Does the Septic Shock Interfere Experimental Acute Pancreatitis in Rats?

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Abstract: ***Introduction*:** Acute pancreatitis is a disease involving pro-inflammatory mediators. Two complex and multifactorial pathogenetic ways lead to edematous or necrotizing pancreatitis. The course of the disease is thought to be the consequence of an acute inflammatory response.

AIM: The authors examined the impact of *Escherichia coli* LPS on the acute cerulein pancreatitis in rats.

Methods: The study was performed on rats using the ceruleine pancreatitis model. The activation status of polymorphonuclear cells, blood IL-6 concentration, oxidative stress parameters, pancreatic enzymes concentration and microscopic alterations were determined at 5th and 9th h observations.

Results: In acute pancreatitis and acute pancreatitis with LPS groups, the peripheral polymorphonuclear cells activity was lower than in control one. Authors noticed the same neutrophil activation in acute pancreatitis after lipopolysaccharide administration although the peripheral blood polymorphonuclear cells count was significantly higher at the 9th h observation. LPS neither changed the oxidative stress within pancreatic gland, nor amylase or serum lipase activity. LPS given to acute pancreatitis animals resulted in significant increase of serum IL-6 concentration at 5th observation turning normal after 9th h.

Conclusions: Collected data supports thesis of early polymorphonuclear cells involvement in acute pancreatitis and oxidative stress evidence in pancreatic parenchyma. However, results did not reveal that administration of LPS amplified inflammatory response during the course of acute pancreatitis.

Key Words: Acute pancreatitis, lipopolysaccharide, septic shock.

INTRODUCTION

The pathomechanism of acute pancreatitis (AP) is complex. AP are classified into two main categories: mild pancreatitis with pancreatic edema or necrotic pancreatitis with hemorrhagic necrosis of the gland. The exact pathogenetic features leading to specific course is unknown. The morbidity of AP depends on the disease course and complications resulted from organ dysfunction. Treatment of acute pancreatitis is focused on the pathogenesis cascade blocking, disease limiting and organ dysfunction prevention. Autodigestion theory resulted in several protease inhibitors engagement in acute pancreatitis treatment [19]. Although experimental data supports that point of view, clinical treatment results were disappointing. Several authors suggested that the AP dependent organ damages caused by the systemic inflammatory response syndrome (SIRS) in both courses of the disease. Activated neutrophils were thought to be the important mediator of SIRS [1-3]. PMN are involved in AP dependent acute respiratory distress syndrome (ARDS) [16, 17]. Last 50 years have brought the evolution of AP treatment strategy from antiprotease to anti-inflammatory agents. Different substances modulating the inflammatory response replaced the former treatment concept of autodigestion in AP. A number of inflammatory modulators were evaluated in AP treatment [4-8]. The PAF antagonist, TNF-α receptor antagonist, adhesion molecule monoclonal antibodies were used as anti-inflammatory therapeutic agents in AP. On the other hand several inflammatory stimulants were investigated to reveal their influence on inflammatory cascades involved in the pathomechanism of the disease [35, 36, 38]. Endotoxins and large bowel bacteria are suspected to be responsible for exacerbation of the AP and may change the course of the disease from mild to severe one [9, 10]. Some investigators support the thesis of exacerbation of AP as e result of endotoxemia or bacteremia [35]. The aim of the presented study was to investigate the role of Escherichia coli lipopolysaccharide (LPS) on the AP course and PMN activity. Another goal was to evaluate the status of polymorphonuclear cells (PMN) in the early acute pancreatitis in rats and LPS role in their activation during AP.

MATERIAL AND METHODS

The study was approved by the Local Ethics Committee for the use of Experimental Animals of the Medical

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University of Gdansk (Opinion nr 22/71). The study was carried out on male Wistar rats weighting 180 – 200 g, kept on standard rat chow and fasted overnight before the experiment with free access to water. Acute pancreatitis was induced by four i.p. injections of ceruleine (Cn) (15 μg/kg) in 1 ml of saline at 1 h intervals. LPS (Sigma Chemical Co., St. Louis, USA) was given at the beginning of the experiment i.p. in a dose of 10 mg/kg. Control animals received i.p. the equal volume of 0.9% saline solution. Animals were divided into four groups:

Group 1 (n = 14) control

Group 2 (n = 16) acute pancreatitis (AP)

Group 3 (n = 11) acute pancreatitis (AP) + lipopolysaccharide (LPS)

Group 4 (n = 11) control + lipopolysaccharide (LPS)

Five and nine hours after the first Cn dose, rats were anesthetized with sodium pentobarbital (25 mg/kg i.p.). Following the anesthesia, a thoracolaparotomy was performed and blood was aspirated on heparin 30 U/ml from left ventricle, the pancreas was removed, and animals were sacrificed by exsanguination. The pancreas after extraction was divided. Half was taken for microscopic examination and half was homogenized for malonyldialdehyde (MDA), total sulphydryl groups (-SH) and myeloperoxidase (MPO) determination.

WHOLE BLOOD CHEMILUMINESCENCE ASSAY

Chemiluminescence (CL) Reagent Preparation

Blood diluent was prepared with phosphate buffered saline (PBS) mixed with calcium and magnesium chloride (BIOMED - Poland) and glucose (0.1%). Granulocyte chemiluminescence was started by the phorbol-12-myrystate-13-acetate (PMA) (ICN Biomedicals Inc., USA). PMA (1 mg) was diluted with 1 ml dimethyl-sulpho dioxide (DMSO) (SIGMA) to final concentration 1.6×10^{-3} M. PMA solution was diluted with PBS to final concentration 1.6×10^{-6} M just before the measurement. Luminol solution was prepared with 1.77 mg of dry substance (ICN Biomedicals Inc., USA) combined with 1 ml 0.4% NaOH and filled to 3 ml with PBS to obtain 3×10^{-5} M concentration.

Whole Blood Chemiluminescence Measurement

Transparent tubes containing 100 μl heparinized whole blood diluted 1:20 with PBS, 200 μl luminol (3×10^{-5} M), 500 μl PBS and 200 μl PMA (1.6×10^{-5} M) were analysed by FB 12 Luminometer (BERTHOLD DETECTION SYSTEMS, Germany) at 460 nm. The measurement was carried out in 10 s intervals for 20 min. All reagents were kept in 22° C during the procedure. The CL intensity was calculated in relative light units (RLU) per 2000 whole blood polymorphonuclear cells (PMN), per minute (RLU/2000 pmn x min).

Serum Polymorphonuclear Elastase (PMN-E)

The measurement was carried out on the basis of commercial ELISA method (PMN-Elastase, K6840 IMMUNDIAGNOSTIC GmbH, Germany). Method is based on oxidation of orthophenylenediamine. The absorbance was determined at 450 nm (Cecil Super Aquarius CE 9200). The result was compared to standard curve and expressed in (ng/mL).

Pancreatic Myeloperoxidase (MPO)

Part of the pancreatic gland (at least 0.3 g wet mass) was taken during the laparotomy. The tissue was homogenized (Porter-Elvenheim) immediately in 4°C with phosphate-buffered saline (PBS) pH 7.4. The volume of PBS was adequate to obtain 5% homogenate solution. Then the sample was centrifuged at 600 g in 4°C for 10 min. 2 ml of supernatant was mixed with 0.5 ml of 0.1% phenyl-methyl-sulpho-fluoride (SIGMA Chemicals Co.) and 0.5 ml of 0.5% citrimide (MERK). The mixture was centrifuged again in 4°C at 1000 g. MPO was measured using the ELISA method (IMMUNDIAGNOSTIC GmbH, Germany).

Sulphydryl Groups (-SH) and Malondialdehyde (MDA) in Pancreatic Homogenate

Samples were tested for sulfhydryl content by a procedure described in details elsewhere [11] using dithionitrobenzoic acid (DTNB).

MDA content was measured with the use of spectrophotometer using Test Kit LPO-586 (OxisResearch, Portland, USA). 600 μl of homogenate supernatant was mixed with 1950 μl of R1 (10.3 nM N-methyl-2-phenylindole solved in acetonitrile) and 450 μl 37% HCl. Samples were incubated in water bath at 45°C for 1h. Then samples were centrifuged at 3500 rpm/min for 10 min. The absorbance of supernatant was measured at 586 nm (Cecil Super Aquarius CE 9200) and compared to standard curve and presented in International Units per milliliter (U/mL).

Serum α-Amylase and Lipase Activity

The α-amylase serum activity was measured using RTU 63116 (BioMerieux) kit. The method was based on a quantitative enzymatic assay. The absorbance was measured at 405 nm. The test was carried out on serum (15 μl), each test was duplicated and the mean value was treated as a result in International Units per milliliter (U/mL).

Serum lipase activity was determined using commercial kit LIPASE COLOR 63109 (BioMerieux). The quantitative enzymatic assay was used. The absorbance was measured at 550 nm (Cecil Super Aquarius CE 9200). Ten microliters of serum was required for determination, the result was expressed as International Units per milliliter (U/mL).

Microscopic Findings Within Pancreatic Gland

Pancreatic tissue underwent standard H/E staining. An experienced "blinded" pathologist performed the light microscopic examination under 200x magnification. Four criteria were evaluated: oedema, vacuolization and necrosis of pancreatic cells were established in modified Spormann scale (0-3 points each parameter) and polymorphonuclear infiltration within pancreatic parenchyma (calculated as mean number of neutrophils in 10 consecutive microscopic fields).

Complete Blood Count (CBC)

White blood cells (WBC) and neutrophils count (PMN) were analyzed in Bürker's camera following 1:200 dilution with Türk's solution.

Interleukin-6 (IL-6) Concentration

Serum IL-6 concentration was determined using the commercial ELISA kit Rat-IL-6 (BIOSOURCE). The reaction was based on the peroxydation of tetramethylbenzene (TMB).

Statistical Analysis

Statistical analysis was performed using the ANOVA test. The groups were analyzed in pairs. Acute pancreatitis group was compared to the control one. Lipopolysaccharide group was compared to the control one. Acute pancreatitis plus LPS group was compared to acute pancreatitis group. Significant difference in comparison to control group is marked as "*"; compared to AP which is marked as "†" on all figures and tables. Difference was considered as significant with $P<0.05$.

RESULTS

Whole Blood Chemiluminescence (CL)

The authors observed the significant deterioration of peripheral blood PMN chemiluminescence from 1700 to 1086 RLU/min x 2000 PMN in the AP group after 5 hours, and from 1842 to 988 RLU/min x 2000 PMN after 9 hours. The LPS administration resulted in no significant CL deterioration in both observation periods. LPS given to healthy animals resulted in significant CL reduction in 9th hour of experiment Fig. (**1**).

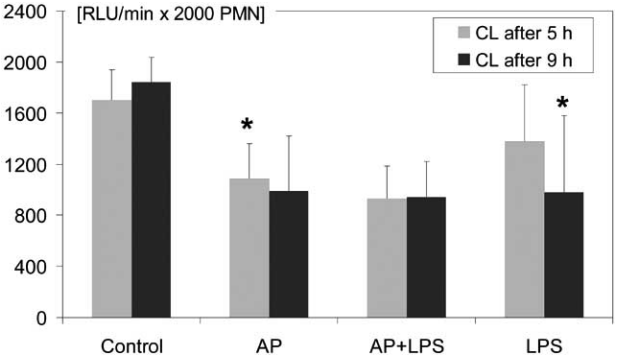

Fig. (1). Peripheral blood PMN chemiluminescence (CL) at 5th and 9th h of observation. Significant difference in comparison to control group is marked as *. Difference was considered as significant with $P<0.05$.

Polymorphonuclear Cells (PMN)

Acute cerulein pancreatitis resulted in significant reduction of PMN count in the fifth hour of experiment, 614 µl-1 as compared to 1230 µl-1 in the control animals. LPS given in AP group in the beginning of experiment changed significantly PMN count in 9th hour of observation from 1286 to 5146 μl^{-1}. LPS given to healthy animals have the same PMN count in both observation periods.

Polymorphonuclear Elastase (PMN-E) Serum Activity

Cerulein acute pancreatitis caused an elevation of serum PMN-E in all acute pancreatitis groups (group 2 and 3). Animals receiving LPS in AP (group 3) has similar PMN-E activity in both periods of observation compared to AP group (Table **1**).

Table 1. Polymorphonuclear Cells (PMN) and Polymorphonuclear Elastase (PMN-Elastase) in two Observation Periods

group	Time [h]	PMN [μl^{-1}]	PMN-elastase [ng/ml]
Control	5	1230 ±490	1.0 ±0.5
	9	1434 ±569	0.0 ±0.0
AP	5	614* ±229	15.0* ±2.0
	9	1286 ±598	17.0* ±1.5
AP + LPS	5	1047 ±560	13.0 ±1.7
	9	5146† ±1124	17.0 ±3.1
Control +LPS	5	2892* ±1372	3.0* ±0.2
	9	2959* ±1553	8.0* ±0.9

Mean values ±SD, * significant difference in comparison to control group $P<0.05$, † significant difference in comparison to AP group $P<0.05$.

EXPRESSION OF OXIDATIVE STRESS WITHIN PANCREATIC TISSUE

Sulphydryl Compounds (–SH) and Malondialdehyde (MDA) in Pancreatic Homogenate

Cerulein induced acute pancreatitis had significant effect on the oxidative stress parameters within the pancreatic gland. The concentration of (–SH) groups in pancreatic homogenate was reduced after 5 h of observation from 60.3 in the control group to 40.2 nmol/mg protein in the AP group. Depletion of (–SH) compounds accompany the elevation of malondialdehyde (MDA) concentration in pancreatic homogenate in AP group to 0.38 nmol/mg protein (control group 0.21 nmol/mg protein) during the same period of time. After 9 hours of observation the concentration of (–SH) groups was reduced from 64.7 in control to 43.5 nmol/mg protein in the AP group. MDA concentration in that period was respectively 0.22 and 0.39 nmol/mg protein. LPS given in AP group did not significantly change oxidative stress parameters within pancreatic gland Fig. (**2**) and Fig. (**3**).

Mieloperoxidase (MPO) Concentration in Pancreatic Homogenate

Acute pancreatitis resulted in significant rise, at 5th h and 9th h of observation, in pancreatic MPO concentration to 602 and 626 ng/g protein; compared to the control group 280 ng/g protein.

Lipopolysaccharide administration in acute pancreatitis did not significantly interfere with the pancreatic MPO concentration (Table **2**).

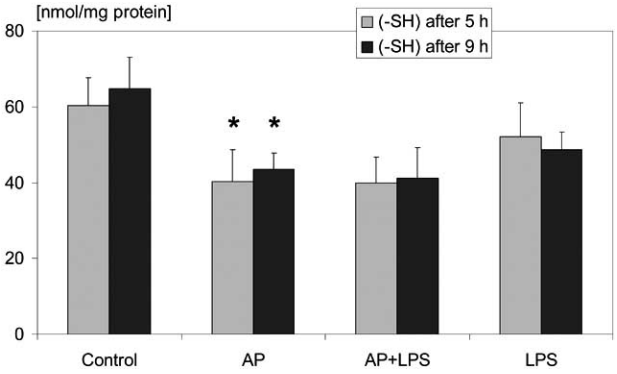

Fig. (2). Pancreatic sulphydryl groups (–SH) concentration at 5th and 9th h of observation. Significant difference in comparison to control group is marked as *. Difference was considered as significant with P<0.05.

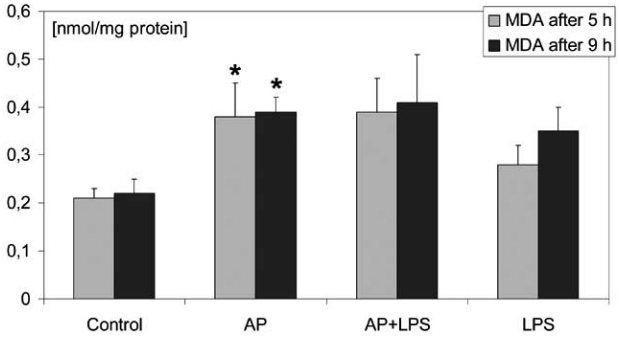

Fig. (3). Pancreatic malondialdehyde (MDA) concentration at 5th and 9th h of observation. Significant difference in comparison to control group is marked as *. Difference was considered as significant with P<0.05.

Table 2. Pancreatic Myeloperoxydase (MPO) in Two Observation Periods

group	Time [h]	MPO [ng/g protein]
Control	5	260 ±80
	9	280 ±93
AP	5	602*±220
	9	626* ±290
AP + LPS	5	508 ±190
	9	587 ±225
Control +LPS	5	307 ±77
	9	418 ±132

Mean values ±SD, * significant difference in comparison to the control group P<0.05.

α-Amylase and Lipase Serum Activity

A dose of cerulein (15 μg/kg for 4 h) caused marked hyperamylasemia (8286 and 5908 U/l) and hyperlipasemia (2140 and 929 U/L) in all acute pancreatitis groups (group 2 and 3) in two observation periods. Animals receiving LPS in AP (group 3) have similar α-amylase and lipase activity and serum activity compared to pure AP group.

Interleukin-6 Serum Concentration

Interleukin-6 (IL-6) was not detectable in acute pancreatitis group at 5 or 9 h of observation. High IL-6 serum concentration was noted at 5th h of observation only in group 3 and group 4 receiving LPS. Acute pancreatitis group receiving LPS did not differ significantly to pure AP group (Table **3**).

Microscopic Findings

The cerulein AP caused marked pancreatic microscopic changes such as interstitial edema, acinar cell vacuolization, neutrophil infiltration and foci of necrosis. The acute cerulein pancreatitis, compared to control animals, resulted in significant increase of edema formation and vacuolization at fifth hour of experiment, with the trend to normalize at nineth hour of experiment. The pancreatic polymorphonuclear infiltration is time dependent (Table **4**).

DISCUSSION

Cerulein acute pancreatitis is a well-known model of acute edematous pancreatitis in small animals [12-14]. Cerulein injection of 15 μg/kg i.p. induced the AP in rats in the presented study. Morphologic criteria have been found: acinar cells vacuolization, interlobular and interstitial edema, leukocyte infiltration and some foci of acinar cell necrosis. Cerulein given in dose 15 μg/kg caused a significant increase of serum pancreatic enzymes activity. Authors select the edematous model of acute pancreatitis to examine the potential deterioration of the disease after lipopolysaccharide administration.

Chemiluminescence (CL) is the process of light emission derived from the generation of chemical reaction in which chemically excited molecules decay to the electronic ground state and emit photons. The light emission is directly related to the reagents concentration. Released oxygen radicals are detected in CL reaction with high effectiveness and accuracy [15, 16]. This method is often used in laboratory medicine to measure the phagocyte function. The method is also introduced as a marker of infection. CL represents a sensitive detection assay for immunology and filter membrane biospheric reactions [15, 17, 18]. The whole blood CL approach is simple, time saving, requires small blood samples and avoids artefacts due to the cell isolation. For quantitative analysis, the white blood cells (WBC) and red blood cells (RBC) counts have to be obtained. 1:200 diluted samples of whole blood reflect activity of individual neutrophil, because the interaction between blood cells and serum components are negligible [19].

The most important pathological feature of the inflammatory process is neutrophils infiltration. The first stage in the neutrophil infiltration is endothelial adherence in the area of inflammatory process (primarily postcapillary venules) [1, 20]. Early neutrophil infiltration in acute

Table 3. Serum α-Amylase, Lipase Activity and Serum Interleukin-6 (IL-6) Concentration in Two Observation Periods

group	Time [h]	α-amylase [U/L]	Lipase [U/L]	IL-6 [pg/mL]
Control	5	690 ±120	6 ±8	0.2 ±0.3
	9	743 ±156	8 ±9	0.1 ±0.3
AP	5	8286* ±1432	2140* ±1123	0.6 ±2.0
	9	5908* ±640	929* ±252	0.0 ±0.0
AP + LPS	5	9310* ±7552	2573* ±2133	982.0† ±749.0
	9	4001* ±1638	1257* ±164	10.0† ±20.0
Control +LPS	5	584* ±182	11 ±8	941.0* ±756
	9	332* ±93	8 ±4	2.0* ±4.0

Mean values ±SD, * significant difference in comparison to the control group P<0.05, † significant difference in comparison to AP group P<0.05.

pancreatitis is documented by several investigators [12, 21, 22].

PMN sequestration within the pancreatic gland is the evidence of activation. In the presented study the authors found neutrophil infiltration at the 5th and 9th h of observation. PMN infiltration within pancreatic parenchyma accompanies PMN chemiluminescence reduction in peripheral blood during acute pancreatitis. PMN sequestration resulted in peripheral PMN count diminished compared to the control group. Blood chemiluminescence reduction might have resulted from activated PMN migration into the inflamed pancreatic parenchyma. At the 9th h of AP, the number of PMN cells in blood stream was almost equal to the control group, although the CL level at the same time, was significantly lower. Reduced PMN chemiluminescence probably was a consequence of immature PMN liberation from bone marrow. Another possible mechanism for that phenomenon may be the influence of serum anti-inflammatory agents released during acute pancreatitis [23]. These observations are in contrast to several other investigators, who observed elevated CL in AP [24, 25]. These authors investigated isolated PMN cells. Each isolation method resulted in PMNs activation status change [15, 26, 27]. The direct full-blood method seems to be more valuable in identifying PMN activation status.

Animals with AP receiving LPS presented as low CL intensity as in AP group in both observation periods. However, the PMN count was significantly higher at 9th h of experiment. Thus the CL intensity does not depend on the PMN count. Locally activated neutrophils migrate into pancreatic parenchyma. LPS resulting significant interleukinemia did not change the status of PMN activity in

Table 4. Microscopic Findings Within Pancreatic Gland. Oedema, Vacuolization and Necrosis of Pancreatic Cells were Established in Modified Spormann Scale (0-3 Points Each Parameter). Polymorphonuclear Infiltration Within Pancreatic Parenchyma was Calculated as Mean Number of Neutrophils in 10 Consecutive Microscopic Fields. All Data Shown in Two Observation Periods

group	Time [h]	Oedema [0-3]	Vacuolization [0-3]	Infiltration [n]	Necrosis [0-3]
Control	5	0.00 ±0.00	0.00±0.00	1.1±0.9	0.00±0.00
	9	0.00 ±0.00	0.00±0.00	1.21±1.03	0.00±0.00
AP	5	1.91 * ±0.90	2.50 * ±0.90	7.40 *±5.29	0.17±0.38
	9	0.50 ±0.57	1.00±1.41	14.60 *±2.37	0.25±0.50
AP +LPS	5	1.28±1.25	1.57±1.39	10.94±3.02	0.14±0.37
	9	2.00 ±1.15	1.25±1.25	17.92±5.98	0.00±0.00
Control +LPS	5	0.00±0.00	0.00±0.00	2.45±2.75	0.00±0.00
	9	0.00±0.00	0.00±0.00	6.00 *±4.11	0.00±0.00

Significant difference between control group and AP is marked as * P<0.05.

CL assay. On the other hand, LPS was given once 5 hours before blood sample collection. The difference in pancreatic infiltration between AP and AP+LPS group is comparable to pure LPS group infiltration. The PMN infiltrating pancreatic gland in pure LPS group did not cause any morphological change in acinar cells. Thus LPS given to AP animals probably activate PMN cells in two different mechanisms.

Oxidative stress was another effect of PMN activity within pancreas observed in AP group. Sulphydryl compounds reduction and simultaneous elevation of MDA concentration in pancreatic homogenate were detected in AP groups. Neutrophils NADPH-oxidase activation leads to superoxide anions production during AP. PMN are not the only one possible source of oxygen radicals. There are several signs of ischemia-reperfusion phenomenon and xanthine oxidase reaction is important in reactive oxygen species (ROS) generation in AP [11, 28-30]. Kusterer *et al*. observed that aggregation of leukocytes forming plaques limited the vein lumen in early stage of acute pancreatitis. Leukocyte adherence started within the first 30 min of AP [31, 32].

The inflammatory reaction exacerbation may occur after Escherichia coli lipopolysaccharide (LPS) administration. Pure LPS has a strong proinflammatory potency used in experimental septic shock induction. 10 mg/kg b.w. is the dose believed to induce septic shock in small animals [34]. Surprisingly, the LPS administration in AP group did not significantly change the pancreatic histological score. Some data confirmed the possibility of AP exacerbation from edematous to severe form after LPS administration [19, 34-36]. Ding *et al*. induced severe acute pancreatitis in mice after LPS administration in AP. They used higher cerulein dose (50 μg/kg) followed by LPS administration [35]. While Widdson *et al*. reported only cytokinemia elevation after LPS administration in AP [25]. Some data supports the reduced neutrophil activation after LPS administration [25]. Low dose of LPS (i.e. 50 μg/kg) resulted in pancreatic cells apoptosis stimulation and protective effect on acute pancreatitis. Our results did not confirm the detrimental effect of pure LPS on the cerulein AP course. The simultaneous intraperitoneal administration of 10 mg/kg of LPS and 15 ug/kg of cerulein resulted in significant IL-6 elevation only.

The cytokinemia after LPS administration in acute experimental pancreatitis were based on biological assay in the past. The ELISA method, used in presented protocol, seems to be more accurate and specific [37]. The effect of LPS on healthy animals seems to be more evident than on AP ones. PMN in acute pancreatitis group, presenting low CL was less potent to react on LPS stimulation. Thus the LPS did not reveal the anticipated biological effect. This observation may be the evidence of PMN activation dysfunction.

The LPS administrated in AP animals produced higher PMN infiltration within pancreatic gland at 5th and 9th h of observation. The authors did not find any necrotic foci within pancreatic parenchyma after LPS administration in cerulein injected animals. Some authors, in different experimental protocol, reported the acute pancreatitis exacerbation from edematous into severe necrotizing form after LPS administration [35].

The lack of IL-6 in edematous form of AP was observed in other studies as well [4, 38]. IL-6 elevated in clinical AP noted by several authors, may not be the acute pancreatitis marker but the early infection or multi organ failure indicator. The clinical course of septic patient with acute pancreatitis seems not to be comparable with presented animal model.

In conclusion, endotoxemia during the early stage of edematous acute pancreatitis did not significantly interfere on the disease course. IL-6 seems not to be the marker of acute pancreatitis severity but only the marker of infection. The PMN are effectors of acute pancreatitis and endotoxic shock, but the pathomechanism of these two reactions seems to be different.

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