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RESEARCH ARTICLE

Serum and Intestinal Tissue Zonulin Levels in the Evaluation of Intestinal Permeability in Rats with Acute Pancreatitis

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Abstract: To evaluate the effects on malondialdehyde (MDA), superoxide dismutase (SOD), lipid hydroperoxide (LOOH), glutathione (GSH) levels as oxidative stress markers, zonulin levels, and histopathological findings of experimental acute pancreatitis (AP). This study was conducted three groups of Sprague-Dawley rats (seven animals in every group). First group was evaluated as control group (sham laparotomy). Group 2 (AP, 48% ethyl alcohol) and group 3 (severe pancreatitis (SP), 80% ethyl alcohol) was performed. The effects of pancreatitis were evaluated by comparing these groups according to histopathologial results of polimorphonuclear leukocytes, infiltration, oedema, haemorrhagie, apoptosis, aciner cellular degeneration in pancreatic and intestinal tissue. In the SP group, completely flattened mucosal surface and severe villi loss (total villous atrophy), disorganization and hyperplasia in the crypts in the lamina propria were detected. MDA, LOOH and zonulin levels in serum and intestinal tissue were found to be significantly higher in the SP group compared to the control group. The serum and intestinal tissue SOD and GSH were found to be significantly lower than the control group. In the AP group, while LOOH and zonulin in tissues were significantly higher than control, only zonulin levels in serum were found higher than control. Zonulin and oxidative stress is basically involved in the pathogenesis of pancreatitis. Biochemical results are also supported by histopatological improvement in intestinal and pancreas tissue. Patients with pancreatitis may be more exposed to impaired gut barrier function. Serum zonulin levels can be used in the evaluation of intestinal permeability in AP.

Keywords: *Experimental acute pancreatitis, Intestinal permeability, Lipid hydroperoxide, Malondialdehyde, Zonulin*

Akut Pankreatitli Sıçanlarda Bağırsak Geçirgenliğinin Değerlendirilmesinde Serum ve Bağırsak Dokusu Zonulin Düzeyleri

Öz: Deneysel akut pankreatitin (AP) histopatolojik bulguları ile zonulin düzeyleri ve oksidatif stres belirteçleri olan malondialdehit (MDA), süperoksit dismutaz (SOD), lipid hidroperoksit (LOOH) ve glutatyon (GSH) düzeyleri arasındaki ilişkinin değerlendirilmesi amaçlanmıştır. Bu çalışmaya, üç grup olacak şekilde Sprague-Dawley sıçanı (her grupta yedi hayvan) alınmıştır. Birinci grup kontrol grubu (sham laparotomi) olarak belirlendi. Grup 2 (%48 etil alkol) AP ve grup 3 (%80 etil alkol) ağır (şiddetli) pankreatit (ŞP) olarak tasarlandı. Pankreatitin etkileri, pankreas ve bağırsak dokusunda bu gruplar karşılaştırılarak histopatolojik sonuçlara göre polimorfonükleer lökositler, infiltrasyon, ödem, hemoraji, apoptoz, asiner hücresel dejenerasyon değerlendirildi. ŞP grubunda tamamen düzleşmiş mukozal yüzey ve ciddi villus kaybı (total villus atrofisi), lamina propriada kriptlerde dezorganizasyon ve hiperplazi tespit edildi. Serum ve bağırsak dokusunda MDA, LOOH ve zonulin düzeyleri kontrol grubuna göre ŞP grubunda anlamlı olarak yüksekti. Serum ve bağırsak dokusu SOD ve GSH değerleri kontrol grubuna göre anlamlı düşük bulundu. AP grubunda dokulardaki LOOH ve zonulin düzeyleri kontrol grubuna göre anlamlı yüksek bulunurken, sadece serum zonulin düzeyleri kontrole göre daha yüksekti. Zonulin ve oksidatif stres esas olarak pankreatit patogenezinde rol oynar. Biyokimyasal sonuçlar ayrıca bağırsak ve pankreas dokusundaki histopatolojik iyileşme ile de desteklenmektedir. Pankreatitli hastalar bozulmuş bağırsak bariyer fonksiyonuna daha fazla maruz kalabilirler. AP'de bağırsak geçirgenliğinin değerlendirilmesinde serum zonulin düzeyleri kullanılabilir.

Anahtar sözcükler: *Bağırsak geçirgenliği, Deneysel akut pankreatit, Lipid hidroperoksit, Malondialdehit, Zonulin*

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Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas associated with tissue damage and necrosis, its incidence continues to increase worldwide and is the leading cause of hospitalizations [1-3]. AP has a mild form involving only the pancreas and a severe form that results in extra pancreatic organ failure associated with systemic inflammatory response syndrome and even death [1]. In addition, it is known that recurrent AP may cause chronic pancreatitis and as a result, exocrine and endocrine insufficiency may develop [4,5]. However, today there are no therapeutic agents that can change the course of the disease [1]. At the same time, our knowledge about the etiology and pathogenesis of the disease is limited. One reason for the limited information about pathogenesis is that lack of access to the human pancreatic organ during illness [1,2]. Nevertheless, significant advances have been made in recent years in elucidating the pathophysiological mechanisms of acute pancreatitis. Studies conducted in this context indicate that intestinal permeability increases in acute pancreatitis, but it is not clear whether this is a mechanism that causes AP or a just a consequence of the disease [3,6-8].

Epithelial tight junctions prevent allergens, toxins, and pathogens from entering the interstitial tissue through the epithelium in various organ systems. In the gastrointestinal tract, disruption of tight junctions and loss of epithelial barrier function increases intestinal permeability to harmful factors that cause inflammation and mucosal damage [9]. It is emphasized that systemic inflammatory syndrome and bacterial translocation developing in AP may develop after intestinal permeability increase [8].

The protein, zonulin, has been identified as an important regulator of intestinal permeability. When bound to surface receptors, intracellular actin filaments polymerize, and this process causes opening of tight junctions and increased intestinal permeability [10]. Dysregulation of the zonulin pathway followed by "intestinal leakage" due to increased intestinal permeability has been associated with the pathogenesis of many gastrointestinal, autoimmune, inflammatory and neoplastic diseases [11].

Another factor that may cause impairment of intestinal permeability is free oxygen radicals, mostly form of reactive oxygen species (ROS). ROS modulate multiple signaling pathways and disrupt tight junctions and damage the epithelial and endothelial barrier [9]. In an animal study, it was reported that in the early stage of acute pancreatitis, free radicals derived from cytotoxic oxygen can contribute to the improvement of changes in intestinal permeability and absorption function [12].

Our aim in this study is to investigate the possible dysfunction in the zonulin-tight junction mechanism and also possible role of oxidative stress markers as malondialdehyde (MDA), lipid hydroperoxide (LOOH), Cu, Zn-superoxide dismutase (Cu-Zn-SOD) and glutathione (GSH) in acute pancreatitis. In addition, possible changes in serum zonulin levels in the early stages of these diseases may bring clinical use as a biomarker. Level changes in later stages may guide the follow-up of the disease in chronic stages.

Material and Methods

Ethical Statement

This study was approved by the Yeditepe University Animal Experiments Local Ethics Committee (Approval no: 2019/10-3).

Animals

Care and handling of the animals was in accordance with the Helsinki Declaration of 1975, as revised in 2000. Animals were housed in individual cages in a temperature-controlled room (23±1°C) and a light-dark cycle-controlled environment (12 h) with free access to food and water. Experiments were performed on 21 Sprague-Dawley rats with an average body weight of 250-320 g.

Experimental Design

In this study, a total of 21 rats, 7 in each group, were used. Groups were determined as group 1 (control, sham laparotomy), group 2 (acute pancreatitis (AP), 48% ethyl alcohol), and group 3 (severe pancreatitis (SP), 80% ethyl alcohol). AP and SP were performed adhering to the experimental model previously created by us [13]. Our study was carried out in accordance with the Animal Care and Use Committee (ACUC) criteria. Animals were fed with standard lab diet and *ad libitum* water before and after surgery. Animals had access to standard laboratory feed and water *ad libitum* and were not subjected to any restrictions.

Surgical Procedure

After anesthesia, 48% ethyl alcohol was injected into one group at a dose of 1 cm^3 with a fine dental needle into the suspended biliopancreatic duct to be opened with a midline incision after anesthesia, and 80% ethyl alcohol was injected into the other group. All groups were sacrificed on the 3rd postoperative day. Maximum blood was taken by abdominal midline exploration and cardiac puncture under anesthesia, and in all groups, the 5-6 cm segment of pancreatic tissues, distal ileum and transverse colon were excised. The tissues were divided into two, fixed in 10% buffered formalin, embedded in paraffin for standard histologic examinations and immediately frozen at -80°C for biochemical analysis.

Tissue Preparation and Biochemical Analysis

The intestinal tissue sample was diluted to a 20% w/v solution

in 20 mM ice-cold Tris HCl (pH 7.4) and homogenized with a Bosch Scintilla SA (Switzerland). The homogenate was centrifuged at 5000×*g* for 10 min; all biochemical parameters were determined in the same supernatant fraction of each homogenized pancreatic sample.

Assay of Zonulin

Determination of zonulin levels in intestinal tissue and serum samples were done using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) technique (Sunred Biological Technology®, Shanghai, China) according to the manufacturer's protocol. Assay range and sensitivity for zonulin levels were 0.3-90 ng/mL and 0.287 ng/mL, respectively.

Assay of Malondialdehyde (MDA)

One of the end products of lipid peroxidation is MDA. MDA levels were determined as previously described by Ohkawa et al.[14] with a minor modification. The intra- and inter-assay CV values were 3.1% (n=20) and 4.0% (n=20), respectively.

Assay of Lipid Hydroperoxide (LOOH)

LOOH levels were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange version 2 (FOX2) [15]. The intra- and inter-assay CV values were 3.5% (n=20) and 4.3% (n=20), respectively.

Assay of Cu, Zn, Superoxide Dismutase (Cu, Zn-SOD)

Cu, Zn-SOD activity were determined in terms of the inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine/xanthine oxidase used as a superoxide generator [16]. The intra- and inter-assay CV values were 3.6% (n=20) and 4.8% (n=20), respectively.

Assay of Glutathione (GSH)

Intestinal tissue GSH levels were measured as per the method of Beutler et al.[17]. The intra- and inter-assay CV values were 3.2% (n=20) and 4.4% (n=20), respectively.

Histopathological Examination

Tissue samples were routinely %10 formalin-fixed and paraffin-embedded. For histological evaluation each 5 µm section prepared and stained with H&E in Health Sciences University Haydarpasa Numune Training and Research Hospital Pathology Laboratory.

Statistical Analysis

Results of biochemical parameters were expressed as mean ± standard deviation. For comparing three groups comparison, one-way ANOVA test and post-hoc Tukey test were applied. Relationships between variables were assessed with Pearson's correlation coefficient. A P value equal to or lower than 0.05 was considered statistically significant. All analyzes were performed with the SPSS 22.0 (IBM Corp., Armank, USA) statistical package program.

Results

Biochemical Findings

Oxidative stress parameters (MDA, LOOH, Cu, Zn-SOD and GSH) and zonulin levels were examined in intestinal tissue and serum samples belonging to the experimental groups. Biochemical results in control, AP and SP groups were shown in *Table 1*.

In correlation analysis, it was found that the correlations in both AP and SP groups were largely in the same direction. Correlations of biochemical parameters in AP and SP were documented in *Table 2* and *Table 3*.

Histopathological Findings

As a result of histopathological examinations of pancreatic tissues belonging to the control groups, it was found that there were densely packed pancreatic acinar glands

Table 1. *Biochemical results in control, acute and severe pancreatitis group*

Parameters		Control (n=7)	Acute pancreatitis (n=7)	Severe pancreatitis (n=7)
		Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Tissue	MDA (µmol/g wet tissue)	57.31±8.46	68.28±6.64	72.71±9.62 a**
	LOOH (nmol/g wet tissue)	2.14±0.42	2.62±0.26 a*	2.88±0.32 a**
	Cu-Zn SOD (U/g wet tissue)	28.80±2.77	24.35±3.75	20.00±3.54 a***
	GSH (µmol/g wet tissue)	66.36±5.44	60.96±4.49	54.74±5.26 a***
	Zonulin (ng/g wet tissue)	3.38±0.84	11.37±1.76 a***	14.66±3.02 a***, b*
Serum	MDA (µmol/mL)	3.17±0.37	3.53±0.45	3.95±0.45 a**
	LOOH (nmol/mL)	0.47±0.08	0.58±0.09	0.76±0.13 a***, b*
	Cu-Zn SOD (U/mL)	20.16±2.49	17.53±1.99	16.27±2.05 a**
	Zonulin (ng/mL)	13.84±2.11	17.55±2.73 a*	20.47±2.37 a***, b*

MDA: Malondialdehyde; ***LOOH:*** Lipid hydroperoxide; ***Cu, Zn-SOD:*** *Cu, Zn-speroxide dismutase*; ***GSH:*** *Glutathione*
** P<0.05, ** P<0.01, *** P<0.001;* ***a:*** *Compared to Control,* ***b:*** *Compared to Acute pancreatitis*

Table 2. *Correlations of biochemical parameters in acute pancreatitis*

Parameters		Tissue LOOH	Tissue Cu, Zn-SOD	Tissue GSH	Tissue Zonulin	Serum MDA	Serum LOOH	Serum Cu, Zn-SOD	Serum Zonulin
Tissue MDA	r	0.926**	-0.865*	-0.860*	0.810*	0.935**	0.929**	-0.912**	0.815*
	p	0.003	0.012	0.013	0.027	0.002	0.003	0.004	0.025
Tissue LOOH	r	-	-0.861*	-0.945**	0.752	0.904**	0.855*	-0.918**	0.761*
	p	-	0.013	0.001	0.051	0.005	0.014	0.004	0.047
Tissue Cu, Zn-SOD	r		-	0.907**	-0.940**	-0.887**	-0.976**	0.938**	-0.972**
	p		-	0.005	0.002	0.008	0.000	0.002	0.000
Tissue GSH	r			-	-0.823*	-0.820*	-0.869*	0.872*	-0.816*
	p			-	0.023	0.024	0.011	0.010	0.025
Tissue Zonulin	r				-	0.799*	0.926**	-0.884**	0.976**
	p				-	0.031	0.003	0.008	0.000
Serum MDA	r					-	0.910**	-0.898**	0.819*
	p					-	0.004	0.006	0.024
Serum LOOH	r						-	-0.941**	0.960**
	p						-	0.002	0.001
Serum Cu, Zn-SOD	r							-	-0.920**
	p							-	0.003

MDA: Malondialdehyde; ***LOOH:*** Lipid hydroperoxide; ***Cu, Zn-SOD:*** *Cu, Zn-speroxide dismutase;* ***GSH:*** *Glutathione; * P<0.05 ** P<0.01 *** P<0.001*

Table 3. *Correlations of biochemical parameters in severe pancreatitis*

Parameters		Tissue LOOH	Tissue Cu, Zn-SOD	Tissue GSH	Tissue Zonulin	Serum MDA	Serum LOOH	Serum Cu, Zn-SOD	Serum Zonulin
Tissue MDA	r	0.929**	-0.773*	-0.976**	0.722	0.831*	0.962**	-0.935**	0.798*
	p	0.002	0.041	0.000	0.067	0.020	0.001	0.002	0.031
Tissue LOOH	r	-	-0.673	-0.939**	0.699	0.838*	0.899**	-0.759*	0.809*
	p	-	0.097	0.002	0.081	0.019	0.006	0.048	0.028
Tissue Cu, Zn-SOD	r		-	0.816*	-0.939**	-0.904**	-0.792*	0.764*	-0.930**
	p		-	0.025	0.002	0.005	0.034	0.045	0.002
Tissue GSH	r			-	-0.822*	-0.920**	-0.985**	0.910**	-0.888**
	p			-	0.023	0.003	0.000	0.004	0.008
Tissue Zonulin	r				-	0.940**	0.814*	-0.692	0.979**
	p				-	0.002	0.026	0.085	0.000
Serum MDA	r					-	0.879**	-0.764*	0.982**
	p					-	0.009	0.046	0.000
Serum LOOH	r						-	-0.918**	0.863*
	p						-	0.004	0.012
Serum Cu, Zn-SOD	r							-	-0.718
	p							-	0.069

MDA: Malondialdehyde; ***LOOH:*** Lipid hydroperoxide; ***Cu, Zn-SOD:*** *Cu, Zn-speroxide dismutase;* ***GSH:*** *Glutathione; * P<0.05 ** P<0.01 *** P<0.001*

(Fig. 1); in the AP group, focal segmental fibrosis among intact parenchymal areas were selected. Plasma cell-predominant moderate inflammatory cell infiltration in which rare eosinophil leukocytes and ductal proliferation, disorganization, irregularity was observed in the fibrosis area in the pancreatic ducts. Regenerative changes were also detected in the ductal epithelium *(Fig. 2)*. In the SP group, distinct acinar atrophy, extensive fibrosis, hemorrhage, intense inflammation which rich in polymorphous leukocytes, fat necrosis and acinar necrosis were seen in the parenchyma *(Fig. 3)*.

As a result of the histopathological examinations of the intestinal tissues, evenly distributed villi on the luminal surface and mucosal layers of usual morphology were observed in the control group *(Fig. 4)*. In the AP group,

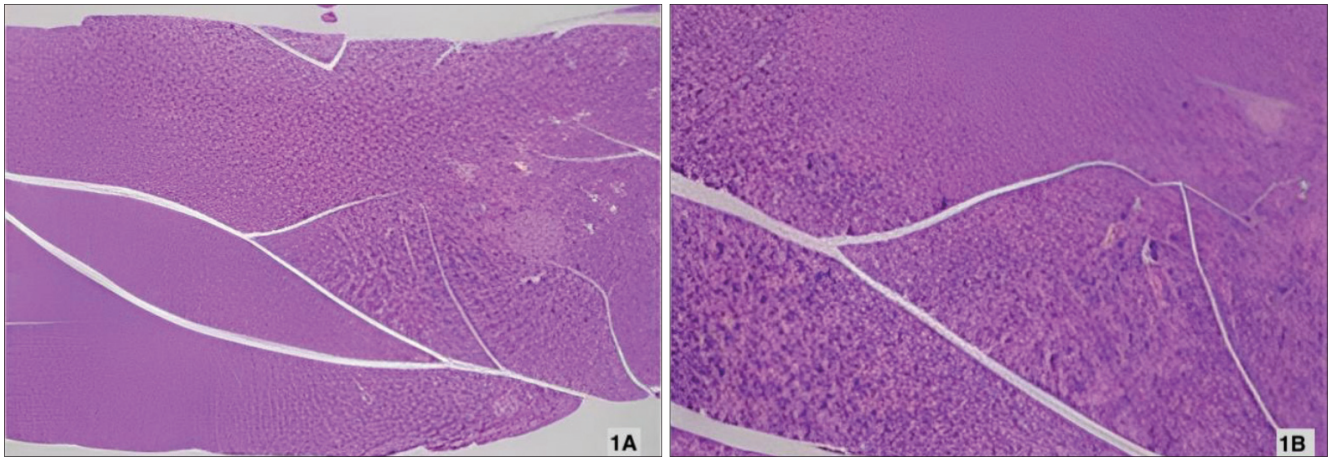

Fig 1. Pancreatic tissue of the control group; **A.** Densely packed pancreatic acini (H&E X200) **B.** Normally pancreatic tubuloacinar glands and 1 Langerhans Islet (H&E X40) in the right midline

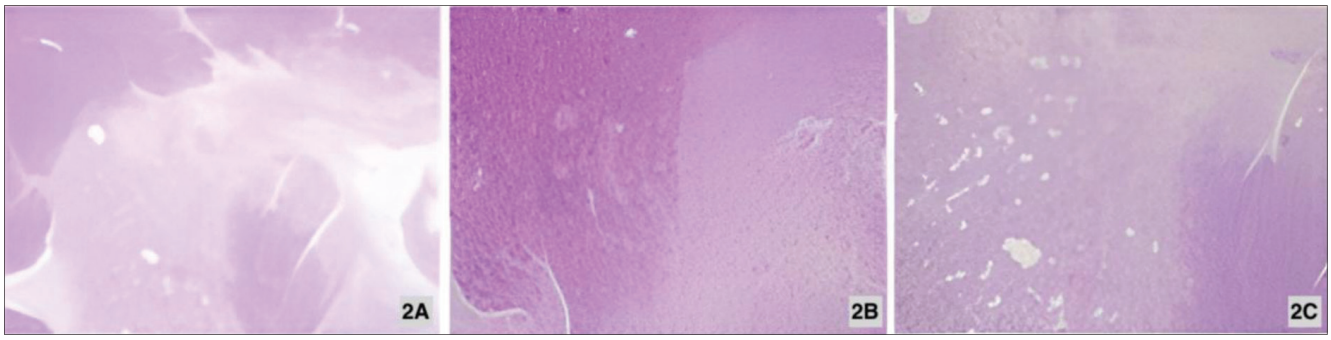

Fig 2. Pancreatic tissue of the acute pancreatitis group; **A.** Focal segmental fibrosis between intact parenchymal areas, moderate inflammatory cell infiltration, and irregularity of the pancreatic ducts (H&E X40) **B.** Plasma cell predominant chronic inflammation in which sparse eosinophil leukocytes are selected in the fibrosis area (H&E X200). **C.** Periductal fibrosis and moderate chronic inflammation. Proliferation, disorganization and irregularity in the ducts. Regenerative changes in the ductal epithelium (H&E X200)

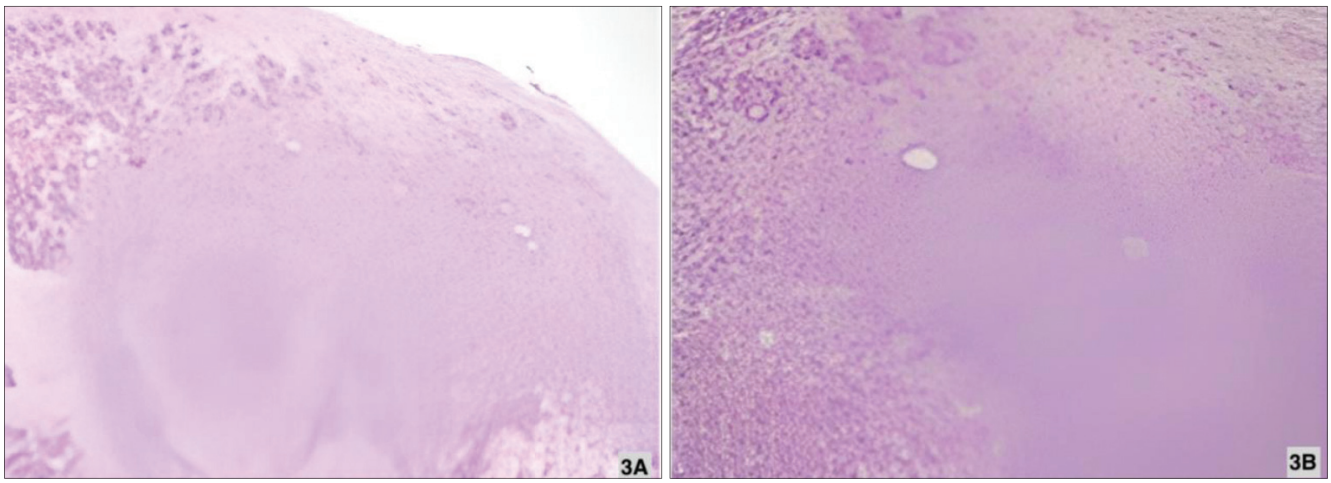

Fig 3. Pancreatic tissue of the severe pancreatitis group; **A.** Significant acinar atrophy, extensive fibrosis and intense inflammation in the parenchyma. Fat necrosis (lower right) and acinar necrosis (middle) (H&E X40). **B.** Intraparenchymal extensive fibrosis, intense inflammation rich in polymorphous leukocytes and hemorrhage (H&E X400)

moderately blunting of the villus size, moderate degeneration and desquamation in the intestinal epithelium and loss of intestinal epithelial cells, irregularity and disorganization in the crypts in the lamina propria were detected. Mild inflammatory cell increase accompanied by eosinophil leukocytes in the lamina propria was observed *(Fig. 5)*. In the SP group, completely flattened mucosal surface and severe villi loss (total villous atrophy), disorganization and hyperplasia in the crypts in the lamina propria were detected *(Fig. 6)*.

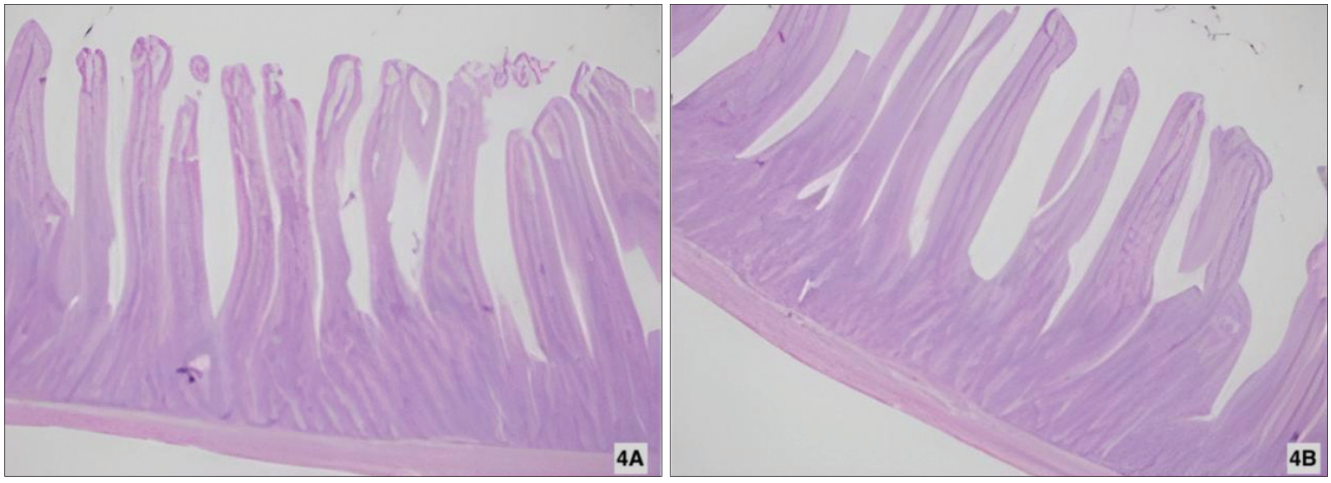

Fig 4. Intestinal tissue of the control group; **A.** Regularly distributed villus on the luminal surface and mucosal layers of usual morphology (H&E X200). **B.** Villus that are parallel to each other and distributed regularly (H&E X200)

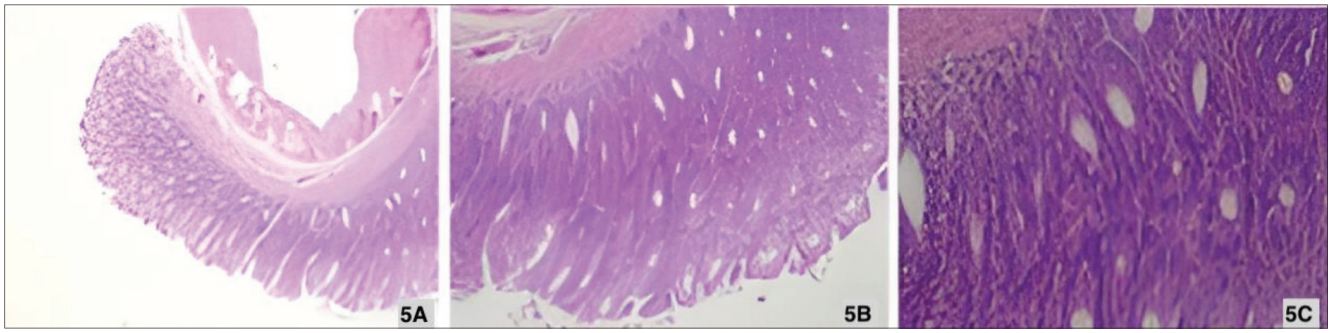

Fig 5. Intestinal tissue of the acute pancreatitis group; **A.** Moderate blunting in villus sizes and loss of intestinal epithelial cells (H&E X100). **B.** Moderate degeneration and desquamation of the intestinal epithelium. Irregularity in the crypts in the lamina propria (H&E X200). **C.** Disorganization in crypts and mild inflammatory cell increase in lamina propria accompanied by eosinophil leukocytes (H&E X400)

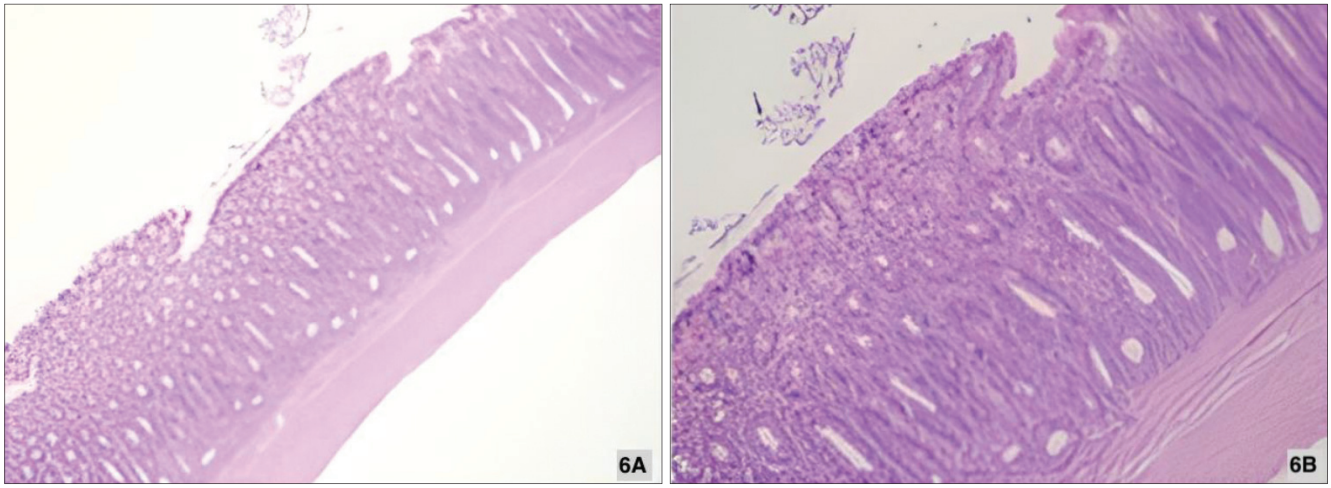

Fig 6. Intestinal tissue of the severe pancreatitis group; **A.** Completely flattened mucosal surface and severe villi loss (total villous atrophy). Crypt hyperplasia in the lamina propria (H&E X200). **B.** Severe villus atrophy on the surface. Disorganization and hyperplasia of the crypts in the lamina propria (H&E X400)

Discussion

ROS and reactive nitrogen species (RNS), which play an active role in the early and late stages of AP, cause deterioration in cell membrane and functions by direct action on lipids and proteins, and damage in pancreatic cells with the release of lysosomal enzymes in experimental and human studies [18-20]. However, to date no information about the possible dysfunction in the zonulin-tight junction mechanism in AP has been reported. The main findings of this study were that (i) in the AP group, while LOOH and zonulin levels in intestinal tissues were significantly

higher than control, only zonulin levels in serum samples were found higher than control, (ii) while zonulin levels in intestinal tissue samples of the SP group were found to be significantly higher compared to the AP group, in serum samples, only LOOH levels were found to be significantly higher, (iii) the serum zonulin could be used to distinguish AP from SP was serum zonulin. Zonulin is basically involved in the pathogenesis of pancreatitis. Patients with pancreatitis may be more exposed to impaired gut barrier function [21]. Zonulin can be used as a biomarker of impaired gut barrier function for pancreatitis and can be a potential therapeutic target for the treatment of these disturbing situations.

Lipid peroxidation and free oxygen radicals may also be effective in the pathogenesis of AP as a result of oxidative stress. In experimental pancreatitis studies, while MDA levels, the final product of lipid peroxidation, increased, it was observed that glutathione peroxidase (GPaz), catalase, and SOD, which are protective enzymes from free oxygen radicals and GSH decreased [19-21]. In experimental pancreatitis studies, while MDA levels, the final product of lipid peroxidation increased, glutathione peroxidase (GPaz), catalase and SOD, which are protective enzymes from free oxygen radicals, and GSH were found to decrease [22-27]. Intestinal epithelial barrier dysfunction and increased permeability have been described in many human diseases, including SP [28-33]. In current study, while LOOH and zonulin levels increased in AP compared to control group, it was found to be increased in SP group compared to AP in pancreas tissue. Serum zonulin levels increased significantly in AP compared to the control group, while it was found to be increased in SP compared to AP. Moreover, a positive correlation was found between serum zonulin and tissue zonulin, tissue and serum MDA, and serum LOOH. The increase in tissue and serum zonulin levels in the study confirms the increase in intestinal permeability in AP. While there was a negative correlation between tissue zonulin and tissue GSH and Cu, Zn-SOD activity, a negative correlation was found between serum zonulin and tissue and serum Cu, Zn-SOD, and tissue GSH in AP. It indicates that oxidative stress increases in AP and a decrease in antioxidant capacity eliminates the increased levels of free radicals. The impairment of intestinal permeability in pancreatitis was demonstrated by the increase of oxidant substances such as LOOH and MDA, and the decrease of antioxidants such as GSH and Cu, Zn-SOD. All of results support the hypothesis of increased oxidative stress and decreased antioxidant capacity during increased intestinal permeability with increased zonulin levels with AP. Zonulin secretion seems to be one of the most important causes of increased intestinal permeability in AP and SP. Fishman et al.[34] demonstrated that damage to the unstirred mucus layer with evidence of oxidative stress occurs during AP-induced gut barrier failure in rats. Liu et al.[28] suggests that gut mucosal damage takes place in early phase of AP and more severe in SP than mild AP patients. The results of our study are supported by the results of Fishman et al.[34]'s study.

In the SP group, distinct acinar atrophy, extensive fibrosis, hemorrhage, intense inflammation which rich in polymorphous leukocytes, fat necrosis and acinar necrosis were seen in the parenchyma in pancreatic tissues. In the SP group, completely flattened mucosal surface and severe villi loss (total villous atrophy), disorganization and hyperplasia in the crypts in the lamina propria were detected in intestinal tissue. Our biochemical findings are also supported by histopathological improvement in pancreatic and intestinal tissues. These results suggest that breakdown of intestinal mucosa via intense inflammation and necrosis may increase in intestinal permeability in AP and SP.

The severity of disease, high zonulin, LOOH, and MDA, low GSH and Cu, Zn-SOD activity are associated with increased intestinal permeability in early phase of AP. The inflammatory signaling and response in pancreatitis is mediated in part through ROS as important mediators of oxidative stress. Serum zonulin levels may be a promising clinical marker for differentiation AP and SP in clinical practice. Patients with pancreatitis may be more exposed to impaired gut barrier function. Serum zonulin levels can be used in the evaluation of intestinal permeability in acute pancreatitis. In order to use our results in clinical practice, clinical studies showing the relationship between impaired intestinal permeability in pancreatitis and zonulin are also required.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (H. Uzun) on reasonable request.

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Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

AY, BPK, AT, RG, EU and HU conceived and supervised the study. AY, SY, SD and ZKS collected and analyzed data. SD made laboratory measurements. ZKS applied the histopathological examination of the study. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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