The role of allopurinol in experimental acute necrotizing pancreatitis

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Background & objectives: Acute pancreatitis (AP) in its severe form can lead to severe complications and death. Translocation of bacteria from the gut is one of the most important factors in the development of septic complications and mortality in acute pancreatitis. Oxygen-derived free radicals have been suggested to play a major role in the pathogenesis of AP. Xanthine oxidase enzyme is an important source of reactive oxygen metabolites. We undertook this study to evaluate the effect of allopurinol on bacterial translocation, oxidative stress and the course of AP in a rat model.

Methods: Male Sprague-Dawley rats (n=48) were randomly allocated into three equal groups. Acute pancreatitis (AP) was induced in group II (AP+Saline), and group III (AP+allopurinol) by retrograde infusion of taurocholate into the common biliopancreatic duct. Group I rats (Sham) received normal saline infusion into the common biliopancreatic duct for mimicking pressure effect. Group III rats were treated with allopurinol intraperitoneally for 48 h after induction of pancreatitis. Blood samples were drawn from all animals for biochemical analyses and pancreatic tissues were examined for bacterial translocation.

Results: Acute pancreatitis was developed in all groups, but not in group I (Sham), as indicated by microscopic parenchymal necrosis, fat necrosis and abundant turbid peritoneal fluid. Pathologic score of the pancreatitis in the allopurinol group (14.0 ± 0.5) was lower when compared with group II (19.2 ± 0.6) (P<0.001). Bacterial translocation to pancreas in group treated with allopurinol was significantly lower when compared with control group (p<0.02). Plasma glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) levels were higher and malondialdehyde (MDA) levels were lower in allopurinol group when compared with those in control groups.

Interpretation & conclusion: Our findings suggested that addition of allopurinol to the treatment protocol in the acute pancreatitis might improve the pathologic score, bacterial translocation and oxidative stress parameters. However, more studies need to be done to confirm these findings.

Key words Acute necrotizing pancreatitis - allopurinol - bacterial translocation

Acute pancreatitis (AP) exhibits a broad clinical spectrum and its severity may range from a mild, self-limited illness to a catastrophic one with multiple potentially severe complications and risk of death1,2. In the severe form of the disease, marked pathophysiological changes in the pancreas eventually lead to multiple organ failure and high mortality rates3. Patients with infected pancreatic and
peripancreatic tissue have an increased risk of developing septic complications which have emerged as the major cause of morbidity and mortality\textsuperscript{1,2,4}. Contamination of necrotic tissue occurs primarily due to translocation of enteric microorganisms\textsuperscript{5}. Bacterial translocation is not only specific to AP but is responsible for the development of infection and sepsis in other diseases as well\textsuperscript{6-8}. Prevention of bacterial translocation may play a major role in reversing the outcome after severe pancreatitis\textsuperscript{5,9}. Though various experimental models of AP have been developed, the exact mechanisms leading to this disease are not well understood.

Oxygen-derived free radicals have been suggested to play a decisive role in the pathogenesis of acute pancreatitis. Studies have demonstrated that the reactive oxygen metabolites, generated by activated xanthine oxidase enzyme, are released at an early stage of the disease\textsuperscript{10-13}. Allopurinol has been shown to prevent the initial development of acute pancreatitis by inhibiting xanthine oxidase activity\textsuperscript{10-13}. In addition, xanthine oxidase, an important source of endothelial cell-derived superoxide and hydrogen peroxide, has a main role in ischaemia-reperfusion injury, a mechanism contributing to intestinal barrier failure\textsuperscript{14}. Hypoxia or ischaemia promotes the conversion of the NAD-reducing dehydrogenase form of the enzyme to the oxygen-reducing oxidase form. The accumulation of hypoxanthine during ischaemia allows for a burst of superoxide and hydrogen peroxide production by the enzyme when oxygen is reintroduced into the blood vessel at the time of reperfusion\textsuperscript{15}.

The present study was undertaken to evaluate the effect of the allopurinol, a xanthine oxidase inhibitor, on bacterial translocation, oxidative stress and the course of acute necrotizing pancreatitis in a rat model.

**Material & Methods**

The experimental protocol was approved by the Institutional Animal Use and Care Committee of the Gulhane Medical Academy, Turkey and performed in accordance with the standard guidelines for the care and handling of animals.

**Animals**: Male Sprague-Dawley rats weighing from 280 to 350 g were obtained from Gulhane School of Medicine Research Center (Ankara, Turkey). Animals were fed standard rat chow and water ad libitum and housed in metabolic cages at controlled temperature and 12 h light/dark cycles for at least 1 wk before the experiments.

**Induction of pancreatitis**: Animals were anaesthetized with sevoflurane (Sevorane\textsuperscript{®} Liquid 250 ml, Abbott, Istanbul, Turkey) inhalation, laparotomy was performed through a midline incision. The common biliopancreatic duct was cannulated with a 28 gauge ½-inch, microfine 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 3 per cent sodium taurocholate (Sigma, USA) was slowly infused into the common biliopancreatic duct, and the infusion pressure was kept below 30 mmHg, as measured with a mercury manometer calibrated system\textsuperscript{16}, and monitored with a monitoring kit (Transpac IV Safeset, Abbott, Rep. of Ireland) attached to the infusion line with a three-way stopcock. When the infusion was finished, the microclips were removed, and the abdomen was closed in two layers.

**Study protocol**: After the stabilization period of 1 wk, 48 male rats were randomly divided into three groups. Group I (sham+saline, n=16) underwent laparotomy with manipulation of the pancreas. Groups II, and III underwent laparotomy with induction of pancreatitis. Group II (AP+saline, n=16) received saline injection after the induction of pancreatitis. In the saline groups, sterile saline 10 ml/kg, ip every 12 h was given for 2 days. Group III (AP+allopurinol, n=16) received allopurinol (Sigma, USA) injection (200mg/kg, ip every 12 h, 2 h after the induction of AP). After 48 h of the induction all
surviving animals were killed with intracardiac pentobarbital (200 mg/kg) injection. Blood samples were taken from the heart before killing, to measure serum amylase. Pancreatic tissue samples also taken for oxidative stress, histopathology and bacterial translocation.

**Laboratory tests:** A Hitachi 917 autoanalyzer (Boehringer Mannheim, Mannheim, Germany) was used for the amylase assay. Amylase level was expressed as U/l.

**Histopathologic analysis:** A portion of the pancreatic tissue from each rat was fixed in 10 per cent neutral buffered formalin and embedded in paraffin. Two pathologists who were blinded to the treatment protocol scored the tissue sections stained with haematoxylin and eosin for oedema, acinar necrosis, inflammatory infiltrate, haemorrhage, fat necrosis, and perivascular inflammation in 20 fields. The scores of each histological examination were summed up, with a maximum score of 24 as defined by Schmidt et al.

**Evaluation of oxidative stress:** Pancreatic tissue samples were homogenized in cold KCl solution (1.5%) in a glass homogenizer on ice, centrifuged and supernatant was used for various estimations.

<table>
<thead>
<tr>
<th>Tissue malondialdehyde (MDA) concentration</th>
<th>Group I (n=16)</th>
<th>Group II (n=11)</th>
<th>Group III (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>12.3 ± 0.4</td>
<td>28.3 ± 0.6*</td>
<td>18.7 ± 0.5***</td>
</tr>
<tr>
<td>SOD (U/g)</td>
<td>395 ± 7</td>
<td>252 ± 5*</td>
<td>353 ± 13***</td>
</tr>
<tr>
<td>GSH-Px (U/g)</td>
<td>52 ± 2</td>
<td>31 ± 1*</td>
<td>17 ± 1**</td>
</tr>
<tr>
<td>Amylase (U/l)</td>
<td>322 ± 46</td>
<td>1972 ± 269*</td>
<td>1221 ± 105***</td>
</tr>
</tbody>
</table>

Values are mean ± SEM
MDA, malondialdehyde; SOD, superoxide dismutase
GSH-Px, glutathione peroxidase

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were estimated by method of Ohkawa et al. MDA level was expressed as nmol/g tissue.

**Superoxide dismutase (SOD) activity measurement** was based on the generation of superoxide radicals produced by xanthine-xanthine oxidase system that reacts with 2 (4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form red formosan dye. The SOD activity is then measured by the degree of inhibition of this reaction. The SOD activity was expressed as U/g tissue.

Glutathione peroxidase (GSH-Px) activity was measured by the method of Paglia & Valentine, and was expressed as U/g tissue.

**Quantitative cultures and bacterial identification:** The portions of the pancreas with macroscopic necrosis were excised, weighed, and homogenized. The homogenate was diluted serially, quantitatively plated in duplicate on phenylethyl alcohol and MacConkey II agar, and then incubated aerobically at 37°C for 24 h. Bacterial counts were expressed as colony forming units (cfu/g tissue), and counts of 1,000 cfu/g and higher were considered to be indicative of a positive culture. Gram-negative bacteria were identified with the API-20E system (BioMerieux Vittek, Hazelwood, MO, USA). Gram-

<table>
<thead>
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<th>Table II. Histopathologic scores of the groups</th>
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<tbody>
<tr>
<td>Group I (n=6)</td>
</tr>
<tr>
<td>Histopathology score</td>
</tr>
<tr>
<td>Oedema</td>
</tr>
<tr>
<td>Acinar necrosis</td>
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<tr>
<td>Inflammatory infiltrate</td>
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<tr>
<td>Haemorrhage</td>
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<tr>
<td>Fat necrosis</td>
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<tr>
<td>Perivascular infiltration</td>
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</table>

Values are mean ± SEM
Mann-Whitney U test. *P<0.001 compared to group I 
P<0.05, **<0.01, ***<0.001 compared to group II
positive bacteria were identified to the genus level by means of standard microbiologic methods\textsuperscript{21,22}.

**Statistical analysis:** Results were expressed as mean ± SEM. Translocation incidence was evaluated by $\chi^2$ test and Fisher’s exact test where appropriate. The significance of differences in oxidative stress parameters, and serum amylase levels were assessed by One way ANOVA and Tukey’s HSD as Post Hoc comparisons. Histopathological scores were assessed by Kruskal-Wallis and subgroup analysis was assessed by Mann-Whitney U test, because of the data distribution in groups was not homogeneous. $P < 0.05$ was considered significant. All statistical measurements were made by using SPSS PC ver. 11.05 (SPSS Inc. USA).

**Results**

Acute pancreatitis developed in groups II and III rats, demonstrated by macroscopic parenchymal necrosis, and abundant turbid peritoneal fluid. All animals except five in group II and three in group III survived the experiment period of 48 h.

Serum amylase levels were significantly reduced ($P < 0.01$) in group III rats compared with group II rats. MDA level was lower, and SOD and GSH-Px levels were higher in group III than group II (Table I).

The total histopathologic score was significantly reduced in the group III (14.0 ± 0.5) compared to group II (19.2 ± 0.6), ($P < 0.001$). Oedema, acinar necrosis, inflammatory infiltration, haemorrhage, fat necrosis and perivascular inflammation in group III were also significantly decreased with allopurinol treatment when compared with group II (Table II).

Bacterial translocations to pancreatic tissue were found in 10 of 16 (62.5\%) rats treated with allopurinol whereas all pancreatic tissues were infected in rats treated with saline. Decrease of translocation to pancreas in group treated with allopurinol (group III) was statistically significant compared with the group II ($P < 0.02$). Bacteria isolated from pancreas of rats with acute pancreatitis included *Escherichia coli*, *Enterococcus* sp., *Staphylococcus* sp., *Klebsiella oxytoca* and *Proteus* sp. The most common bacteria were *E. coli*.

**Discussion**

During the course of necrotizing pancreatitis, pancreatic infection may be predicted to develop in 40 to 70 per cent of patients and is associated with significant morbidity and mortality in up to 50 per cent of such individuals\textsuperscript{1,2}. The most important factor determining the prognosis in the acute pancreatitis is infection\textsuperscript{5,21}. The risk of the development of infection increases with the amount of pancreatic glandular necrosis. Contamination of necrotic tissue occurs primarily due to translocation of enteric microorganisms\textsuperscript{5}. Bacterial translocation is not only specific to AP but it is responsible for the development of infection and sepsis in several diseases such as shock, malnutrition, and burn\textsuperscript{6-8}. Although pathogenesis of translocation is not known completely, it is thought to be related to multiple factors. Previous studies have confirmed that mucosal injury, ceacal bacterial overgrowth, decreased gut motility, and compromised host immune function are underlying mechanisms of bacterial translocation\textsuperscript{24,25}.

The failure of gut mucosa to act as a barrier against bacterial translocation has been accepted as a potential origin of sepsis and subsequent organ failure after pancreatitis\textsuperscript{23,24}. The effects of both systemic prophylactic antibiotic usage and selective gut decontamination by antibiotics have not been clearly demonstrated. The attention has been now focused on prevention of translocation as a key to improving the outcome after severe pancreatitis\textsuperscript{5,9}.

The first mechanism facilitating the loss of gut barrier function is increased nitric oxide (NO) synthesis\textsuperscript{14,24}. NO, a highly reactive free radical, is produced in the cell by the action of isoenzymes. Normally, there are three isoforms of nitric oxide synthase (NOS) in the intestine: a constitutive enzyme normally found in the endothelium lining (ecNOS), a neurally associated constitutive form found in the neurons of enteric nervous system (ncNOS), and an inducible enzyme (iNOS) that is not a normal cellular constituent but can be expressed in response to various inflammatory stimuli\textsuperscript{26,27}. In
the AP, once induced, iNOS generates large amounts of NO in a largely uncontrolled fashion different from cNOS. It has been thought that peroxynitrite, consisting of reaction of NO and superoxide, is responsible for intestinal barrier dysfunction\textsuperscript{28-30}. Simsek et al\textsuperscript{27} demonstrated that S-methylisothiourea, an iNOS inhibitor, decreased the incidence of bacterial translocation in an animal model of AP. In a previous study, we also demonstrated that S-methylisothiourea improved both oxidative stress and course of pancreatitis in an experimental rat model\textsuperscript{31}.

The second mechanism being responsible for intestinal barrier dysfunction is ischaemia-reperfusion injury\textsuperscript{4,24,32}. Rahman et al\textsuperscript{32} demonstrated increased urinary excretion of free fatty acid binding protein in patients with AP.

Hotz et al\textsuperscript{4} demonstrated that systemic blood pressure was normal whereas there was 74 per cent decrease in capillary blood flow in the colon, 6 h after induction of pancreatitis, and that these decreases did not associate with increase in gut permeability. Due to non significant change in intestinal permeability in the early stage of pancreatitis, it is suggested that therapeutic interventions aimed to prevent development of secondary infection by decreasing translocation will be able to improve the prognosis of the disease.

In our study, we demonstrated that allopurinol therapy after induction of pancreatitis decreased the incidence of bacterial translocation. This result was in agreement with the observation of Deitch et al\textsuperscript{9}. Inhibition of xanthine oxidase is thought to be responsible for decrease in bacterial translocation. In the present study, the fact that bacteria species isolated from the infected pancreatic tissue resembled common enteric bacteria, suggest that secondary pancreatic infections have resulted from translocation of colonic flora to this site\textsuperscript{23,33}.

Pancreatic MDA, SOD and GSH-Px levels were used for the evaluation of oxidative stress. All these parameters were better in the rats received allopurinol compared with control group. These results were in agreement with the earlier observation\textsuperscript{34,35}. As xanthine oxidase was a major source of oxygen radical generation, allopurinol treatment prevented the generation of oxidative stress in the acute pancreatitis.

We found that allopurinol caused significant reduction in total histopathologic scores similar to earlier reports\textsuperscript{13,36}. Improvement in the histopathologic score may be due to beneficial effects of allopurinol on bacterial translocation and oxidative stress.

In summary, allopurinol, an inhibitor of xanthine oxidase, has shown beneficial effects on bacterial translocation, oxidative stress and the course of pancreatitis in the experimental necrotizing pancreatitis. These results need to be supported with more comprehensive studies with allopurinol alone or in combination with antibiotics.

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References

8. Deitch EA, Specian RD, Berg RD. Endotoxin-induced bacterial translocation and mucosal permeability: Role of
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