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ORIGINAL ARTICLE

Effects of probiotics on the severity of experimental acute pancreatitis

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Objective: This study was designed to evaluate the effects of probiotics on the severity of experimental acute pancreatitis.
Design: Experimental study.
Setting: Experiments were done in a laboratory at Haydarpasa Numune Teaching and Research Hospital.
Subjects:A total of 50 Wistar rats were randomly divided into five groups.
Interventions: Group 1 was control group. Group 2 received an intraperitoneal injection of a 20% solution in 0.15 mol/l NaCL. Group 3 was injected NaCL and fed with probiotics. Acute pancreatitis was induced in rats by intrperitoneal injection of L-Arginine in groups 4 and 5. The rats in group 5 were treated with probiotics. The pancreas was removed for histologic examination. Evaluation of the pathologic changes was done by a new combined histopathologic grading scale.
Results: The mean scores of fibrosis, acinar cell loss, oedema, parenchymal necrosis, mononuclear cells infiltration, polymorphonuclear leucocytes infiltration, ductal damage and atypical reactive regeneration in group 5 were significantly lower than group 4.
Conclusions: We demonstrated that enteral feedings with added probiotics can reduce the severity of acute pancreatitis.
Sponsorship: None.
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Introduction

Acute pancreatitis is characterized by inflammation of pancreatic and peri-pancreatic tissues and ranges from a mild illness to a life threatening condition. The treatment of acute pancreatitis depends on the severity of the attack. If no complications occur, acute pancreatitis usually improves on its own. Treatment, in general, is designed to support vital bodily functions and prevent complications. The parenteral nutrition has theoretical advantage over enteral feeding, because it does not stimulate pancreatic secretions. With the understanding of bacterial translocation and other septic complication on the progress of acute pancreatitis, enteral feeding became more popular. Nowadays, it has been accepted by many authors that early enteral feeding has many positive impact on the acute pancreatitis. Probiotics can be defined as nonpathogenic microorganisms which when ingested play a key role in human nutrition and health in balancing the intestinal microflora naturally. These living bacteria can influence the human in many aspect (Dugas *et al*., 1999; Isolauri *et al*., 2001; Marteau *et al*., 2001). It has been shown that when probiotics supplemented early enteral nutrition they reduced the septic complications in acute pancreatitis (Dugas *et al*., 1999; Oláh *et al*., 2002a, b). Several advantages of enteral feeding over parenteral feeding have been demonstrated but no experimental study has documented the histopathologic effects of probiotics in acute pancreatitis (Sahin *et al*., 1999; Qin *et al*., 2002).

The present study was designed to analyse the effects of probiotics on the progress of experimental acute pancreatitis. Histopathologic features of experimental acute pancreatitis were seen and compared in two groups that did and did not receive intraperitoneal L-arginine.

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Materials and methods

The experimental animal model used in this present study was approved by the Animal Ethics Committee of Haydarpasa Numune Research and Training Hospital and followed EU guidelines for the handling and care of the laboratory animals. An experimental investigation was set up using Wistar Albino rats weighing 250–300 g. Each rat was housed in one cage in an appropriate environment (controlled with 12-hourlight and dark cycles). The animals were fasted for 12 h before surgery.

Experimental groups
A total of 50 rats were randomly divided into five groups. Group 1 was the control group, and no treatment was given. The rats in group 2 (Sham group) received an intraperitoneal injection of a 20% solution in 0.15 mol/l NaCL twice, at an interval of 1 h. The rats in group 3 (Sham and probiotics) were injected equal volume of NaCL plus fed with probiotic via ora-gastric tube once a day for 5 days. The rats in group 4 (acute pancreatitis group and enteral feeding) were given intraperitoneal injection of 250 mg/100 g body weight of L-arginine (Sigma Chemical Co., St Louis, Missouri, USA) as a 20% solution in 0.15 mol/l NaCL twice, at an interval of 1 h. The rats in group 5 (acute pancreatitits and enteral feeding with probitics) were applied same procedure than in group 4 and probiotics was given by oro-gastric tube. All rats in groups were fed with standard rat food and had free access to water throughout the study. All groups were treated for 5 days. At the end of experiments, a midline incision was done to all rats and pancreas and mesenteric lymph nodes were taken out for analysis.

Preparation of probiotics
The freeze-dried probiotics were transported to our laboratory according to the cold chain transfer rules. Rats in group 3 and 5 received 200 mg containing probiotic culture each day for 5 days. A measure of 200 mg of probiotic cultures were prepared into small sacs and stored at -20°C. A 200 mg probiotics culture consisting of 1.0×10^9 organisms of live *Streptococcus Thermophilus,* 2×10^8 organisms of live *Lactobacillus Acidophillus* and 1.2×10^9 *Bifidobacterium Lactis* (MSK-Mix ABD V 1–54/DaniscoCultor/Denmark) was diluted with 1 ml of 1 M NaCl. Prepared probiotics was immediately given by gavage.

Bacteriology
Lymph nodes were preserved in sterile brain–heart infusion broth and transferred to bacteriology laboratory. They were homogenized with sterile teflon-coated tissue grinding rods (Fischer Scientific, Pittsburg, Pennsylvania, USA). Each homogenate was innoculated into blood agar and MacConkey'agar culture palates and neomycine blood containing culture for anaerobic incubation. All culture palates were incubated at 37°C and examined 18–24 h, with organisms identified by standard bacteriological techniques.

Histologic examination
Pancreas samples were harvested, fixed for 24 h in 10% neutral buffered formalin, embedded in paraffin, sectioned at 3–4 μm thickness, and stained with hematoxylin and Eosin by a pathologist. Two pathologists then evaluated the slides with light microscope. The pathologists were blinded about the animal groups. The slides were randomly distributed between two pathologists. Both pathologists used the same grading system. Because currently there is no adequate grading system for evaluating acute pancreatitis, a new combined histopathologic grading scale for pancreatitis was designed and detailed in Table 1. This system was created on the basis of histologic features of acute pancreatitis and basically based on scales described by Tito and Freiburghaus (Tito *et al*., 1993; Freiburghaus *et al*., 1995).

Statistical methods
Differences between groups were tested using Kruskal–Wallis analysis of variance and Fisher's exact test when appropriate. A value of $P<0.05$ was considered statistically significant.

Results

Experimental pancreatitis did not developed in groups 1, 2 and 3. These groups did not show any evidence of pancreatitis. Experimental acute pancreatitis was seen in those animals receiving an intraperitoneal injection of 250 mg/100 g body weight of L-arginine. The rats with experimental acute pancreatitis had swollen and oedematous pancreas at the laparotomy. These gross appearances were correlated with the histological findings of pancreas specimens. The scores of histological grades for each rat were added up in each group separately and we found the scores for each histopathologic change. Mean scores were shown in Table 2. Comparison between group 4 (acute pancreatitis group and enteral feeding) and group 5 (acute pancreatitis and enteral feeding with probitics) disclosed a significant ($P<0.05$) difference in fibrosis, acinar cell loss, oedema, parenchymal necrosis, mononuclear cells (MNL) cell infiltration, polymorphonuclear leucocytes (PMNL) cell infiltration, ductal damage, atypical reactive regeneration and vacuolization. The mean scores of fat necrosis in group 5 was lower than group 4, but a statistical difference were not found. It was not possible to analyse the data of haemorrhage because group five's standard deviation was zero. As a result, the experimental pancreatitis in group 5 (Figure 1) had a milder form of pancreatitis than group 4 (Figure 2).

The bacterial translocation to the mesenteric lymph nodes occurred in group 4 and 5 (Table 3). Bacterial translocation

Table 1 Combined histopathologic grading scale for pancreatitis

Fibrosis
0 No fibrosis
1 Focal, <10% of the pancreas parenchyma
2 Mild, between 11 to 50% of the pancreas parenchyma
3 Diffuse, between 51 to 75% of the pancreas parenchyma
4 Severe, >76% of the pancreas parenchyma

Acinar cell loss
1 No acinar cell desruction
2 Acinar cell destruction <25% of acinar cells
3 Acinar cell destruction <26–50% of acinar cells
4 Acinar cell destruction <51–75% of acinar cells
5 Acinar cell destruction >75% of acinar cells

Edema
0 No oedema
0.5 Focal expansion of interlobar septa
1 Difffuse expansion of interlobar septa
1.5 Same as 1 + focal expansion of interlobar septa
2 Same as 1 + diffuse expansion of interlobar septa
2.5 Same as 2 + focal expansion of interacinar septa
3 Same as 2 + diffuse expansion of interacinar septa
3.5 Same as 3 + focal expansion of intercellular spaces
4 Same as 3 + diffuse expansion of intercellular spaces

Fat necrosis
0 Absent
1 Present

Parenchymal necrosis
0 No necrosis
0.5 Focal occurence of 1–4 necrotic cells/HPF
1 Diffuse occurence of 1–4 necrotic cells/HPF
1.5 Same as 1 + focal occurence of 5–10 necrotic cells/HPF
2 Diffuse occurence of 5–10 necrotic cells/HPF
2.5 Same as 2+ focal occurence of 11–16 necrotic cells/HPF
3 Diffuse occurence of 11–16 necrotic cells/HPF
3.5 Same as 3 focal occurence of >16 necrotic cells/HPF
4 ⩾16 necrotic cells

Inflammation and perivascular infiltration of polymorphonuclearleucocyte
0 No inflammation
0.5 2–5 PMNL/HPF
1 6–10 PMNL/HPF
1.5 11–25 PMNL/HPF
2 16–20 PMNL/HPF
2.5 21–25 PMNL/HPF
3 26–30 PMNL/HPF
3.5 ⩾30 PMNL/HPF or focal microabscesses
4 ⩾35 PMNL/HPF or confluent microabscesses

Inflammation and perivascular infiltration of mononuclear cells
0 0–20/HPF
0.5 21–100/HPF
1 101–300/HPF
1.6 301–500/HPF
2 501–700/HPF
2.5 701–900/HPF
3 901–1100/HPF
3.5 1101–1300/HPF
4 ⩾1301/HPF

Ductal damage
0 Absent
1 Present

Atypical reactive regeneration
0 Absent
1 Present

Vacuolization
0 No vacuolization
0.5 Less than one eight of cells with vacuoles
1 Between one and two eights
1.5 Between two and three eights
2 Between three and four eights
2.5 Between four and five eights
3 Between five and six eights
3.5 Between six and seven eights
4 All cells with vacuole formation

Hemorrhage
0 Absent
1 Present

HPF: High power field.

was not confirmed in other groups. Five of the ten (50%) rats showed positive culture and the predominant organism was *Escherichia coli* and the genera *Protues* and *Klebsiella* were identified in group 4. Only one rat showed the evidence of the bacterial translocation and *E. coli* was cultured in group 5. Two monomicrobial infections in group 4 and one in group 5 occurred and which was *E. coli*. There was not a significant difference between group 4 and group 5 with regard to the rate of bacterial translocation. No rat died during the experiment. Surprisingly, four rats in group 5 suffered diarrhea and two rats had soften stool for 2 days. They simultaneously recovered.

Discussion

Acute pancreatitis is an acute inflammatory process of pancreas. It is characterized by varying degrees of oedema, haemorrhage and necrosis of the pancreas and surrounding fat. The mortality differs according to the severity of disease, oedematous pancreatitis is a self-limited disease and it generally responds to medical treatment if it is uncomplicated. The development of pancreatic necrosis is a serious consequence of acute pancreatitis in 40–70% of patients (Buchler *et al*., 2000). If pancreatic necrosis is infected, patients with necrotizing pancreatitis have a great chance of progression to systemic inflammatory response syndrome, multi-organ dysfunction and sepsis. So, the infection and the extent of necrosis play a crucial importance in the progression of acute pancreatitis (Isenmann *et al*., 1999). Bacterial endotoxins and antigens gain access to portal blood and activate pancreatic macrophages to release inflammatory cytokines like interleukin 1, interleukin 6, tumor necrosis factor in necrotizing. The inflammatory cytokines are one of the major reason for developing sepsis and multi-organ failure (Kusske *et al*., 1996; Mayer *et al*., 2000). The question to be answered, what is the source of infection in acute pancreatitis. Beger *et al*. (1986) stated that the microorgan-

Table 2 Comparisons of histopathological changes of acute pancreatitis between two groups

	Group 4 *Experimental pancreatitis + enteral feeding (n = 10)* *Mean score (± s.d.)*	*Group 5* *Experimental pancreatitis + (enteral feeding + probiotics)* *(n = 10)* *Mean score (± s.d.)*	P
Fibrosis	1.6 ± 0.5	0.5 ± 0.5	<0.01
Acinar loss	2.5 ± 0.5	0.87 ± 0.5	0.02
Edema	3.2 ± 0.5	1.2 ± 0.4	<0.01
Fat necrosis	0.1 ± 0.3	0.1 ± 0.3	1.0
Parenchymal necrosis	1.1 ± 0.5	0.4 ± 0.5	0.01
Inflammation with infiltration of leukocytes	3.62 ± 0.3	1.2 ± 0.4	<0.01
Inflammation with infiltration of macrophages	2.6 ± 0.4	1.0 ± 0.4	<0.01
Ductal damage	0.7 ± 0.4	0.1 ± 0.3	0.04
Atypical reactive regeneration	0.8 ± 0.4	0.2 ± 0.4	0.04
Vacuolization	0.7 ± 0.5	0.2 ± 0.2	<0.01
Hemorrhage	0.1 ± 0.3	0	—

Table 3 Comparison of bacterial translocation

	Positive translocation (%)	*Negative translocation (%)*
Group 4	5/10 (50)	5/10 (50)
Group 5	1/10 (10)	9/10 (90)

isms identified in pancreatic necrosis were mostly gram-negative enteric bacteria and predominant organisms grown from those patients were E. *coli*. It was also reported that gram-negative organisms, anaerobes and fungi were isolated from the pancreatic necrosis (Beger *et al*., 1986, 1997). Experimental studies of bacterial translocation have also shown that bacterial translocation occured in 40–70% of necrotizing pancreatitis (Oláh *et al*., 2002b). These findings suggested that the source of infection was the intestine in necrotizing pancreatitis. It is, now, widely accepted that enteric microorganisms can penetrate the intact intestinal wall in case of severe acute pancreatitis. Many factors have been proposed to explain the bacterial translocation. The absence of enteral nutrition and prolonged total parenteral nutrition may be the most important contributing factor in the occurrence of bacterial translocation. The importance of enteral nutrition was accepted by several authors in recent years. It is shown that the nutrition by enteral route is better utilized compared by parenteral route in many studies (Oláh *et al*., 2002b). The main advantages of enteral feeding are to stop the progression of gastrointestinal mucosal atrophy and to help maintaining of immune status and normal gut flora (Nakasaki *et al*., 1988). It was showed that enteral feeding was effective in preventing the occurrence of multiple organ failure (Austrums *et al*., 2003). Enteral nutrition containing

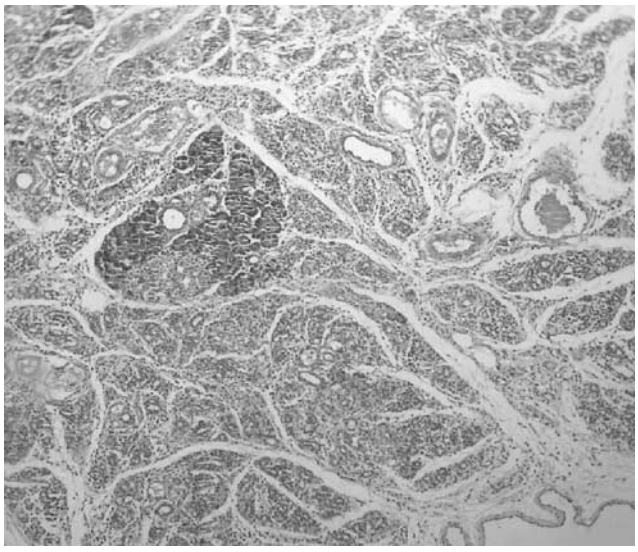

Figure 1 Photomicrograph of experimental acute pancreatitis with enteral feeding (Group 4). This section shows extensive mononuclear cells and polymorphonuclear leucocytes infiltrate the pancreatic parenchyma. It is characterized with diffuse acinar loss and fibrosis. Islet of Langerhans and surrounding acinus are spared.

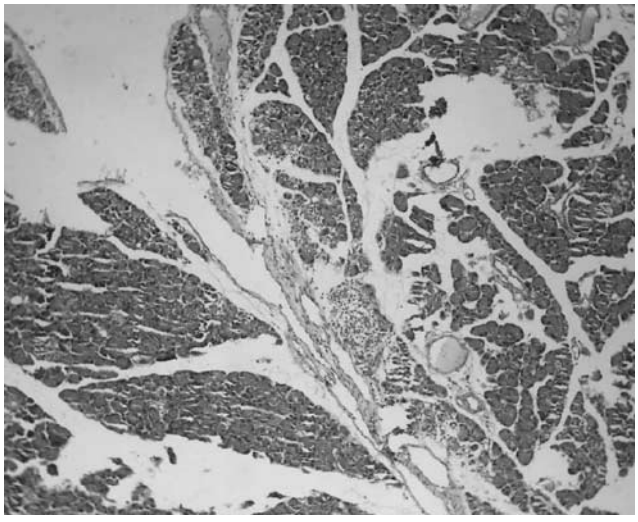

Figure 2 Photomicrograph of experimental acute pancreatitis with enteral feeding and probiotics (Group 5). Note inflammatory cells destruct focal area of pancreatic parenchyma.

arginine, omega-3 fatty acids, glutamine and nucleotides has been advocated for patients requiring nutritional support.

In our study, we tested the effects of probiotics by evaluating histopathologic changes on the rat model of L-arginine-induced experimental acute pancreatitis. L-arginine-induced pancreatitis model in rats was firstly described by Mizunuma *et al*. (1984). Tani *et al*. (1990) demonstrated a model of acute necrotizing pancreatitis induced by L-arginine in rats This experimental model has been widely used so far. We noticed the fibrosis in the slides of acute

experimental pancreatitis. Fibrosis is not characteristic of acute pancreatitits. Delaney *et al*. also observed histological findings of chronic pancreatitis in rats induced with L-arginine, but the reason is still obscure. So, 'Experimental acute pancreatitis' is an appropriate term describing the histopathology.

A probiotic is a live organism that contribute to many health aspects (nutrition, gut motility, prevention of cancer, immunity, etc.) and balance of the intestinal tract. Probiotic bacteria favourably adjust the intestinal microflora balance, inhibit the growth of harmful bacteria, promote digestion, boost immune function and increase resistance to infection. These features of friendly live microorganisms make it possible to use it as a therapeutic application. Probiotics, the supplement form of microorganisms, have been used for many years to increase the proportion of protective microflora and the most known form of probiotics is yogurt. Probiotics should be of human origin, be safe for human use, be stable in acid and bile and adhere to the intestinal mucosa. There are quite a lot of probiotics available and the most frequently used probiotic genera are *Lactobacillus* and *Bifidobacterium* (Holzapfel *et al*., 2001). Oláh *et al*. (2002a) reported that when patients with acute pancreatitis received *Lactobacillus plantarum* 299, they had lower incidence of pancreatic sepsis and needed less surgical interventions compared with control patients. It was also reported that the treatment with probiotics was effective in reducing microbacterial translocation in an experimental pancreatitis model (Mangiante *et al*., 2001). The rate of bacterial translocation was also affected by administration of probiotics in experimental acute pancreatitis in our study. Probiotics significantly reduced the percentage of positive culture in lymph nodes from 50 to 12.5%. We think that it is a very strong positive impact on bacterial translocation in spite of not having a statistical significance. This is the first experimental study which demonstrated histopathological changes of acute pancreatitis, whereas other human studies lacked these data.

We found that acinar cells were destroyed, whereas the Langerhans islet cells were spared in rats treated with intraperitoneal L-arginine. Added probiotics to enteral feeding statistically reduced the severity of fibrosis, acinar cell loss, oedema, parenchymal necrosis, inflammation and perivascular infiltration of PMNL, inflammation and perivascular infiltration of MNL, ductal damage, atypical reactive regeneration and vacuolization. Intrapancreatic haemorrhage did not occur in group 5 and only one pancreatic specimen showed haemorrhage in group 4. Fat necrosis was found in one pancreas in each group. So, fat necrosis did not reach any statistical significance.

When looking at the results, it seems that, probiotics lowered the severity of the histological injury of acute pancreatitis. The reduced bacterial translocation without a statistical significance is the only explanation we have at this moment. This means, the exact mechanism is still unclear and needs further studies.

As a conclusion, we demonstrated that enteral feeding with the supplement of probiotics can ameliorate the severity of experimental acute pancreatitis.

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