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Nitric Oxide, Heparin and Procaine Treatment in Experimental Ceruleine-Induced Acute Pancreatitis in Rats

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Abstract. The aim of the study was to investigate the impact of L-arginine (nitric oxide donor), L-NNA (NO synthase inhibitor), heparin and procaine on the pancreas' microcirculation, serum interleukin 6 (IL-6) level, and microscopic alterations of the pancreatic gland in acute pancreatitis (AP) in rats. AP was induced by 4 i.p. injections of cerulein (15 μg/kg/h). Microcirculatory values of the pancreas were measured by means of laser Doppler flowmetry 5 h after the first cerulein injection. Remarkable morphologic changes in the pancreas, including parenchymal necrosis, an elevation of serum IL-6 activity, and significant drop of pancreatic capillary perfusion was observed in rats with NO synthase inhibition. L-arginine improved the pancreatic microcirculation but worsened the microscopic alterations within the pancreas. Heparin had a beneficial effect on the microcirculatory values, serum IL-6 activity, and morphologic changes. Procaine had no effect on the course of AP. Authors conclude that heparin, improving the pancreatic capillary blood perfusion, may be considered as a promising therapeutic agent in acute pancreatitis.

Key words: acute pancreatitis; microcirculation; interleukin 6; nitric oxide; heparin; procaine.

Introduction

Acute pancreatitis (AP) remains still an enigmatic clinical problem. Although the majority of patients present mild edematous form of the disease, characterized by satysfying results of treatment, some patients develop progressive pancreatic necrosis with high morbidity and mortality rate. The factors which regulate the progression from edematous to necrotizing form of pancreatitis are still obscure. Pancreatic ischemia seems to be one of the earliest events observed in AP[6, 32]. Some authors underline that microcirculatory disturbances superimposed upon pancreatic edema may be the crucial pathological factor leading to the development of necrotizing pancreatitis[16, 27].

The therapeutic goal to improve affected capillary blood flow of the pancreas in AP was experimentally undertaken by some investigators[8, 9, 13, 19, 21, 23]. In the present study, for the amelioration of pancreatic perfusion, the authors used L-arginine (nitric oxide donor), heparin and procaine.

Nitric oxide (NO) is an endogenous vasodilator, it may act as an anticoagulant, preventing platelet aggregation, and may function as a biological scavenger or inactivator of oxygen free radicals[28, 29]. On the other hand, excessive amounts of NO and superoxide may combine to form the potentially cytotoxic substance peroxynitrite[2].

Heparin is a well known therapeutic agent which besides its anticoagulant properties, also inhibits complement activity, histamine, serotonine, and endothelin-1

release[14, 24, 31]. Procaine is an analgetic drug, which has been used in AP treatment in order to protect from pancreatic vasoconstriction by means of coeliac trunk blockade. Moreover, procaine is phospholipase A2 inhibitor, a crucial enzyme in AP pathogenesis[4, 26].

The aim of this study was to compare the influence of L-arginine (NO-donor), NG-Nitro-L-arginine (NO synthase inhibitor), heparin, and procaine administration in cerulein-induced acute pancreatitis in respect of microcirculatory values, serum IL-6 activity, and microscopic alterations of the pancreas.

Materials and Methods

Animal model. The study was carried out on 110 male Wistar rats weighing 150–200 g, kept on a standard rat chow and fasted overnight before the experiment with water allowed *ad libitum*. Acute pancreatitis was induced by four intraperitoneal injections of cerulein (Cn) (Sigma, St. Louis, USA; 15 μg/kg) in 1 ml of saline at 1 h intervals: at the beginning, and consecutively after the first, second, and third hour of the experiment. Five hours after the first cerulein injection, rats were anesthetized with pentobarbital sodium (40 mg/kg). Following the anesthesia, a laparotomy was performed, and pancreatic blood flow was estimated by means of laser Doppler flowmetry (Periflux 4001, Perimed Jarfalla, Sweden). A fiberooptic probe of the flowmeter was positioned against the surface of pancreatic serosa. Blood flow was measured in 3 different portions of the pancreas. The microcirculatory values were presented as percent change from basal value obtained in control rats. After the measurements, blood was aspirated from the inferior vena cava, the pancreas was removed, and the animals were exsanquinated.

The animals were divided into the following groups:
– with pancreatitis
group I (n=12) – acute Cn-induced pancreatitis without treatment;
group II (n=12) – AP + L-NNA (Calbiochem, Lucerne, Switzerland) 2×25 mg/kg;
group III (n=12) – AP + L-arginine (Calbiochem, Lucerne, Switzerland) 2×100 mg/kg;
group IV (n=12) – AP + heparin (Polfa, Warszawa, Poland) 2×2.5 mg/kg;
group V (n=12) – AP + procaine (Polfa, Starogard, Poland) 2×25 mg/kg.
– control groups
group VI (n=10) – rats receiving saline only;
group VII (n=10) – rats receiving L-NNA 2×25 mg/kg;
group VIII (n=10) – rats receiving L-arginine 2×100 mg/kg;
group IX (n=10) – rats receiving heparin 2×2.5 mg/kg;
group X (n=10) – rats receiving procaine 2×25 mg/kg.

All the medicaments were administered intraperitoneally in 0.5 ml of the saline, 1 and 2 h after the first Cn injection.

Interleukin 6 functional assay. The IL-6 bioassay was performed using the IL-6 dependent mouse hybridoma cell line B9 obtained from Dr. Lucien Aarden, Netherland Red Cross, Amsterdam. B9 cells were cultured in flat bottom microtitre plates (10 000 cells/well) in the presence of serial dilutions of test sera. Starting serum dilutions were 1:10 for all specimens. After 48 h of incubation, proliferation of the B9 cells was measured using a rapid colorimetric MTT (tetrazolium) assay. The concentration of recombinant IL-6 giving rise to half maximal proliferation was defined as one unit of activity, and the quantity of IL-6 in a serum sample was calculated by reference to a standard curve. Polyclonal antibody anty-IL-6 (Boehringer-Mannheim) was added into selected wells in concentrations: 1:10, 1:20, 1:50 in order to confirm the specifity of the assay. The antibody completely blocked IL-6 activity.

Histologic examination. Portions of the pancreas were fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin and eosin, and microscopically examined by a pathologist. Interstitial edema was scored as follows: 0 – absent, 1 – expanded interlobular septa, 2 – expanded intralobular septa, 3 – separated individual acini. Grading of vacuolization was based on the percentage of cell involved: 0 – absent, 1 – less than 25%, 2 – 25–50%, 3 – more than 50%. Inflammatory infiltration was graded from 0 (absent) to 3 for maximal alterations (diffuse infiltration within the entire pancreatic gland). Parenchymal necrosis was graded according to the approximate percentage of the involved area: 0 – absent, 1 – less than 5%, 2 – 5–20%, 3 – more than 20%.

Statistical analysis. Data are presented as mean ± standard deviation (SD). The differences between the groups were analyzed by means of ANOVA test. Groups II–V were compared to the group I, while group I was compared to the control group VI. Probability values less than 0.05 were considered significant.

Results

Microcirculation of the pancreas

Acute pancreatitis resulted in a significant drop of microcirculatory values to 37% in the group I, as com-

pared to the control group (vasal value 100%). Intraperitoneal administration of NO-synthase inhibitor (L-NNA) in the group II caused slight, insignificant decrease of the pancreatic blood flow in comparison to the group I (34% of the control value). The heparin and L-arginine treatment in the groups III and IV significantly improved the pancreatic microperfusion (76 and 72% of the control values, respectively). The procaine treatment had no effect on the pancreas' perfusion in AP (36%).

In the control groups without pancreatitis, the microcirculatory values were estimated as follows: control + L-NNA 70%, control + L-arginine 110%, control + heparin 112%, control + procaine 102% (Fig. 1).

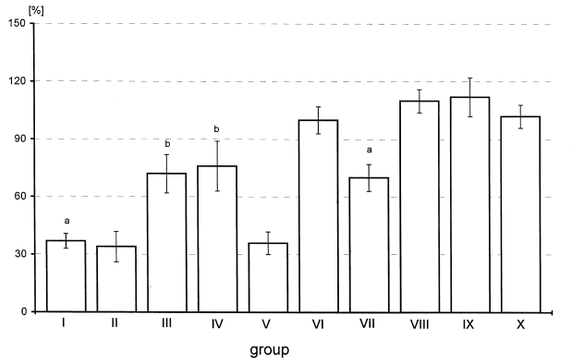

Fig. 1. Microcirculatory values of the pancreas in groups of rats. Mean values ± SD. Group I – AP without treatment, group II – AP + L-NNA, group III – AP + L-arginine, group IV – AP + heparin, group V – AP + procaine, group VI – control, group VII – control + L-NNA, group VIII – control + L-arginine, group IX – control + heparin, group X – control + procaine. Values statistically significant: a – in comparison to group VI; b – in comparison to group I ($p<0.05$)

Serum interleukin 6 assay

Cn-induced acute pancreatitis caused significant increase of serum IL-6 activity up to 359 U/ml. The NO-synthase inhibition by L-NNA resulted in a further augmentation of this cytokine level up to 409 U/ml. The treatment with L-arginine or procaine had no effect on the IL-6 level (352.5 and 371 U/ml, respectively) but the heparin administration significantly diminished IL-6 concentration to 288 U/ml. Among the control groups, the IL-6 concentrations were low, apart from the group VII, in which L-NNA injections increased IL-6 level to 79 U/ml (Fig. 2).

Morphologic alterations

Four i.p. injections of cerulein resulted in the group I edematous form of AP with inter- and intralobular

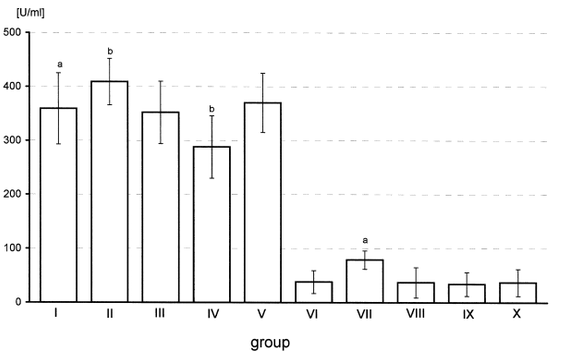

Fig. 2. Serum interleukin 6 activity in groups of rats. Explanations see under Fig. 1

edema, vacuolization of acinar cells, and leukocyte infiltration within the pancreatic gland. Focal parenchymal necrosis was observed in 4 animals with AP receiving L-NNA, and 2 animals treated with L-arginine. Apart from the necrosis, in 5 animals of the group II, focal hemorrhage was noticed. The histologic grading, concerning the severity of pancreatic tissue lesions was the most pronounced in the AP group treated with L-NNA (total score 8.0 patients). In rats receiving L-arginine, the total score was calculated as 6.0, and in the rats treated with procaine as 5.58. The least advanced inflammatory changes were observed in the AP group treated with heparin (total score 4.25 patients; Table 1).

Discussion

This study indicates that in Cn-induced experimental acute pancreatitis, the microperfusion of the pancreas became decreased. These observations are in accordance with other investigators, who revealed the drop of local pancreatic blood flow in Cn-induced pancreatitis in rats[19, 22, 23], however these authors did not observe as strong deterioration of pancreas perfusion as noticed in the present study. Two studies of LIU et al.[22, 23], by means of hydrogen gas clearance, demonstrated the drop of pancreatic microcirculation in Cn-induced AP to 70 and 80% only of the basal value in healthy animals. Contrary to these observations, KLAR et al.[18] described a homogenous capillary perfusion as well as a significant increase of capillary blood cell velocity in Cn-induced AP in rabbits, quantified by intravital microscopy. In Klar's study, the animals received fluid replacement (Ringer's solution to maintain hematocrit and central venous pressure in the control range), and dextran i.v. to facilitate fluorescent microscopy, what might have had an advantageous impact on

Table 1. "Histologic grading" of morphologic alterations of pancreatic gland in groups of rats

Group	Edema	Vacuolization	Infiltration	Necrosis	Total
I	$2.08^{a} \pm 0.28$	$1.75^{a} \pm 0.72$	$1.50^{a} \pm 0.50$	$0.00 \pm 0{,}00$	$5.33^{a} \pm 1.03$
II	2.33 ± 0.47	$2.75^{b} \pm 0.60$	$2.50^{b} \pm 0.50$	$0{,}42^{b} \pm 0.64$	$8.00^{b} \pm 1.08$
III	2.00 ± 0.41	2.25 ± 0.72	1.58 ± 0.64	0.17 ± 0.37	6.00 ± 1.47
IV	$1.58^{b} \pm 0.49$	1.67 ± 0.62	$1.00^{b} \pm 0.00$	0.00 ± 0.00	$4.25^{b} \pm 0.92$
V	2.00 ± 0.00	1.92 ± 0.64	1.67 ± 0.47	0.00 ± 0.00	5.58 ± 0.49
VI	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
VII	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
VIII	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IX	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
X	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Mean values ± SD; group I – AP without treatment, group II – AP + L-NNA, group III – AP + L-arginine, group IV – AP + heparin, group V – AP + procaine, group VI – control, group VII – control + L-NNA, group VIII – control + L-arginine, group IX – control + heparin, group X – control + procaine. Values statistically significant: [a] – in comparison to group VI; [b] – in comparison to group I ($p<0.05$).

the pancreatic tissue perfusion. Moreover, the pancreatic blood flow was assessed up to 3 h only. In a study of KELLY et al.[15], local pancreatic vascular abnormalities in Cn-induced AP were found early in the disease, and progressed during the study period. The most advanced changes were observed after the 4 h study period, however, the authors suggested that due to a mild form of pancreatitis, the vessel obstruction was rather functional.

Such a difference of microcirculatory values between these results and those of other studies may be due to the method applied. Laser Doppler flowmetry, used in our study, is an established noninvasive technique for measuring blood perfusion. This method enables measurement of relative changes in red blood cell flux, but not absolute values[3]. In the study of KONTUREK et al.[19], the pancreatic blood flow in Cn-induced pancreatitis, calculated by laser Doppler flowmetry, was reduced to 50% of the basal values. A stronger decline of the pancreatic microperfusion, observed in our study in the group with pancreatitis, could be due to an additional stress caused by 6 i.p. injections of the drugs in conscious animals. In the Liu's study, the water immersion stress in rats with Cn-induced AP caused further pancreatic ischemia and diminished the pancreatic blood flow to 37% of the normal value, and was considered as an aggravating factor in acute pancreatitis[23].

The inhibition of NO-synthase by L-NNA aggravated the course of acute pancreatitis. In spite of slight and insignificant further reduction of pancreatic perfusion in comparison to the pancreatitis group, NO blockade augmented the histologic alterations of the pancreas (focal necrosis and hemorrhages were observed), and increased the IL-6 serum level. Interleukin 6 is a proinflammatory cytokine. Its serum concentrations rise more rapidly and to a greater degree than any other acute phase protein[12]. Therefore, it has been proposed that IL-6 may be used as an early marker of the severity of acute pancreatitis[11].

Besides the vasoconstriction[1], the mechanisms aggravating pancreatitis after blockade of NO synthase may reflect deletion of the beneficial effects usually ascribed to NO, including inhibition of platelet aggregation, reduced adherence, and activation of neutrophils and scavenging of superoxide and other oxygen-derived free radicals[25, 28, 29]. The detrimental effect of decreased pancreatic blood flow on experimental pancreatitis induced by the NO-synthase inhibition[14], endothelin-1 administration[22], hemorrhagic shock[20], water immersion stress[23], and phenoxybenzamine treatment[17] was observed in several studies. The mechanisms by which the microcirculatory disturbance could aggravate pancreatitis are as follows: a) plasma protease inhibitors are unable to circulate through acinar cells and protect them; b) a local pancreatic metabolic acidosis activates proteases (for example, cathepsin B) to exacerbate pancreatic autodigestion and interfere with the metabolism of acinar cells; c) free radicals following ischemia inhibit binding of serum protease inhibitor to activated protease, and free radicals together with activated protease might damage the vascular endothelium[22].

The L-arginine treatment of pancreatitis in rats improved the pancreatic microperfusion, what is in accordance with KONTUREK et al.[19] and LIU et al.[23] studies. Contrary to these observations, we did not noticed an improvement of microscopic alterations in rats receiving cerulein and L-arginine. In this group the vacuolization was more pronounced, and in 2 animals focal necrosis was observed. This discrepancy could be explained by the route of L-arginine administration. In the studies mentioned above, L-arginine was infused intravenously, while in our investigation NO synthase substrate was injected intraperitoneally, what might result in a higher local nitric oxide concentration. Ex-

cessive amounts of NO, produced by inducible form of NO synthase, together with superoxide may combine to form the potentially cytotoxic substance peroxynitrite[2].

The advantageous effect of heparin in acute pancreatitis treatment was underlined in several studies. In GOULBORNE et al. experimental work[10], pretreatment with heparin reduced fibrinogen and platelets entrapment in the lungs observed in pancreatitis. Increased lung wet weight associated with pancreatitis was also reduced by heparin treatment. The favourable effect of heparin in experimental pancreatitis was shown by Gabryelewicz's group in a number of studies. This effect was related to prevention by heparin of disseminated intravascular clotting syndrome, inhibition of trypsinogen conversion to active enzyme in the pancreatic tissue, stabilisation of pancreatic lyzosomes, and preserved or even increased serum activity of alpha-1 antitrypsin, alpha-1 antichymotrypsin and antithrombin III[5, 8]. The improvement of pancreatic tissue perfusion observed in our investigation after heparin treatment might be explained by its anticoagulation effect which diminishes blood viscosity and facilities capillary blood flow. Besides its anticoagulant properties, heparin may play the advantagenous role by the inhibition of complement activity[31], and histamine release[24]. It was also shown by STERNBERG et al.[30] that heparin prevented postischemic endothelial cell dysfunction independently of its anticoagulant activity. Moreover, heparin has an inhibitory effect on the biosynthesis and release of endothelin-1, an endogenous vasoconstrictor[14].

Summarizing, our observation suggests that in Cn-induced acute pancreatitis heparin administration improves the microcirculatory dysfunction in the pancreas and diminished the severity of pancreatitis. Inhibition of NO-synthase has negative impact on the course of AP. Although the treatment with NO synthase substrate, L-arginine, protects from the pancreatic blood flow impairment, we did not find morphologic evidences that NO has a protective effect on acute pancreatitis. Procaine, used in the present study, does not seem to have any effect on the course of acute pancreatitis.

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