Does the 3-aminobenzamide effect on bacterial translocation affect experimental acute necrotizing pancreatitis?

3-amino benzamid deneysel akut nekrotizan pankreatitte bakteriyel translokasyonda etkili midir?

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Background/aims: *One of the most important complications of acute pancreatitis is the secondary bacterial infections of the pancreas and gut. Translocation of bacteria from the gut is accepted as being responsible for the development of septic complications in acute pancreatitis. In this study, our aim was to investigate the effect of PARP inhibition via 3-aminobenzamide on the bacterial translocation in acute pancreatitis.* ***Methods:*** *45 male Sprague-Dawley rats were randomly allocated into three groups. Group I (Sham+saline) received normal saline infusion into the common biliopancreatic duct. Acute pancreatitis was induced in Group II (acute pancreatitis+saline) and Group III (acute pancreatitis+3-aminobenzamide) by the retrograde injection of taurocholate into the common biliopancreatic duct. Six hours after induction of pancreatitis, the rats in Group I and II were treated with saline (1 ml, every 12 hours), while the rats in Group III were treated with 3-aminobenzamide (10 mg/kg/day every 12 hours), intraperitoneally. In the 54th hour of the study, blood and tissue samples were taken for biochemical, microbiological and histopathological analysis.* ***Results:*** *Acute pancreatitis developed in Groups II and III. Pathologic score [median (25-75% percentiles)] of the pancreatitis in Group III [8 (7-9)] was significantly lower than in Group II [19 (18-21)] (p<0.001). Bacterial translocation to mesenteric lymph node (53.3%), peritoneum (60%) and pancreas (46.7%) in Group III was significantly lower than in Group II (100% for all) (p<0.02, p<0.03, p<0.005, respectively). Pancreatic tissue glutathione peroxidase, superoxide dismutase, and malondialdehyde levels were better in Group III compared to Group II (p<0.001 for all). Comparison of Groups II and III demonstrated reduced severity of inflammation of the gut in Group III (p>0.05). Improvement in bacterial translocation was correlated with reducing oxidative stress.* ***Conclusions:*** *We demonstrated that 3-aminobenzamide therapy improved histopathologic score and oxidative stress in experimental pancreatitis. In addition, it was demonstrated microbiologically and histopathologically that 3-aminobenzamide therapy improves bacterial translocation. Further survival studies demonstrating the efficacy of 3-aminobenzamide therapy and explaining the potential mechanisms of bacterial translocation prevention in acute necrotizing pancreatitis will be beneficial.*

Key words: Acute pancreatitis, poly (ADP-ribose) polymerase/synthetase, oxidative stress, bacterial translocation

Amaç: *Akut pankreatitte mortalitenin en önemli nedeni barsakların ve pankreasın sekonder enfeksiyonudur. Barsaklardan kaynaklanan bakteriyel translokasyon akut pankreatitteki septik komplikasyonların nedeni olarak kabul edilmektedir. Bu çalışmada, akut nekrotizan pankreatitte bir PARS inhibitörü olan 3 amino-benzamidin pankreatitte bakteriyel translokasyon etkisinin değerlendirilmesi amaçlanmıştır.* ***Yöntem:*** *Erkek cins 45 adet Sprague-Dawley ratı rastgele üç gruba ayrıldı. Grup I(sham+salin), biliopankretik kanala salin infüzyonu yapıldı. Grup II (akut pankreatit+Salin) ve Grup III (akut pankreatit+3-aminobenzamid)'e ise biliopankretik kanala taurokolikasit uygulandı. Akut pankreatit indüksiyonundan 6 saat sonra Grup I ve II salin (12 saatte bir 1ml) ve Grup III'e 3-aminobenzamid (12 saatte bir 10mg/kg) intraperitoneal olarak uygulandı. 3-aminobenzamid pankreatit indüksiyonundan hemen sonra ve 10 mg/gün tek dozda intraperitoneal olarak uygulandı. İndüksiyondan 54 saat sonra mikrobiyolojik, histopatolojik ve biyokimyasal incelemeler için kan ve doku örnekleri alındı.* ***Bulgular:*** *Grup I dışında bütün gruplarda akut pankreatit gelişti. Grup III'ün patolojik skoru belirgin bir şekilde Grup II'ten daha düşüktü (p<0.001). Grup III'te mezenter lenf nodunda %53.3, peritonda %60 ve pankreasta %46.7 olan bakteriyel translokasyon grup II'den daha düşüktü (p<0.02, p<0.03, p<0.005, sırasıyla). Pankreas doku glutatyon peroksidaz, superoksid dismutaz ve malondialdehid düzeyleri Grup II-I'te Grup II'den daha iyi olarak saptandı (p<0.001 bütün karşılaştırmalar için). Grup II ile kıyaslandığında Group III de intestinal inflamasyon şiddeti daha düşüktü (p>0.05). Bakteriyel translokasyondaki iyileşme oksidatif stresteki iyileşme ile koreleydi.* ***Sonuç:*** *Akut nekrotizan pankreatitte 3-aminobenzamid uygulaması ile patolojik skorun ve oksidatif stres parametrelerinin iyileşmesinin yanısıra bakteriyel translokasyonda anlamlı bir şekilde azaldığı gösterildi. Ayrıca 3-aminobenzamid, bakteriyel translokasyonu iyileştirdiği hem mikrobiyolojik hem de histopatolojik olarak gösterildi. Akut nekrotizan pankreatitte yeni bir tedavi seçeneği oluşturabilir. Akut pankreatitte bakteriyel translokasyonu önlemede rol oynayan muhtemel mekanizmaları açıklayan ve 3-aminobenzamid'nin etkinliğini inceleyen ileri survi çalışmaların yapılması faydalı olacaktır.*

Anahtar kelimeler: Akut pankreatit, poli (ADP-riboz) polimeraz/sentetaz, oksidatif stres, bakteriyel translokasyon

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INTRODUCTION

Acute pancreatitis (AP), which exhibits a broad clinical spectrum and has various clinical manifestations, has long been considered to be an autodigestive disease, in which the parenchymal tissue of the organ is destroyed by its own proteolytic enzymes (1, 2). Pancreatic infections are a serious complication of acute necrotizing pancreatitis, and intestinal bacterial translocation (BT) is an important development of these problems (3). Normally, there is a homeostasis among the intestinal bacteria, their products and the gut mucosal barrier (4). AP is known to be associated with gut barrier dysfunction (5). It has been suggested that oxygen-derived free radicals play a crucial role in the pathogenesis of AP (5,6). Poly (ADP-ribose) synthetase/polymerase (PARP) activation, which is most likely due to peroxynitrite production, depletes the intracellular concentration of the energetic substrate, thus leading to cellular dysfunction and cell death (7).

Pharmacological inhibition of PARP had been demonstrated to have beneficial effects against peroxynitrite-mediated cell injury (8). The 3-aminobenzamide (AB), an endogenous inhibitor of PARP, has long served as a "benchmark" inhibitor of PARP and an experimental agent suitable for laboratory investigations (9). In related studies, it was revealed that 3-AB markedly decreased AP severity and pancreatitis-associated lung injury in mice (10, 11). In addition, in many experimental studies such as hemorrhagic shock and burns, it was demonstrated that inhibition of PARP improved intestinal BT (4, 12). As a result of these features of PARP, the question of whether or not PARP inhibition is effective in preventing BT in acute necrotizing pancreatitis needs to be clarified.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Use and Care Committee of the Gülhane Medical Academy and performed in accordance with the National Institutes of Health guidelines for the care and handling of animals.

Stabilization period for animals

Male Sprague-Dawley rats weighing from 280 to 350 g were obtained from Gülhane School of Medicine Research Center (Ankara, Turkey). For the stabilization, animals were fed with standard rat chow and water *ad libitum* and housed in metabolic cages under controlled temperature and 12-hour light/dark cycles for at least one week before the experiment.

Induction of pancreatitis

Anesthesia was induced with sevoflurane inhalation (Sevorane® Liquid 250 ml, Abbott; İstanbul, Turkey). Laparotomy was performed through a midline incision. The common biliopancreatic duct was cannulated with a 28 gauge $^1/_2$-inch, micro-fine catheter. One microaneurysm clip was placed on the bile duct below the liver and another around the common biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents into the duct. Then, 1 ml/kg of 5% sodium taurocholate (Sigma; St. Louis, MO, USA) was slowly infused into the common biliopancreatic duct, and the infusion pressure was kept below 30 mmHg, as measured with a mercury manometer (13). When the infusion was finished, the microclips were removed, and the abdomen was closed into two layers. All procedures were performed using sterile techniques.

Study protocol

After the stabilization period, 45 male rats were randomly divided into three groups. Group I (sham+saline, n=15) underwent laparotomy with manipulation of the pancreas and received saline injection (saline 1 ml, i.p. every 12 hours) as controls. Groups II and III underwent laparotomy with induction of pancreatitis. Group II (AP+saline, n=15) received saline injection 6 hours after the induction of pancreatitis as in Group I. Group III (AP+3-AB, n=15) received 3-AB (Sigma; St. Louis, MO, USA) injection every 12 hours (10 mg/kg/day, i.p. 6 hours after the induction of pancreatitis) (14). Fifty-four hours after the induction, all surviving animals were killed with intracardiac phenobarbital (200 mg/kg) injection. Rats that died before the end of the experiment were replaced in order to maintain 15 rats in each group.

Laboratory tests

Blood samples were taken from the heart before sacrifice for measurement of serum amylase levels. A Hitachi 917 autoanalyzer (Boehringer Mannheim; Mannheim, Germany) was used for the amylase assay.

Histopathologic analysis

A random portion of the pancreatic and intestinal specimens from each rat was fixed in 10% neutral buffered formalin and embedded in paraffin. One

paraffin section stained with hematoxylin and eosin was examined for each pancreas and gut. Two blinded pathologists scored the tissues for edema, acinar necrosis, inflammatory infiltrate, hemorrhage, fat necrosis, and perivascular inflammation in 20 fields for the pancreas, with a maximum score of 24 as defined by Schmidt et al. (15). Histopathologic examination of the gut for inflammation was defined with a maximum score of 4, as described in Table 2, and was performed in the cecum.

Evaluation of oxidative stress

Pancreatic tissue samples were homogenized in cold KCl (Sigma; USA) solution (1.5%) in a glass homogenizer (117, Thomas; Philadelphia, USA) on ice. These samples were then centrifuged and supernatant was used for following determination.

Tissue malondialdehyde (MDA) concentration was estimated using the method of Ohkawa et al. (16). The supernatant was resuspended in 4 ml water, 0.5 ml glacial acetic acid (Riedel, Germany) and 0.5 ml 0.33% aqueous thiobarbituric acid solution. The mixture was heated in the boiling water bath for 60 min. After cooling of the samples, the complex formed by thiobarbituric acid reactant substances (Sigma; USA) was extracted into an n-butanol phase, and the formed chromogen was measured at 532 nm by a UV-VIS recording spectrophotometer (UV-2100S, Shimadzu Co.; Kyoto, Japan). A standard absorption curve for MDA was prepared by using tetra-methoxy propane (Sigma; USA) solution. MDA level was expressed as nmol/g tissue.

For superoxide dismutase (SOD) (Sigma; USA) activity measurement, each supernatant was diluted 1:400 with 10mM phosphate buffer, pH 7.00. 25 µL of diluted supernatant was mixed with 850 µL of substrate solution containing 0.05 mmol/L xanthine sodium (Sigma; USA) and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) (Sigma; USA) in a buffer solution containing 50 mmol/L CAPS (Sigma; USA) and 0.94 mmol/L EDTA (Aldrich; USA), pH 10.2. Then, 125 µL of xanthine oxidase (Sigma; USA) (80U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 min against air. 25 µL of phosphate buffer or 25 µL of various standard concentrations in place of sample were used as blank or standard determinations. SOD activity was expressed in U/g tissue (17).

For glutathione peroxidase (GPx) (Sigma; USA) activity measurement, the reaction mixture was 50 mmol/L Tris buffer (Sigma; USA), pH 7.6, containing 1 mmol/L of Na_2EDTA, 2 mmol/L of reduced glutathione (GSH) (Sigma; USA), 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide (Sigma; USA), and 1000U of glutathione reductase (GR) (Sigma; USA). 50 µL of supernatant and 950 µL of reaction mixture, or 20 µL of supernatant and 980 µL of reaction mixture, were mixed and incubated for 5 min at 37°C. Then the reaction was initiated with 8 mmol/L H_2O_2 (Merck, Germany) and the decrease in NADPH (Serva, Germany) absorbance was followed at 340 nm for 3 min. Enzyme activities were reported in U/g tissue (18).

Microbiologic assessment

When the animals were sacrificed, the abdomen was opened through a separate incision under aseptic conditions. Samples from pancreatic tissue, mesenteric lymph nodes (MLNs), and peritoneum were taken randomly. They were then harvested, weighed and immediately processed for quantitative bacteriologic examination. Specimens were homogenized and qualitatively plated in duplicate on phenylethyl alcohol agar supplemented

Table 1. Oxidative stress parameters, bacterial translocation, histopathologic scores, and amylase levels in the experimental groups studied

	Group I (n=15)	Group II (n=15)	Group III (n=15)	P *
Histopathology score	2 (1-2)	19 (18-21)	8 (7-9)	p<0.001
Amylase (U/L)	263±16	1657±102	544±43	p<0.001
Oxidative stress parameters				
MDA (nmol/g)	13.1±0.4	28.5±0.6	16.6±0.5	p<0.001
SOD (U/g)	397±6	254±5	315±9	p<0.001
GPx (U/g)	53.5±1.8	31.2±0.7	44.3±1.1	p<0.001
Bacterial translocation (%)				
Pancreas	2/15 (13.3)	15/15 (100)	7/15 (46.7)	p<0.005
MLN	4/15 (26.7)	15/15 (100)	8/15 (53.3)	p<0.02
Peritoneum	6/15 (40)	15/15 (100)	9/15 (60)	p<0.03
Intestinal inflammation				
Cecum	0 (0-1)	4 (4-4)	4 (3-4)	p>0.05

MDA: Malondialdehyde. SOD: Superoxide dismutase. GPx: Glutathione peroxidase. MLN: Mesenteric lymph node.
* : Between Groups II and III

with 5% sheep blood and MacConkey II agar and incubated aerobically at 37°C for 24 h. At the end of the incubation period, bacterial counts were calculated and expressed as colony-forming units (CFU/g of tissue). More than 10^3 CFU per gram of tissue was considered as infection (19, 20).

Statistical analysis

Parametric data were expressed as mean ± SEM. Non-parametric data (histopathologic scores) were expressed as median and percentiles (25-75%). Abnormally distributed observations (histopathologic scores) were assessed by Kruskal-Wallis test and subgroup analyses were assessed by Mann-Whitney U test. Normally distributed observations (serum amylase and oxidative stress parameters) were assessed by one way ANOVA test and Tukey's HSD as post-hoc. The incidences of BT were evaluated by chi-square with Yates' correction. Correlation between BT and oxidative stress was evaluated by Spearman's rank correlation. Probabilities less than 0.05 were considered significant. All statistical measurements were made by using SPSS PC Ver. 9.05 (SPSS Inc., USA).

RESULTS

Stratification of acute pancreatitis

AP developed in Group II and III rats, as shown by macroscopic parenchymal necrosis and abundant turbid peritoneal fluid. All animals except 5 in Group II and 3 in Group III survived the experiment period of 54 hours.

Mean serum amylase levels in Group I (263±16 U/L) were significantly lower than in Group II (1657±102 U/L) and Group III (544±43 U/L) ($p<0.001$, $p<0.02$, respectively). Serum amylase levels were significantly reduced in rats treated with 3-AB (Group III) compared with Group II rats ($p<0.001$) (Table 1).

Table 2. Histopathology and scoring of gut

Inflammation	Score
Absent	0
Focal inflammation to lamina propria	1
Focal inflammation to lamina propria and submucosa	2
Diffuse inflammation to lamina propria	3
Diffuse inflammation to lamina propria and submucosa	4

Mostly edema was observed in the histopathologic examination of Group I rats. Median histopathologic score in Group I was significantly lower than in Group II and Group III ($p<0.001$ for both). The total histopathologic score was significantly reduced in Group III [8 (7-9)] when compared with the rats in Group II [19 (18-21)] ($p<0.001$) (Table 1). Edema, acinar necrosis, inflammatory infiltration, hemorrhage, fat necrosis and perivascular inflammation in Group III (Figure 1A) were decreased with 3-AB treatment when compared with Group II (Figure 1B, C).

Gut morphology

Median histopathologic score in Group I was significantly lower than in Group II and Group III ($p<0.001$ for both). The histopathologic score of cecal inflammation was reduced in Group III when compared with Group II, but the difference was not statistically significant ($p>0.05$) (Table 1) (Figure 2).

Oxidative stress

All of the oxidative stress parameters were improved in rats treated with 3-AB (Group III). Mean MDA level in Group III was lower than in Group II ($p<0.001$). Mean SOD and GPx levels in Group III were higher than in Group II ($p<0.001$ for both). Mean oxidative stress parameters in Group I were better than in the other two groups ($p<0.001$ for all comparisons). MDA, SOD, and GPx levels according to groups are summarized in Table 1.

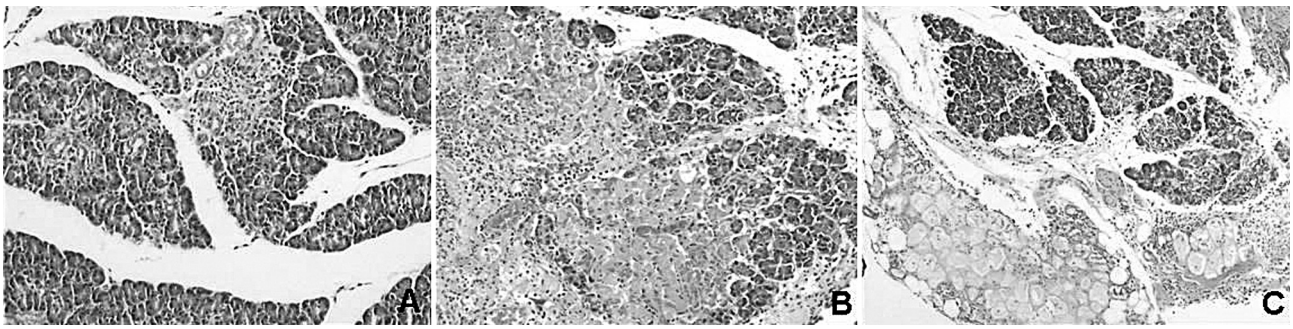

Figure 1. A- Improvement after parenchymal necrosis in Group III (H&E, x100); **B-** hemorrhagic necrosis in Group II (H&E, x200); and **C-** fatty necrosis in Group II (H&E, x100).

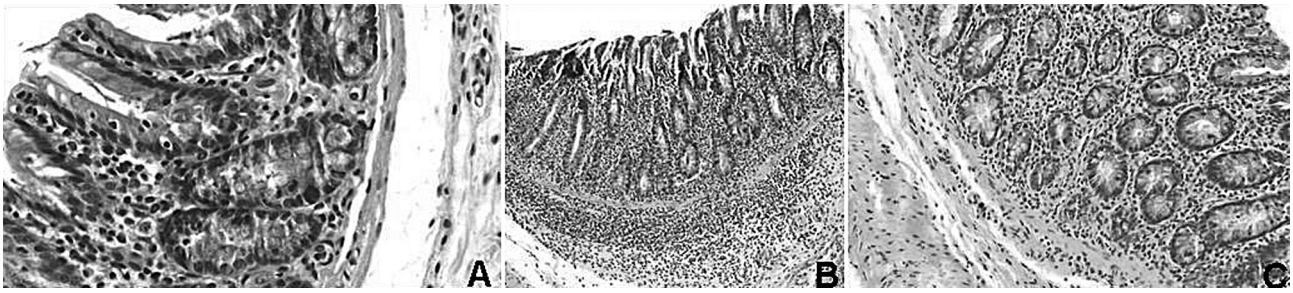

Figure 2. A- Histopathologic examination of the cecum. Minimal focal, almost normal, inflammation to lamina propria in Group I (H&E, x200); **B-** diffuse inflammation to lamina propria and submucosa in Group II (H&E, x50); and **C-** almost diffuse inflammation to lamina propria and focal inflammation to submucosa in Group III (H&E, x100).

Bacterial translocation

BT was detected in 7 of 15 (46.7%) rats in the pancreas, in 8 of 15 (53.3%) rats in MLNs, and in 9 of 15 (60%) in the peritoneum in Group III, whereas all tissue samples were infected in Group II. The differences between translocation ratios in Group II and Group III were significant ($p<0.005$, $p<0.02$, and $p<0.03$, respectively) (Table 1). BT in Group I was less than in Group III, but the differences were not significant. Bacteria isolated from MLN of rats with AP included *Escherichia coli, Enterococcus sp., Staphylococcus sp., Klebsiella oxytoca and Proteus sp*. The most common bacteria were *E. coli.*

When the correlation between BT and oxidative stress was evaluated, MDA was positively correlated with BT to pancreas, MLN, and peritoneum (r:0.832 $p<0.001$; r:0.760 $p<0.001$; r:0.691 $p<0.001$, respectively) and SOD and GPx were negatively correlated with BT to pancreas, MLN, and peritoneum (r:-0.743 $p<0.001$; r:-0.723 $p<0.001$; r:-0.653 $p<0.001$ for SOD; and r:-0.856 $p<0.001$; r:-0.818 $p<0.001$; r:-0.721, $p<0.001$ for GPx, respectively).

DISCUSSION

It has been accepted that the failure of gut mucosa to act as a barrier against BT is the potential origin of septic complications after pancreatitis (21, 22). The special importance of the regulation by PARP is the production of inflammatory mediators, such as the inducible nitric oxide synthase (iNOS) that is known to be so important in BT (23). For this reason, attention has justifiably been addressed to prevention of BT as a key to improving the outcome in patients with severe pancreatitis.

PARP regulates the production of inflammatory mediators, and PARP activation induced with generations of peroxynitrite and hydroxyl radical is important in the progression of pancreatic inflammation (23, 24). In addition, Bowes et al. (25) demonstrated that 3-AB therapy decreases the activity of PARP and reduces the hydrogen peroxide-induced cell injury/necrosis. In accordance with these results, it has been shown that 3-AB therapy has significantly lowered total histopathologic scores when compared with the control group ($p<0.001$). This improvement was likely caused by inhibition of PARP-related activation of the inflammatory process in AP.

In two recent studies, Mota (10) and Mazzon (11) examined the therapeutic application of 3-AB in the development of AP caused by cerulein in mice. In addition, Balachandra (26) demonstrated that reduced PARP activity was related to reduced cytokine production, nitric oxide (NO) and peroxynitrite formation, and tissue injury in cerulein-induced AP. In the present study, besides the therapeutic efficacy of 3-AB, BT was also examined in taurocholate-induced acute necrotizing pancreatitis.

To show the oxidative stress in this study, we examined GPx, SOD and MDA levels in pancreatic tissue. Due to the close interaction between PARP and reactive oxidants such as peroxynitrite and OH^-, it is expected that inhibition of PARP will improve oxidative stress. In accordance with this knowledge, we revealed higher tissue GPx and SOD levels and lower tissue MDA levels in the treatment group compared to Group II ($p<0.001$, for each comparison).

These results are correlated with the literature. Cuzzocrea et al. (27) demonstrated that M40401 improves the increase in PARP in the pancreas from cerulein-treated mice. In addition, they proposed that it was possible that PARP inhibition by

M40401 accounts for its anti-inflammatory effect. In our study, the main difference from Cuzzocrea's study is that taurocholate-induced acute necrotizing pancreatitis was examined, as mentioned above.

Kazantsev et al. (28) demonstrated that infections in AP occurred as a result of translocation of colonic bacteria via lymphogenic route. One of the mechanisms facilitating the loss of gut barrier function is to increase NO synthesis (22, 29). Since PARP has a role in the regulation of iNOS (22), it was thought that 3-AB would be able to inhibit the BT. In fact, we demonstrated that 3-AB therapy decreased the incidence of BT from 100% to 53.3% in MLNs ($p<0.005$). In addition, isolated bacteria in this current study were also of colonic origin, as in the studies of Kazantsev et al. and Beger et al. (28, 30).

This study has shown that 3-AB treatment of AP decreased BT to the MLN, peritoneum and pancreas in rats. The results regarding the decrease in BT with 3-AB suggest that enhancement of PARP activity at least partially contributes to the intestinal barrier failure and the subsequent BT in pancreatitis as in other inflammatory conditions. The effects of 3-AB on BT and intestinal inflammation were not only shown by negative cultures from the MLN, peritoneum and pancreas but also with histopathologic examination of the pancreas and gut at the 54th hour of the experiment. Although the inflammatory process was slowed at the 54th hour histopathologic examination of the gut, the difference was not statistically significant. This suggests that if histopathologic examination regarding the inflammatory process could have been performed at later stages of the experiment other than at the 54th hour, statistically significant results should be obtained. This may be a limitation of our study, but we supported the 54th hour histopathologic examination only with negative cultures. Another evident aspect of the present study was demonstration that decreased BT to the pancreas, MLN and peritoneum was correlated with improvement in oxidative stress parameters.

Because this study did not investigate intestinal barrier functions directly, further studies are needed to determine if 3-AB-induced changes in gut permeability are responsible for reducing BT. The beneficial effect of 3-AB in present study may be explained by two possible mechanisms. Firstly, 3-AB treatment may affect BT directly. Secondly, 3-AB can decrease BT indirectly by improving the severity of pancreatitis. There is no certain data in the literature or in the present study to exclude one of the possible effects. Whether or not 3-AB affects BT directly or through its effect on decreasing the severity of pancreatitis remains obscure.

In present study, we demonstrated marked improvement in histopathologic score, oxidative stress and BT in experimental acute necrotizing pancreatitis with 3-AB therapy. PARP inhibition with 3-AB therapy appears important in preventing the complications of AP. In conclusion, further survival studies demonstrating the efficacy of 3-AB therapy and explaining the potential mechanisms of BT prevention in acute necrotizing pancreatitis will be beneficial.

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