorescence examination of the hepatic tissue samples, were found to be higher in the group fed on wheat gluten compared to the other study groups (TABLE V). It was ascertained that the immunopositivity rates for anti–transglutaminase, and anti–gliadin antibodies increased over time (highest on d 165 of feeding) in all groups with the highest rates having been determined in the group fed on wheat gluten (*P*<0.05)(TABLE V). The images obtained in this study are presented in FIGS. 3 and 4.

The serum levels of the hepatic enzymes investigated in the present study are given in TABLE VI. It was determined that while the levels of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) significantly differed from each other at 65 d of age, the levels of aspartate aminotransferase (AST) and LDH showed significant differences from each other at 185 d of age (*P*<0.05).

Today, gluten–containing foods have become an essential component of the human diet and individuals carrying the genes HLA–DQ2 and DQ8 confront various health problems, which are related to both the digestion of the structural proteins of gluten, and the metabolism of these proteins [9]. The gliadin peptides, which are generated upon the breakdown of gluten in the digestive system, bind to human leukocyte antigen (HLA) molecules, and thereby, induce the onset of clinical signs and immunological processes. It is known that the structural peptides of gliadin induce cellular,

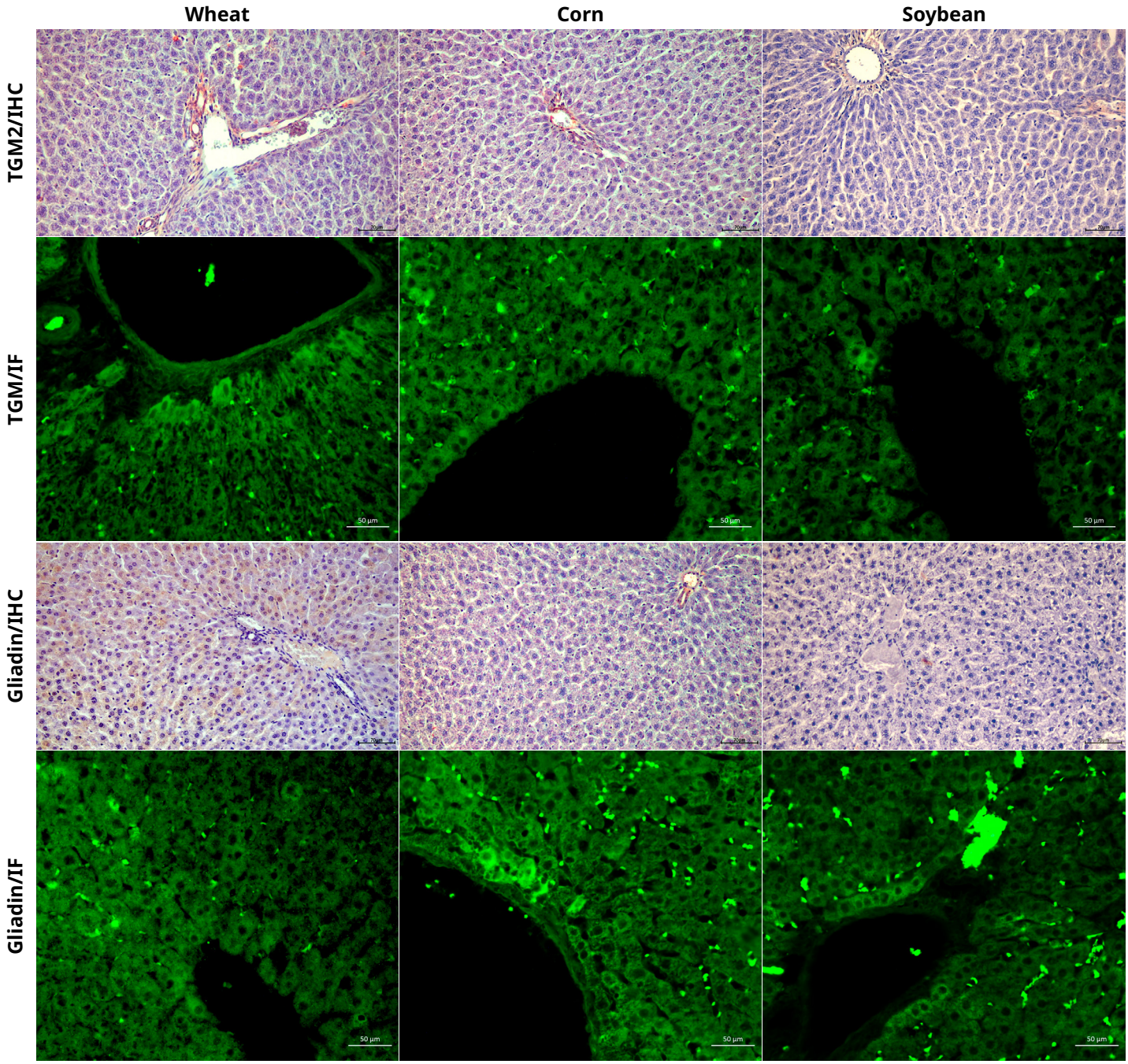

FIGURE 2. Liver tissue, 65–day–old rat, immunohistochemical and immunofluorescence staining results, IHC–P, Bar:70 um, IF, Bar: 50 µm

humoral and inflammatory responses in tissues [15]. Given that its structural peptides are resistant to both proteases and proteolysis in the gastrointestinal tract of coeliac disease patients, gliadin cannot be fully digested, and eventually triggers intestinal T cells [15]. The emergence and advance of autoimmune diseases increases directly proportional to the length of the period of exposure to gluten [16].

Reactions arising from gluten sensitivity are observed primarily in the intestines, but have also been reported in other organs and tissues [7, 17, 18]. In the present study, it was observed that glutens led to histopathological lesions in the hepatic tissue. Examination of hepatic tissue samples demonstrated that gluten exposure had led to the degeneration and necrosis of hepatocytes, biliary hyperplasia, and an increased number of fat droplets. These findings are also observed in the event of intoxication, infection and stress [19]. These findings suggest that, similar histopathological findings may develop in the hepatic tissue of animals fed on various protein sources (wheat gluten, corn gluten, soybean meal).

TABLE V
Statistical values of the histopathological, immunohistochemical, and immunofluorescence parameters of the liver tissue samples taken from average 185 days old rats

Parameters		Groups			*P*-values
		Wheat	Corn	Soybean	
Histopathological					
Deg	X̄ ± SEM	2.00 ± 0.27	1.375 ± 0.183	1.50 ± 0.188	0.168
	Median	2.00	1.00	1.50	
Inf	X̄ ± SEM	1.75 ± 0.164	1.00 ± 0.267	1.125 ± 0.295	0.096
	Median	2.00	1.00	1.00	
Bh	X̄ ± SEM	1.25 ± 0.25	1.125 ± 0.295	1.125 ± 0.295	0.947
	Median	1.00	1.00	1.00	
Fd	X̄ ± SEM	1.75 ± 0.366	0.75 ± 0.313	0.875 ± 0.295	0.110
	Median	2.00	0.50	1.00	
Immunohistochemical and Immunofluorescence					
TG IHC/IF	X̄ ± SEM	2.25 ± 0.313^{a}	0.75 ± 0.313^{b}	0.71 ± 0.360^{b}	0.066
	Median	2.50	0.50	0.00	
Gld IHC/IF	X̄ ± SEM	2.63 ± 0.420^{a}	1.13 ± 0.295^{b}	0.71 ± 0.286^{b}	0.001
	Median	3.00	1.00	1.00	
IgA	X̄ ± SEM	2.75 ± 0.366^{a}	1.75 ± 0.41^{ab}	1.125 ± 0.295^{b}	0.351
	Median	3.00	1.50	1.00	
IgG	X̄ ± SEM	1.375 ± 0.183^{a}	0.63 ± 0.183^{b}	0.50 ± 0.189^{b}	0.390
	Median	1.00	1.00	0.50	
CD4	X̄ ± SEM	1.50 ± 0.189^{a}	0.875 ± 0.295^{b}	0.625 ± 0.183^{b}	0.314
	Median	1.50	1.00	1.00	
CD8	X̄ ± SEM	2.375 ± 0.263^{a}	1.375 ± 0.83^{b}	1.50 ± 0.189^{b}	0.198
	Median	2.00	1.00	1.50	

The values are given as mean ± standard error of the mean (SEM), n=8, $^{a, b}$: Means in the same line with different superscripts differ significantly (*: $P<0.05$), (**: $P<0.01$). Deg: Degeneration, Inf: Inflammation, Bh: Biliary hyperplasia, Fd: Fat drop, TG: Transglutaminase, Gld: Gliadin, IgA: Immunoglobulin A, IgG: Immunoglobulin G

TABLE VI
Statistical values of the liver enzyme levels of the serum tissue samples

Parameters	Groups			*P*-values
	Wheat	Corn	Soybean	
65 days of age				
AST	350.000 ± 35.972	392.000 ± 30.430	364.000 ± 30.100	0.655
ALT	541.000 ± 136.125^{a}	227.000 ± 23.791^{b}	211.000 ± 27.019^{b}	0.023
AST/ALT	0.65	1.73	1.73	
ALP	155.600 ± 21.951	128.800 ± 29.778	184.400 ± 16.690	0.282
LDH	401.800 ± 32.346^{b}	516.600 ± 21.919^{a}	535.600 ± 12.258^{a}	0.004
185 days of age				
AST	400.000 ± 13.928^{b}	516.000 ± 37.736^{a}	490.000 ± 23.749^{a}	0.025
ALT	179.000 ± 73.034	241.000 ± 59.414	213.000 ± 66.888	0.808
AST/ALT	2.24	2.14	2.30	
ALP	133.400 ± 14.497	105.500 ± 13.093	166.800 ± 25.498	0.135
LDH	451.800 ± 19.059^{b}	554.800 ± 26.097^{a}	507.200 ± 10.283^{ab}	0.010

The values are given as mean ± standard error of the mean (SEM). n=8, $^{a, b}$: Means in the same line with different superscripts differ significantly ($P<0.05$, $P<0.01$). AST: aspartate aminotransferase, ALT: alanine aminotransferase and ALP: alkaline phosphatase, LDH: lactate dehydrogenase

It was ascertained that, on days 45 and 165 of the study, the differences between the increases in the histopathological findings of the hepatic tissue (degeneration, necrosis, inflammation, biliary hyperplasia and fat droplets) were statistically insignificant (TABLES III and V). On the other hand, on both d 45 and 165 of the study, values pertaining to the severity of the histopathological findings in the hepatic tissue, including degeneration, necrosis, inflammation, biliary hyperplasia and fat droplets, were numerically higher in Group Wheat, compared to the other study groups, and this suggests that the hepatic tissue is more sensitive to wheat gluten. It is known that the prolonged consumption of gluten-containing foods increases the risk of gluten-related diseases in humans [16, 20].

Immunohistochemical examinations are essential to determining histopathological tissue lesions, which develop in response to gluten sensitivity. Immunohistochemical examinations enable the determination of immunopositivity at cellular and tissue level, as well as, the localization and distribution of specific cell components. These examinations are based on the identification of specific cell or tissue components by means of antigen-antibody reactions [21]. The agreement of the results of these analyses with those of conventional pathological and cytological examinations and other findings is highly important [22, 23].

Tissue anti-transglutaminase antibodies are produced by means of a complicated mechanism. Glutens, found in the structure of cereal proteins (and primarily gliadins), are perceived by tissues as antigens. Thus, T cells are stimulated for the synthesis of antibodies against these antigens. This results in increased levels of anti-transglutaminase antibodies in tissues. A typical example to this is the increased levels of anti-transglutaminase antibodies (tTG) in the hepatic tissue of humans, who carry the genes HLA-DQ2 and DQ8 and consume wheat gluten [9, 24, 25]. In the present study, although no marked increase was detected in the hepatic tissue anti-transglutaminase antibody levels of 65 days old rats fed on glutens, it was observed that sensitivity reactions increased in the hepatic tissue over time and with prolonged feeding on gluten (165 d). Furthermore, the highest tissue levels of anti-transglutaminase antibodies were determined in the group fed on wheat gluten. The present study, which was carried out on healthy rats, demonstrated that gluten sensitivity is not only observed in humans carrying the genes HLA-DQ2 and DQ8, such that similar gluten-induced reactions can be observed in the hepatic tissue of animals not carrying these genes. In a study conducted on rats, it was reported that tissue transglutaminase 2 levels increased in groups given wheat and corn gluten, and immunopositivity for transglutaminase antibody was observed in hepatocytes, inflammatory cells, and epithelial cells of the glands [1]. In another study, immunohistochemical examination of liver tissues in celiac patients revealed that transaminase antibody release was increased, particularly in endothelial cells and periportal hepatocytes [9].

While it is reported that the level of anti-gliadin antibodies generally increases in chronic diseases, 90% of untreated coeliac disease patients also have increased tissue levels of anti-gliadin antibodies [26]. The gliadin protein is presented to reactive CD4+T cells by a T cell receptor, which triggers the production of cytokines that cause tissue damage. CD4 and T cells increase the levels of T Helper 1 and T Helper 2, both of which are proinflammatory cytokines. These cytokines, in return, bring about the generation of firstly B-lymphocytes and then plasma cells. Plasma cells produce anti-gliadin and anti-transglutaminase antibodies [26]. In the present study, the level of immunopositivity for anti-gliadin antibodies in the hepatic

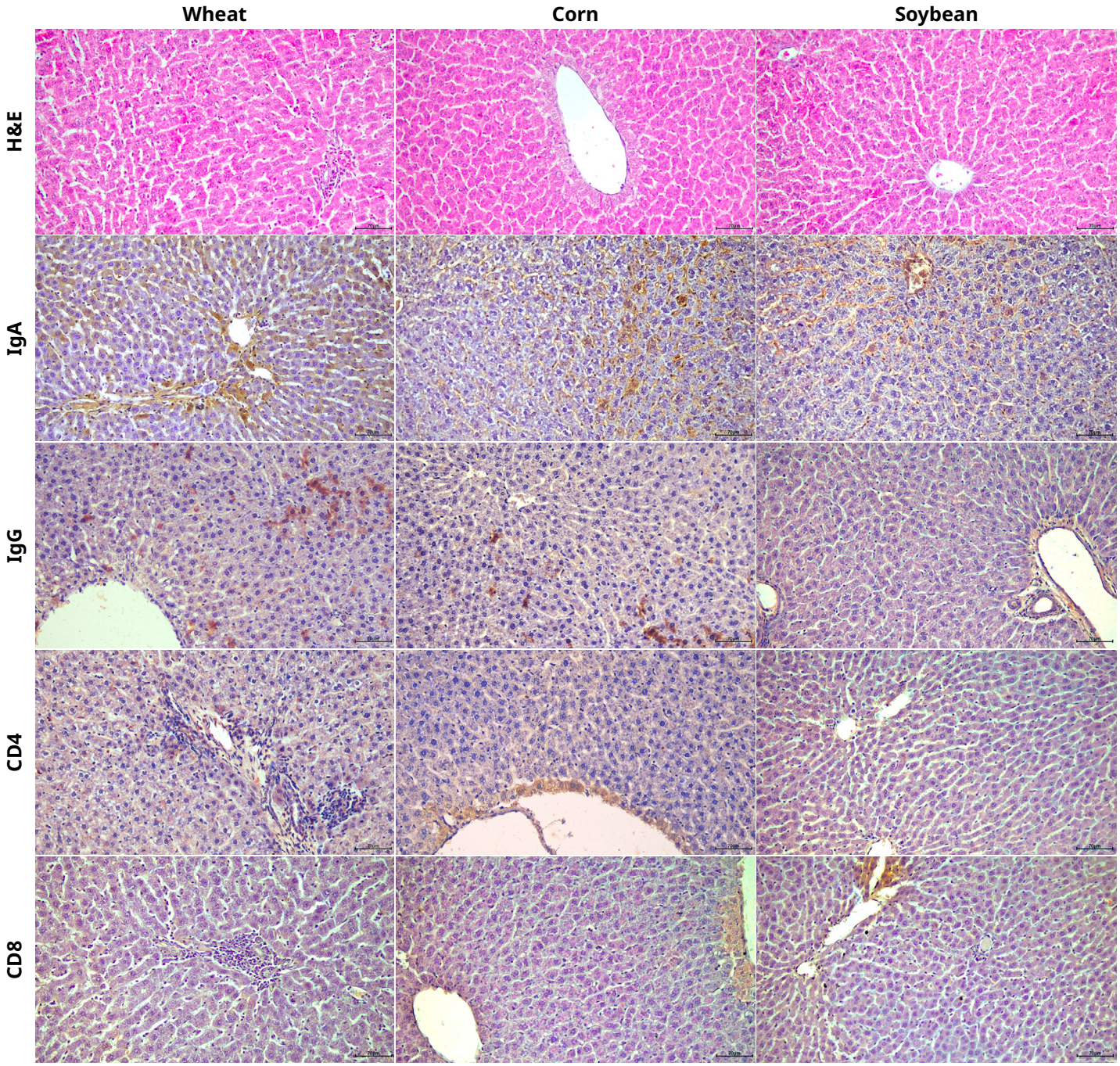

FIGURE 3. Liver tissue, 185–day–old rat, histopathological appearance, H&E, Bar: 70 µm. immunohistochemical staining results, IHC–P, Bar: 70 µm

tissue on d 45 and 165 of the study having observed to differ, points out to the significance of the length of the gluten exposure period in the development of tissue sensitivity. The findings of the present study showed that the group displaying the highest sensitivity level to anti–gliadin antibodies was Group Wheat. An important finding of the present study was the increase that occurred in tissue sensitivity with prolonged exposure to wheat gluten, even in healthy animals.

IgA is a primary component of many external secretions. Secretory IgA molecules are particularly effective against microbial agents on mucosal surfaces, and by preventing bacterial pathogens and their toxins from adhering to mucosal epithelial cells, they neutralize these agents. IgG is produced during the secondary immune response and dominates the extracellular fluid [27]. IgG antibodies act as opsonin and activate the complement system via the classical pathway [28, 29, 30]. On d 45 of the study, the IgA and IgG levels of Group Wheat were observed to be numerically higher. It was determined that the statistical differences observed between the groups increased with the prolongation of the period of exposure (period of feeding on

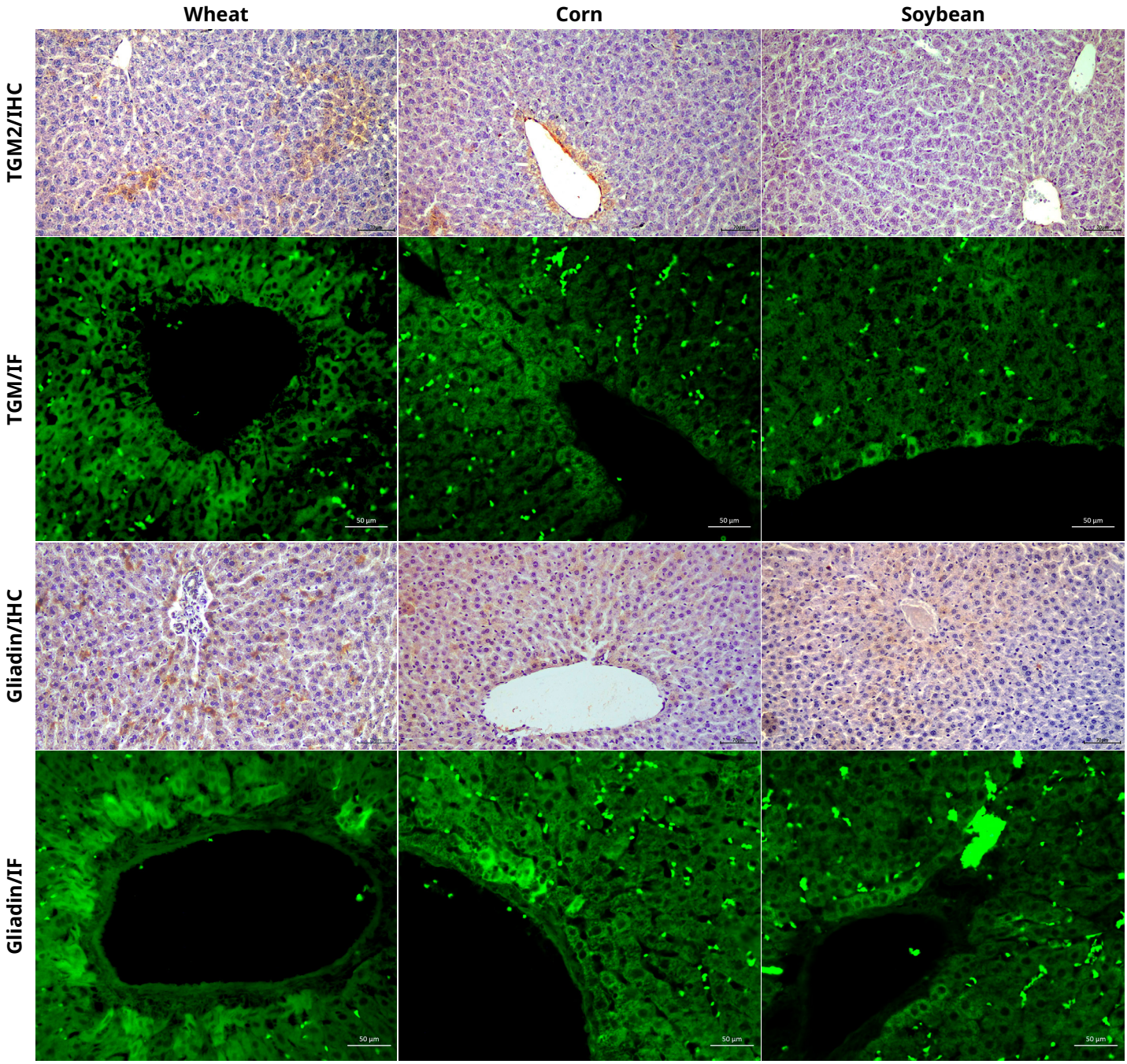

FIGURE 4. Liver tissue, 185–day–old rat, immunohistochemical and immunofluorescence staining results, IHC–P, Bar:70 um, IF, Bar: 50 µm

gluten). Both IgA and IgG levels were observed to have significantly increased in the group fed on wheat gluten.

Hepatic enzyme activities are known to vary with several factors, including among others disease, stress, pharmaceutical treatment, environmental factors and nutrition. Damage to hepatic tissue, either with a symptomatic or asymptomatic clinical course, leads to elevated hepatic enzyme levels [31, 32, 33]. Multiple studies have reported that glutens cause damage to various tissues and organs, initially in the intestines, in individuals carrying the genes HLA–DQ2 and DQ8 [1, 5].

The present study demonstrated the effect of gluten–containing feed rations on liver enzyme activities. The investigation of the effects of dietary wheat gluten, corn gluten and soybean meal on liver enzyme activities in the study groups revealed no effect on AST and ALP activities at 65 days of age, and interestingly, higher AST activity in Group Wheat and higher ALP activity in Groups Wheat and Corn at 185 d of age. The ALT activity of Group Wheat determined to have significantly increased at 65 days of age and to have decreased at 185 d of age suggests the enzyme activity of the hepatic tissue to

have adapted to gluten exposure. In Group Wheat, LDH activity being lower than that of Groups Corn and Soybean at 65 d of age, and being significantly higher than that of Group Soybean demonstrates the impact of diet on liver enzyme activities. Furthermore, the AST/ALT ratio having increased, and having exceeded a value of 2.00 in all study groups shows that liver enzyme activities are not only affected by diet, but also by age. In a study conducted on rats, it was reported that in the group where corn gluten was used as a protein source, serum AST and ALT levels decreased, while ALP levels and AST/ALT ratio were similar to those in the control group [34].

CONCLUSIONS

It was ascertained that, compared to the groups that received dietary corn gluten and soybean meal, the group fed on wheat gluten presented with more severe adverse histopathological effects on the hepatic tissue, including degeneration, necrosis and fat droplets. Furthermore, it was determined that prolonged exposure increased the adverse effects of dietary gluten on the immunohistochemical parameters of the hepatic tissue. Wheat gluten was found to have significantly increased the immunopositivity levels for all of the antibodies (anti-transglutaminase, anti-gliadin, IgA, IgG, anti-CD4 and anti-CD8 antibodies) used in the immunohistochemical examinations. It is considered that the findings of the present study will provide a reference for future studies on gluten metabolism and autoimmune disorders.

Ethical approval

The experimental protocol of the study was approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee's decision dated 2024 and numbered 08.

Conflicts of interest

The authors have no declaration of competing interests.

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