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Basic & Translational

Efficacy of tocilizumab treatment in cerulein-induced experimental acute pancreatitis model in rats

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Original Article

ABSTRACT

Background/Aims: Acute pancreatitis (AP) is a disease that can cause local and systemic complications that may have high morbidity and mortality. Currently, there is not any specific treatment for AP. In this study, we created an experimental model of AP in rats, and we aimed to demonstrate the histological effectiveness of tocilizumab treatment that antagonizes interleukin-6 (IL-6), one of the key cytokines in the development of AP.

Materials and Methods: Forty-eight rats were divided into six groups for this study. AP model was created by subcutaneous injections of cerulein (20 µg/kg) four times at 1-h intervals. Tocilizumab 4 mg/kg was administered to one of the treatment groups and 8 mg/kg to the other treatment group intraperitoneally. The effects of tocilizumab were revealed by examining pancreatic tissue of the rats histopathologically according to the Schonberg scoring system.

Results: A comparison between tocilizumab treatment group and AP control group provides statistically significant improvement in AP ($p<0.0001$). Furthermore, the dose of 8 mg/kg is shown to be more effective than 4 mg/kg ($p=0.004$).

Conclusion: Our study points out that tocilizumab may be an effective agent for pancreatitis treatment.

Keywords: Acute pancreatitis, cerulein, interleukin 6 (IL-6), tocilizumab

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of the pancreas, which is one of the most common gastrointestinal disorders requiring acute hospitalization and causes high mortality and morbidity. It has been shown that 80% of AP cases present as mild disease, whereas 20% of cases eventually have severe morbidity and mortality (1).

The mechanism of pancreatitis remains unclear, but recent studies demonstrated that the uncontrolled activation of inflammatory signaling and the release of potent inflammatory cytokines strain patients to severe disease (2,3). These cytokines have been shown to increase capillary permeability, leukocyte adhesion, and extravasation, which leads to aggravation of AP and systemic complications. Regardless of the trigger factor at baseline, inflammatory activation and acute injury cause a large spectrum of severity of pancreatic injury with pathologic findings such as mild interstitial edema, severe hemorrhagic gangrene, and necrosis (4,5).

Different ways of experimental pancreatitis models have been constructed and studied to define the parameters of this activity and the efficacy of various therapeutic agents. There are several models for inducing pancreatitis in animals. One of the most commonly used agents for generating the experimental pancreatitis model is cerulein, an analogue of cholecystokinin.

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Table 1. Experimental protocol of the rat groups. Each group contained eight Wistar rats. To determine the effect of caerulein on pancreatitis model, we compared the histopathological changes with sham group. To analyze the efficacy of tocilizumab on acute pancreatitis, we compare the changes with placebo group (Group 2). Finally, one group of rats were infected only tocilizumab to determine the effect of drug to pancreas. (s.c.:subcutaneously; i.p.:intraperitoneally)

Experiment group	Number of rats
Group 1. Acute pancreatitis control group (20 µg/kg caerulein s.c. injection)	8
Group 2. Acute pancreatitis placebo group (20 µg/kg caerulein s.c.+1 cc saline i.p.)	8
Group 3. Tocilizumab 4 mg/kg. treatment group (20 µg/kg caerulein s.c. + tocilizumab 4 mg/kg i.p.)	8
Group 4. Tocilizumab 8 mg/kg. treatment group (20 µg/kg caerulein s.c.+tocilizumab 8 mg/kg i.p.)	8
Group 5. Sham group	8
Group 6. Tocilizumab control group (Tocilizumab 8 mg/kg i.p.)	8
Total	48

It stimulates pancreatic exocrine secretions together with relaxation of sphincter and contractions of the gallbladder. They finally cause premature proteolysis of zymogens. As a result, the active trypsin converts other proteolytic enzymes ensuing as pancreatitis.

Acute pancreatitis caused by cerulein has been shown to be histologically similar to early phase of AP. Cerulein has been used via intraperitoneal, intravenous, and subcutaneous administrations for induction of pancreatitis in animal models (6,7). Irrespective of the route of administration, many studies have demonstrated that cerulein leads to edematous pancreatitis resulting in pancreatic edema, vacuolization of the acinar cells, leukocyte infiltration, and elevated serum amylase levels within a few hours. Furthermore, necrotizing pancreatitis might occur with administration of supramaximal doses of cerulein (8).

Tocilizumab is a monoclonal recombinant human antibody that acts as an interleukin-6 (IL-6) receptor antagonist. Studies indicate that it suppresses inflammation via IL-6 receptors. Currently, tocilizumab is approved to treat inflammatory diseases such as rheumatoid arthritis, polyarticular juvenile rheumatoid arthritis, and the systemic form of juvenile rheumatoid arthritis (9).

Acute pancreatitis still has a lethal outcome despite the investigations and has no definite treatment except supportive therapies. Most of the studies of the pancreatitis treatment are made on experimental animal models due to the difficulties of ethical constraints for humans. In this study, we aimed to investigate the therapeutic properties of tocilizumab by evaluating histopathological changes in experimental cerulein-induced rat pancreatitis model.

MATERIALS AND METHODS

Study Design

Forty-eight male Wistar albino rats, weighing 250-350 g, were maintained in stainless steel cages in a room at a constant temperature of 24°C with 12-h light/dark cycles and were fed a standard pellet diet and tap water *ad libitum*. Our experimental study was performed in according to the recommendations of the national guidelines for the care and handling of laboratory animals and followed a protocol approved by the Animal Ethics Committee of GATA Haydarpaşa Training Hospital. The study was conducted in GATA Haydarpaşa Training Hospital Experimental Research Animal Laboratory Center.

Experimental Protocol

Experimental acute edematous pancreatitis was induced with subcutaneous cerulein (Sigma, St. Louis, MO, USA) injection (20 µg/kg) (6). Rats were divided into six groups, each containing eight animals (Table 1). Food intake was withdrawn before 12 h from the first injection.

Acute pancreatitis was induced with subcutaneous 20 µg/kg cerulein injection four times at 1-h intervals (0^{th}, 1^{st}, 2^{nd}, and 3^{rd} h). The rats that were not given any treatment were accepted as control group. To determine the efficacy of the treatment, the placebo group was intraperitoneally (i.p.) injected 1-cc saline (9^{th} h). In the treatment groups, tocilizumab was administered i.p. after 6 h from the last injection of cerulein (9^{th} h) at doses of 4 and 8 mg/kg, respectively (Table 1). The rats without any intervention were determined as sham group, and rats administered tocilizumab 8 mg/kg without cerulein-induced pancreatitis were determined as tocilizumab control group (Table 1). All the animals were sacrificed 18 h after the last injection for histopathological analysis.

Histologic Evaluation

Rats were sacrificed 24 h after the last injection of cerulein (14 h after tocilizumab) under anesthesia with xylazine/ketamine. After midline laparotomy, pancreatic tissues were extracted and fixed in 10% formaldehyde solution, dehydrated, passed through an upgraded ethanol series, and embedded in paraffin blocks. Five-micrometer pancreatic sections were dewaxed by xylene, hydrated through a degraded ethanol series, and stained with hematoxylin and eosin. Specimens were evaluated with light microscopy by an expert pathologist who was unaware of groups and rated samples between 0 and 4 for the presence of edema, acinar necrosis, hemorrhage, inflammation, and perivascular infiltration as described by Schoenberg et al. (10).

Statistical Analysis

Results are given as mean±standard deviation. Data were analyzed using the Statistical Package for Social Sciences Software version 15.0 (SPSS Inc.; Chicago, IL, USA). Kolmogorov-Smirnov test was used for determining normal distribution of the data.

Table 2. The histopathological scores in animals according to Schoenberg scores. The total score in group 3 and group 4 were significantly lower than placebo group (2.63±0.52 vs 6.38±0.92, p<0.0001 and 1.25±0.89 vs 6.38±0.92, p<0.0001, respectively)

Groups	Edema	Inflammation	Vacuolization	Necrosis	Score
Group 1	1	2	2	0	5
A. Pancreatitis	1	2	2	1	6
control	2	3	2	1	8
	2	2	2	1	7
	1	2	2	1	6
	1	2	2	1	6
	2	2	2	1	7
	1	2	2	1	6
Group 2	2	2	1	1	6
A. Pancreatitis-	2	2	1	0	5
placebo	2	2	1	1	6
	2	2	1	1	6
	1	2	2	1	6
	2	2	2	1	7
	2	2	1	0	5
	2	2	1	1	6
Group 3	1	1	1	0	3
A. Pancreatitis-	1	1	0	1	3
Tocilizumab	1	2	0	0	3
4 mg/kg	1	1	1	0	3
	1	1	1	0	3
	1	1	0	0	2
	1	1	0	0	2
	1	1	0	0	2
Group 4	1	1	0	0	2
A. Pancreatitis-	1	1	0	0	2
Tocilizumab	0	1	0	0	1
8 mg/kg	0	0	0	0	0
	1	1	0	0	2
	0	1	0	0	1
	1	1	0	0	2
	0	0	0	0	0
Group 5	0	0	0	0	0
Sham group	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
Group 6	0	0	0	0	0
Tocilizumab	0	0	0	0	0
8 mg/kg	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0

Table 3. Comparison of Schoenberg scores of groups

Groups	Schoenberg Scores
Group 1-2	p>0.05
Group 1-3	p<0.05
Group 1-4	p<0.05
Group 1-5	p<0.05
Group 1-6	p<0.05
Group 2-3	p<0.05
Group 2-4	p<0.05
Group 2-5	p<0.05
Group 2-6	p<0.05
Group 3-4	p<0.05
Group 3-5	p<0.05
Group 3-6	p<0.05
Group 4-5	p<0.05
Group 4-6	p<0.05
Group 5-6	p>0.05

Paired samples correlations and paired samples tests were used to determine associations and differences. The findings were evaluated in 95% confidence interval and 5% significance level. P<0.05 was considered statistically significant.

RESULTS

Pancreatitis was histologically demonstrated in cerulein-administered groups (four times of 20 µg/kg dose with 1-h intervals). Compared with the sham group, AP groups indicated fat necrosis, interstitial edema, vacuolization, and inflammation on histopathologic examinations (Figure 1).

Schoenberg index was calculated by inflammation, vacuolization, fat necrosis, and edema on the basis of the histopathological examination of AP model (Figure 2). For Group 5 (sham group) and Group 6 (tocilizumab control group), all the scores were null "0" (Table 2). In the control group, the mean score was 6.38±0.92, and the difference between control group and placebo group was not significant (6.38±0.92 vs. 5.88±0.64, p=NS). The score was significantly lower in tocilizumab 4 mg/kg treatment group (2.63±0.52 vs. 6.38±0.92, p<0.0001) and in tocilizumab 8 mg/kg treatment group when compared with control (1.25±0.89 vs. 6.38±0.92, p<0.0001). Additionally, we observed necrosis in most of the animals in control (7/8) and placebo (6/8) groups, whereas there was no sign of necrosis observed in animals treated with tocilizumab 8 mg/kg (Table 2). The comparison of Schoenberg scores of groups is shown in Table 3.

As described before, Schoenberg scores were significantly decreased in the tocilizumab-treated groups (Groups 3 and 4) when compared with the control group (Group 1) (p<0.0001), and the score was decreased more with tocilizumab at the dose of 8 mg/kg than 4 mg/kg (2.63±0.52 vs. 1.25±0.89, p=0.004) (Figure 3). The histopathological presentations of cerulein-induced pancreatitis with administration of tocilizumab at doses of 4 and 8 mg/kg are demonstrated in Figure 4.

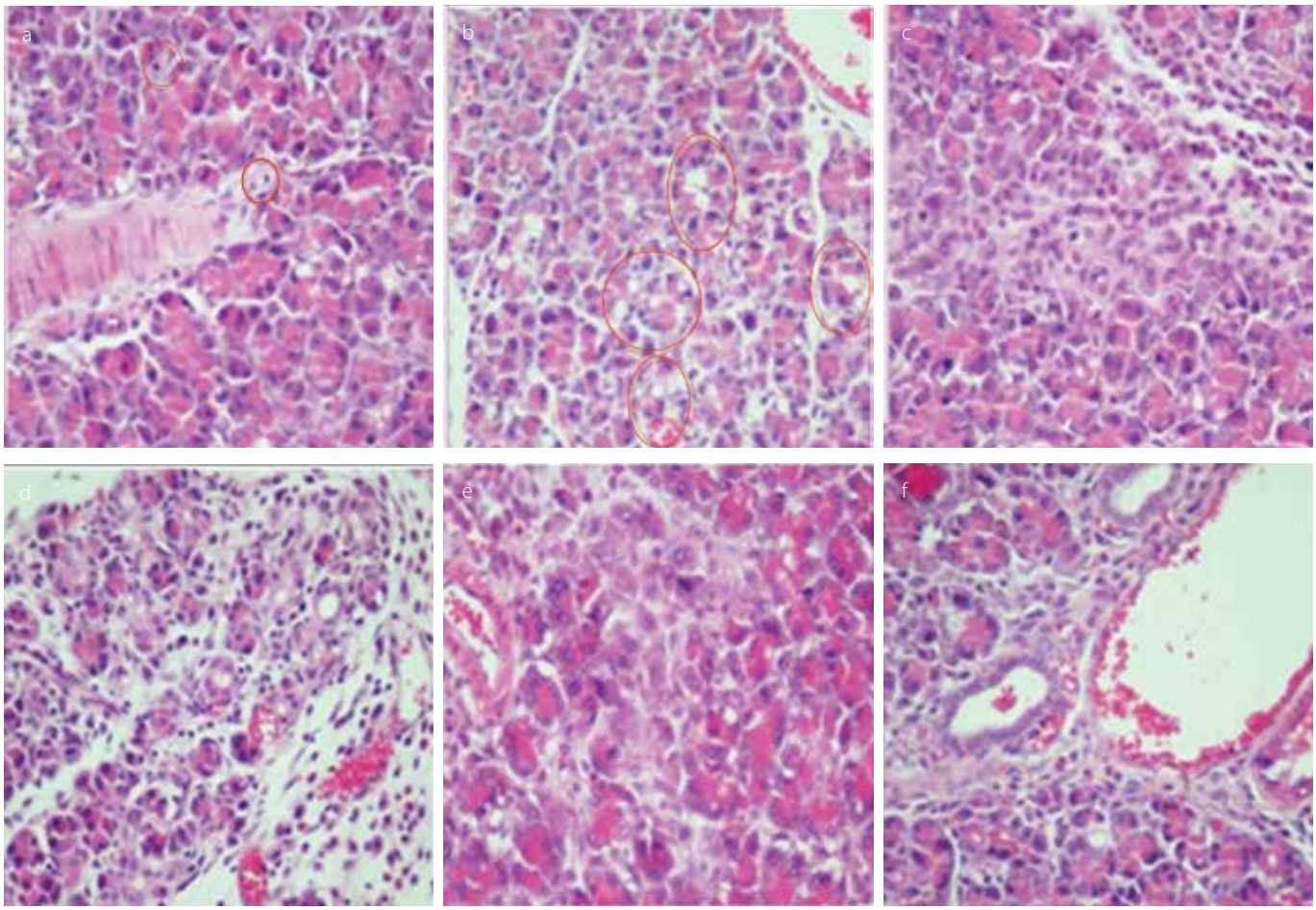

Figure 1. a-f. Histopathologic changes of AP stained with hematoxylin and eosin (×400). (a) Increased mitotic activity in the exocrine pancreas, (b) vacuolization in the exocrine pancreas, (c, d) increase of interlobular connective tissue and inflammatory cell infiltration after exocrine pancreatic injury, (e) Loss of gland formation, (f) Vascular dilatation, connective tissue enlargement between perivascular area and acini, and inflammatory cell infiltration

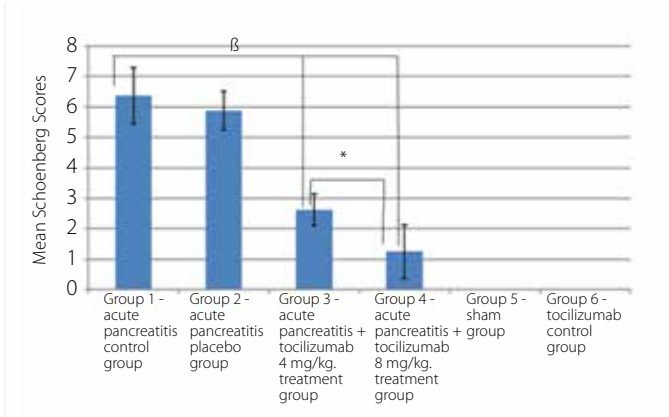

Figure 2. Mean Schoenberg scores of study groups (*, $p<0.01$, β, $p<0.0001$). Tocilizumab decreased the score dose dependently

DISCUSSION

The prognosis of AP can range from self-limiting mild disease to severe disease with life-threatening conditions. Despite improvement in the development of diagnostic tools, access to care, and interventional therapeutic options, AP continues to be associated with high morbidity and mortality (11). Therefore, recent experimental studies have been intensified on the pathogenic mechanisms of AP and especially on its inflammatory pathways to limit disease severity.

Cerulein is one of the most preferred models for experimental pancreatitis (10). The effect of cerulein is dependent on dose and frequency. The dose and duration of administration are determined according to the required pancreatitis severity (12,13). It has been shown that the administration of cerulein subcutaneously at the dose of 10-20 µg/kg causes acute edematous pancreatitis (14,15). In our study, acute edematous pancreatitis was formed by subcutaneous administration of 20 µg/kg cerulein four times at hourly intervals.

The pathological mechanism of the AP is extensive inflammation exceeding rapidly and affecting all systems by time. IL-6, one of the key cytokines in this process, increases the release of acute-phase proteins that have an important role in the onset and severity of the process. IL-6 has been shown to be effective in the development of many diseases. Inagaki et al. (16) found that IL-6 is one of the early markers of pancreatitis severity. In the present experimental study, we evaluated the histopathological effects of tocilizumab as a putative new agent for the treatment of AP.

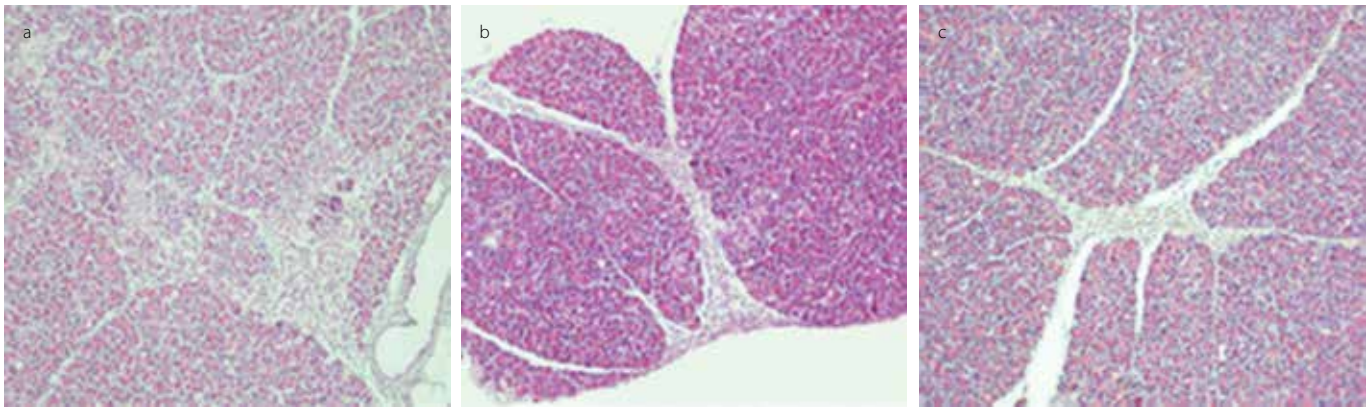

Figure 3. a-c. Histopathological comparison of treatment groups with AP group stained with hematoxylin and eosin (×100). (a) In the AP control group, interlobular connective tissue and inflammatory cell infiltration were increased, (b) in the tocilizumab 4 mg/kg group, s inflammatory cell infiltration was significantly reduced compared with the control group that was restricted to the interlobular region, (c) in the tocilizumab 8 mg/kg group, rare inflammatory cells were observed compared with control and 4 mg/kg groups

AP: acute pancreatitis

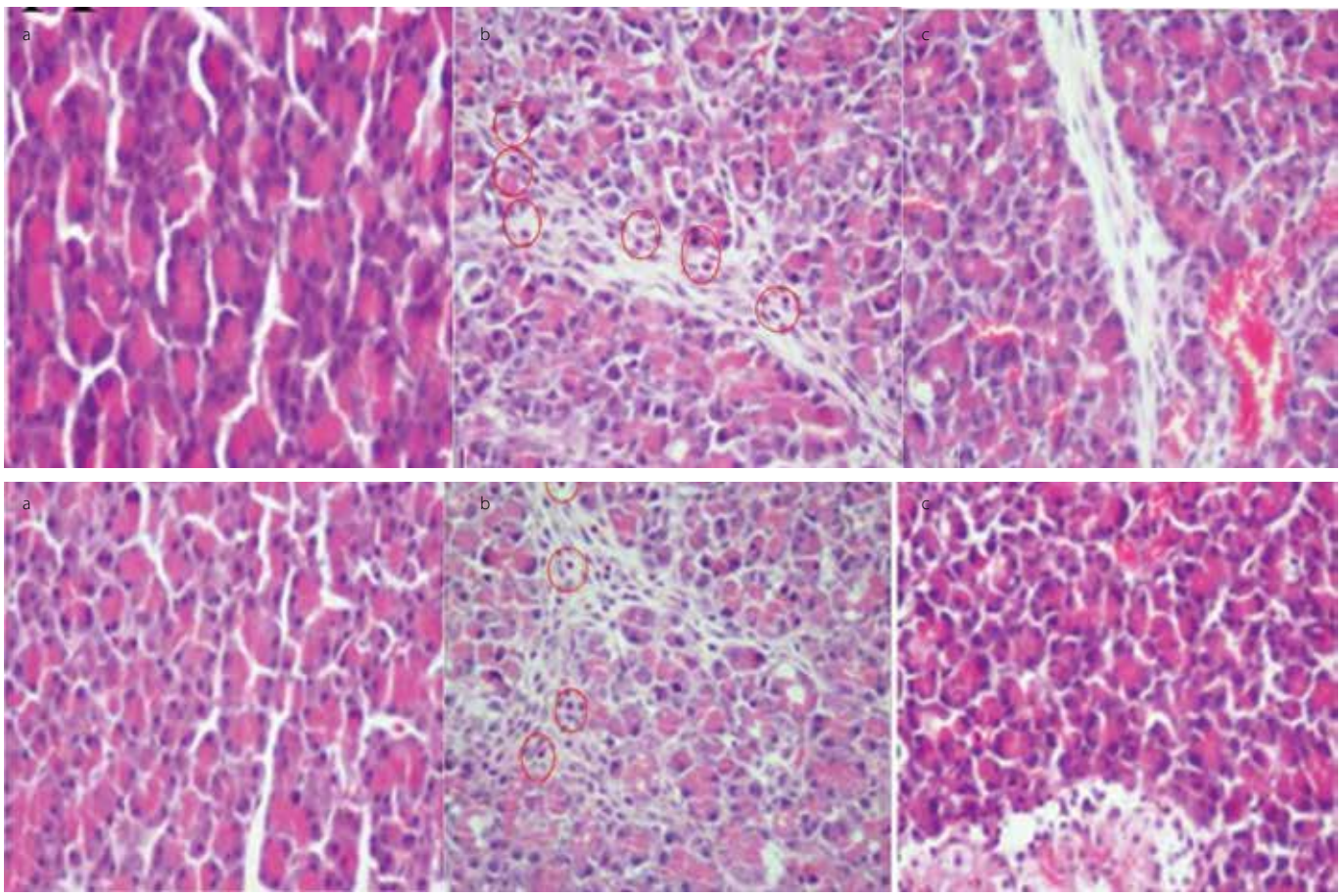

Figure 4. a-c. Histopathological comparison of tocilizumab 4 mg/kg (a) and 8 mg/kg (b) treatment group with sham group and AP control group stained with hematoxylin eosin (×400). (a) Normal exocrine pancreas tissue, (b) AP control group, (c) interlobular connective tissue increase and inflammatory cell infiltration. In figure A, tocilizumab 4 mg/kg group, the neutrophil infiltration disappeared whereas the interlobular connective tissue growth continued. In figure B, tocilizumab 8 mg/kg group after treatment, inflammatory cells disappeared and normal pancreas tissue reconstituted

AP: acute pancreatitis

In the course of AP, systemic complications may develop in addition to localized events by triggering acute inflammatory mediators, intensifying oxidative stress, compromising the microcirculation, and activating neurogenic feedback (17). Free oxygen radicals have been shown to play an important role in the pathogenesis of AP (18). They react with phospholipids in the cell membrane, leading to peroxidation of lipids, disruption of the membrane, and eventually cell death (19). Consequently, a further increase of free oxygen radicals and inflammatory mediators such as IL-1, IL-6, IL-8, and TNF-α occurs. Leukocytes

become active with the effect of these mediators. Through the interaction of adhesion molecules on the surface of neutrophils via vascular endothelium, many more cytokine, chemokine, and free oxygen radicals release (20,21). The deterioration of the microcirculation results as increased permeability of the endothelial layer of the arterioles and venules, leading to extravasation of plasma and erythrocytes presented as edema and microhemorrhagia (19). Damaged tissue releases chemotactic factors that accumulate neutrophils. Gathered neutrophils blocks the lumen and increase the release of enzymes, free oxygen radicals, and other inflammatory mediators such as protease and elastase (22). All these events incessantly trigger each other, leading to a vicious cycle. The main mechanism responsible for systemic complications is neutrophil accumulation in the pancreas and other organs (23). The mortality rate is 50%-90% in this severe clinical course. Until usage of the biological agents, the agents tested in experimental pancreatitis models were the antioxidant agents. In studies conducted with these agents, the inflammatory process was suppressed at an early stage to prevent systemic complications (24,25). Although our study model is mild edematous pancreatitis, tocilizumab was administered at an early stage at sixth hour, suggesting that IL-6 blockade might also be effective in severe pancreatitis with systemic complications.

By the introduction of biological agents, researches aiming to demonstrate the efficacy of these agents in animal pancreatitis models have increased. Previously, Kaplan et al. (26) performed an experimental cerulein-induced acute edematous pancreatitis by intraperitoneally administering cerulein as a total of 4 doses in 20 µg/kg/h intervals and investigated the effects of anakinra, an IL-1 receptor antagonist. They demonstrated the histopathologic healing effects of anakinra on the Schonberg scoring system by comparison with the AP control group. In our study, we also demonstrated the effects of tocilizumab and histopathological recovery according to the Schonberg scoring system. IL-1 and IL-6 receptor antagonists, whose cytokines have leading role in the pathological formation of AP, have produced similar results in a similar experimental pancreatitis model.

Kosekli et al. (27) have also conducted a study to demonstrate the efficacy of certolizumab, a TNF-a blocker, in an experimental AP model in which 20 µg/kg cerulein was administered intraperitoneally four times in 1-h intervals. They also reached the conclusions of our study and the results reported by Kaplan et al. (26).

Recently, Chen et al. (28) demonstrated the effects of tocilizumab on experimental severe AP and associated acute lung injury in taurocholic acid model. They induced AP by retrograde injection of sodium taurocholate (50 mg/kg) into the biliopancreatic duct and applied different doses of tocilizumab (1, 2, 4, 8, and 16 mg/kg) through the tail vein for dose study. They showed that the histopathological scores of pancreas were decreased with tocilizumab treatment without any toxic and adverse effects, even with higher doses. Consistently, we found dose-dependent improvement at histopathological evaluation of pancreas and observed no toxic or adverse effects histologically and physically in our tocilizumab administered groups (two treatment groups of 4 and 8 mg/kg and one tocilizumab control group of 8 mg/kg). Differently, although they found that 2 mg/kg tocilizumab was the optimal treatment dose for rat models, we found that tocilizumab at the dose of 8 mg/kg was more effective than 4 mg/kg at histopathological evaluation. More studies are needed in different animal and pancreatitis models to solve this discrepancy.

As conclusion, we support that the IL-6 receptor antagonist in tocilizumab treatment can positively regulate the histopathological changes in pancreatitis, thus restoring disease activity and severity. The recommended that the initial dose of tocilizumab in humans, 8 mg/kg, was significantly more effective than the 4 mg/kg dose. Our findings demonstrated that tocilizumab appears to be helpful in obtaining favorable results in experimental AP.

Ethics Committee Approval: Ethics committee approval was received for this study from the Animal Ethics Committee of GATA Haydarpaşa Training Hospital.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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