PANCREATIC DAMAGE AND REGENERATION IN THE COURSE OF ISCHEMIA-REPERFUSION INDUCED PANCREATITIS IN RATS

*Department of Physiology and ^bDepartment of Clinical and Experimental Pathomorphology, Jagiellonian University School of Medicine, Kraków, Poland

The objective of this study was to assess the biochemical and histological signs of pancreatic damage development and pancreatic recovery in the course of ischemia-reperfusion induced pancreatitis. Acute pancreatitis was induced in rats by limitation of pancreatic blood flow (PBF) in inferior splenic artery for 30 min using microvascular clips, followed by reperfusion. Rats were sacrificed at the time: 1 h, 12 h, 24 h, and 2, 3, 5, 7, 10, 14, 21 and 28 days after ischemia. PBF was measured using laser Doppler flowmeter. Plasma amylase, interleukin 1β (IL-1β) and interleukin 10 (IL-10) concentration, pancreatic DNA synthesis, as well as, morphological features of pancreatic damage were examined. Ischemia with reperfusion caused acute necrotizing pancreatitis followed by pancreatic regeneration. After removal of microvascular clips, PBF was reduced and the maximal fall of PBF was observed 24 h after ischemia, then PBF grew reaching the control value at 28th day. Plasma amylase activity was increased between 12th h and 3rd day with maximum at 24 h after ischemia. Also plasma IL-1β and IL-10 were elevated with maximal value at the first and second day after ischemia, respectively. DNA synthesis was maximally reduced at the first day (by 70%) and from second day the reversion of this tendency was observed with full restoration of pancreatic DNA synthesis within four weeks. Morphological features of pancreatic tissue showed necrosis, strongly pronounced edema and leukocyte infiltration. Maximal intensity of morphological signs of pancreatic damage was observed between first and second day of reperfusion. During pancreatic regeneration between second and tenth day after ischemia the temporary appearance of chronic pancreatitis-like features such as fibrosis, acinar cell loss, formation of tubular complexes and dilatation of ducts was observed. The regeneration was completed within four weeks after pancreatitis development. We conclude that partial and temporary pancreatic ischemia followed by reperfusion causes acute necrotizing pancreatitis with subsequent regeneration within four weeks. Pancreatic repair after necrotizing pancreatitis is connected with the increase in plasma IL-10 concentration and transitory formation of tubular complexes.

Key words: acute pancreatitis, pancreatic blood flow, tubular complexes, interleukin-1β, interleukin-10.

INTRODUCTION

Acute pancreatitis is a pathological process dependent on autodigestion caused by premature activation of zymogens to active enzymes, but there is
increasing evidence that pancreatic ischemia plays a important role in the initiation of pancreatitis, or the progression to necrotizing pancreatitis (1). Necrosis of pancreatic and peripancreatic tissue is recognized as a key factor in the evolution of the disease from mild to severe. Microvascular perfusion failure is essential for the development of clinical pancreatitis after cardiac (2, 3) or aortic (4, 5) surgery, hypovolemic shock (6), hypothermia (7) and transplantation of the pancreas (8). Also, the diffuse damage of pancreatic vascular network leading to disturbances of an adequate blood supply, may induce acute pancreatitis. Clinically, this situation is observed in generalized atheromatous disease (4), amphetamine-induced vasculitis (9), malignant hypertension (10), periarteriitis nodosa (10, 11) and systemic lupus erythematosus (12). Experimental studies show that ischemia alone may initiate pancreatitis and always aggravates pancreatic damage (1, 13, 14), whereas vasodilatation and improvement of pancreatic blood flow reduces development of acute pancreatitis (15, 16). In acute pancreatitis caused by other, non-vascular factors, the early disturbance of pancreatic circulation is observed (17, 18, 19). It is well known that reduction in pancreatic microcirculation leads to formation of thrombi in capillaries, activation of leukocytes, release of proteolytic enzymes, formation of oxygen-derived free radicals and proinflammatory cytokines (1). The latter two groups of factors act locally and passing into circulation, may cause multiple organ failure and death (20).

Main experimental models of vascular-induced pancreatitis include (14, 21): (a) intravascular injection of microspheres (22, 23); (b) occlusion of pancreatic capillaries and venules by hyaline or thrombi formed in the evolution of the Arthus reaction (24); (c) ligation of pancreatic ducts and occlusion of gastrointestinal artery combined with administration of secretin (25); (d) thrombosis in pancreatic veins combined with ligation of pancreatic ducts (26); (e) pancreatic hypoperfusion evoked by hypovolemic shock (27); (f) clamping of pancreatic arteries followed by reperfusion (28).

In the present study we induced acute, hemorrhagic pancreatitis by temporary clamping of inferior splenic artery followed by reperfusion. The objective of this study was to determine the course of pancreatic regeneration after ischemia-reperfusion-induced pancreatitis.

**MATERIALS AND METHODS**

*Animals and treatment*

Studies were performed on 112 male Wistar rats weighing 180—200 g and were conducted following the experimental protocol approved by the Committee for Research and Animal Ethics of Jagiellonian University.
Animals were housed in cages with wire mesh bottoms, with normal room temperature and a 12-hour light-dark cycle. After fasting for 24 h, but free access to water, rats were anesthetized with ketamine (50 mg/kg intraperitoneally, Bioketan, Biowet, Gorzów, Poland). After longitudinal laparotomy, ischemia in the splenic region of the pancreas was induced by clamping of inferior splenic artery for 30 min using microvascular clips. In sham operated-control animals longitudinal laparotomy and mobilization of pancreas without clamping any arteries was performed. After 30 min ischemia microvascular clips were removed for reperfusion and the abdominal cavity was closed. Animals were anesthetized again immediately before being sacrificed at the time 1 h, 12 h, 24 h, and 2, 3, 5, 7, 10, 14, 21 and 28 days after release of the microvascular clamp (n = 8—10 rats at the each time of observation).

**Determination of pancreatic blood flow**

At the time of experiment cessation animals were anesthetized with ketamine and the abdominal cavity was opened. The pancreas was exposed for the measurement of the blood flow in the pancreatic tissue by laser Doppler flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Järfälla, Sweden), as described previously (29). The pancreatic blood flow was presented as percent change from control value obtained in sham-operated rats.

**Determination of plasma amylase activity and IL-β and IL-10 concentration**

Immediately after measurement of pancreatic blood flow the abdominal aorta was exposed and blood was taken for plasma amylase, IL-1β and IL-10 determination. Plasma amylase activity was determined by an enzymatic method [Amylase reagent set (kinetic), Alpha Diagnostic sp. z o.o., Warszawa, Poland]. The values were expressed as units/liter. Plasma IL-1β and IL-10 were measured in duplicate using the BioSource Cytoscreen rat IL-1β and IL-10 kit based on a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) (BioSource International, Camarillo, California, USA). Concentration was expressed as pg/ml.

**Determination of pancreatic DNA synthesis**

After blood withdrawal the pancreas was carefully dissected from its attachment to the stomach, the duodenum and the spleen. Fat and peripancreatic tissue were trimmed away. The pancreas was rinsed with saline, blotted on paper and weighed. The rate of DNA synthesis in the portion of minced pancreatic tissue was determined by incubating the tissue at 37° for 45 min in 2 ml of medium containing 8 μCi /ml of [3H]thymidine ([6-3H]-thymidine, 20—30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic). The reaction was stopped with 0.4 M perchloric acid containing carrier thymidine (5 mM). Tissue samples were centrifuged and the precipitate washed twice in cold 0.2 M perchloric acid and recentrifuged. RNA was hydrolyzed in 0.3 M KOH incubated for 90 min at 37°C. DNA and protein were reprecipitated with 10% perchloric acid. After standing for 10 min on ice, the tubes were centrifuged and the supernatant was discarded. DNA in the residual pellets was solubilized in 10% perchloric acid by heating at 70°C for 20 min. Denatured protein was removed by centrifugation for 20 min. Using calf thymus as a standard, the DNA concentration was determined by Giles
and Myers procedure (30). The incorporation of $[^3]$Hthymidine into DNA was determined by counting 0.5 ml DNA-containing supernatant in a liquid scintillation system. DNA synthesis was expressed as $[^3]$Hthymidine disintegrations per minute per microgram DNA (dpm/μg DNA).

**Histological examination**

Samples of pancreatic tissue were excised, fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin. The slides were examined histologically by two experienced pathologists without knowledge of treatment given. The histological grading of edema was made using own scale ranging from 0 to 3; 0 = no edema, 1 = interlobular edema, 2 = interlobular and moderate intralobular edema, and 3 = severe interlobular and intralobular edema. Leukocytic infiltration was graded from 0 (absent) to 3 for maximal alterations (diffuse infiltration in the entire pancreatic gland). Grading of vacuolization was based on the percentage of cells involved: 0 = absent, 1 = less than 1 = 5%, 2 = 25—50% and 3 = more than 50%. Findings of acinar necrosis were graded: 0 = absent, 1 = less than 15% of cells involved, 2 = form 15 to 35% of cells involved, 3 = more than 35% of cells involved. Grading of hemorrhages: 0 = absent, 1 = from 1 to 2 foci per slide, 2 = from 3 to 5 foci per slide, 3 = more than 5 foci per slide. Morphological features typical for chronic pancreatitis such as fibrosis, acinar cell loss, formation of tubular complexes and dilatation of ducts, were graded: 0 = absent, 1 = focal changes, 2 = prominent changes involving less than half of the slide, 3 = prominent changes involving more than half of picture.

**Statistical analysis**

The differences between mean values from various groups of experiments were compared by variance analysis and Student's t-test for unpaired data. A difference with a P value of less than 0.05 was considered statistically significant. Results are expressed as means ± S.E.M.

**RESULTS**

The pancreas of sham-operated animals showed macroscopically no tissue alteration and at light microscopic level minimal edema and no inflammation (*Table 1*). In contrast, pancreatic ischemia followed by reperfusion produced acute necrotizing pancreatitis in all tested rats. One hour after ischemia, severe inter- and intralobular edema was accompanied with 1 to 5 foci of hemorrhages per slide. Inflammatory leukocyte infiltration was scarce, predominantly perivascular. Necrosis of acinar cells was observed in all cases but less than 15% of cells was involved. Vacuolization was observed in less than 15% of acinar cells. Pancreatic ducts were unchanged. During the observation period, maximal intensity of edema (severe inter- and intralobular) was found between 1 and 24 h after ischemia, then tended to regress throughout the rest of experiment time reaching the control value 21 days after ischemia.
**Table 1. Morphological features of pancreatic damage in ischemia-reperfusion induced pancreatitis**

<table>
<thead>
<tr>
<th>Time after cessation of ischemia</th>
<th>Edema</th>
<th>Hemorrhages</th>
<th>Inflammatory infiltration</th>
<th>Necrosis</th>
<th>Vacuolization</th>
<th>Pseudo-chronic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shamoperated (control)</td>
<td>0/1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>1 h</td>
<td>3</td>
<td>1/2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
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<td>12 h</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1/2</td>
<td>1</td>
<td>0</td>
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<tr>
<td>24 h</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1/2</td>
<td>1/2</td>
<td>0</td>
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<tr>
<td>5 days</td>
<td>1</td>
<td>0/1</td>
<td>2</td>
<td>0/1</td>
<td>0/1</td>
<td>1/2</td>
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<tr>
<td>7 days</td>
<td>1</td>
<td>0/1</td>
<td>2</td>
<td>0</td>
<td>0/1</td>
<td>2</td>
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<tr>
<td>10 days</td>
<td>1</td>
<td>0/1</td>
<td>2</td>
<td>0</td>
<td>0/1</td>
<td>0/1</td>
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<tr>
<td>14 days</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0/1</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>28 days</td>
<td>0/1</td>
<td>0</td>
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</table>

Numbers represent the predominant histological grading in each group.

The initial number of hemorrhagic foci observed 1 h after reperfusion was followed by an increase in this parameter reaching the maximal number of hemorrhages 24 h after ischemia (more than 5 foci per slide). From the second day after ischemia number of hemorrhages was reduced and starting from the fourteenth day after ischemia, no hemorrhages were observed (Table 1). Maximal, abundant and diffuse inflammatory leukocyte infiltration was present between 12 h and third day after ischemia. From fifth day leukocyte infiltration was diminished and 28 days after ischemia, in the most cases lack of inflammatory infiltration was found.

Acinar necrosis reached maximal grade between 12th h and 2nd day (Table 1). At this time, necrosis in half of cases was observed in less than 15% of cells, in the remaining cases, necrosis was found in 15 to 35% of acinar cells. From 3rd day after ischemia acinar necrosis was reduced and at 5th day necrosis of acinar cells was observed for the last time. 12 h after ischemia the percentage of cells with vacuolization reached the same value as after one hour of reperfusion (Table 1). After 24 h from ischemia vacuolization was present in the maximal number of acinar cells (from less that 25% to 50% of cells) and then percentage of acinar cell with vacuolization was reduced. After 21 days from induction of ischemia no vacuolization was found.
From second day after ischemia induced pancreatitis, some features typical for chronic pancreatitis were observed. We found the acinar cell loss, proliferation of fibroblasts, dilatation of ducts and formation of tubular complexes (Fig. 1 and Fig. 2). Concentrically arranged fibrosis was seen around the ducts and between tubular structures. The tubular complexes appeared as cylindrical tubes, sometimes connected, with wide empty lumen. This lumen was bordered by a monolayer epithelium of flattened duct-like cells. The maximal intensity of pseudo-chronic lesions was found at the seventh day after ischemia and then tended to regress (Table I). Fourteen days after ischemia, tubular complexes and fibrosis disappeared.

![Image](image_url)

*Fig. 1.* Histological findings on third day after induction of ischemia. Only some cells have kept acinar features. Most cells have ductal features with formation of tubular complexes. HE 300×.

1 h after removal of microvascular clips the pancreatic blood flow was reduced by 59% when compared to control (Fig. 3). Maximal fall of pancreatic blood flow was observed 24 h after ischemia; then pancreatic blood flow grew reaching the control value at 28th day.

Ischemia caused the cumulative decrease in pancreatic DNA synthesis reaching the lowest value 24 h after reperfusion (Fig. 4). From fifth day after ischemia the pancreatic DNA synthesis was partially restored and 28 days after ischemia, DNA synthesis reached control group value.
Fig. 2. Acinar cell loss, proliferation of fibroblasts with fibrosis, inflammatory infiltration, dilatation of ducts and formation of tubular complexes seen on fifth day after induction of ischemia. HE 160 x.

Fig. 3. Pancreatic blood flow during reperfusion after pancreatic damage induced by ischemia. Mean ± S.E.M. of 8–10 observations. aP < 0.05 compared with control.
Fig. 4. Pancreatic DNA synthesis during reperfusion after pancreatic damage induced by ischemia. Mean ± S.E.M. of 8–10 observations. *P < 0.05 compared with control.

Fig. 5. Plasma amylase activity during reperfusion after pancreatic damage induced by ischemia. Mean ± S.E.M. of 8–10 observations. *P < 0.05 compared with control.
One hour after removal of vascular clamps, the plasma amylase activity remained unchanged when compared to control (Fig. 5). Significant increase of plasma amylase was observed from 12 h to third day from pancreatitis development with maximal value increase at 24 h after reperfusion. Plasma amylase activity reached the same value as in control group from fifth day after ischemia.

Plasma IL-1β concentration was increased starting from first hour after ischemia (Fig. 6) with maximal increase at 24 h of reperfusion. Plasma IL-1β concentration tended to decrease from second day but remained elevated above control value till 28th day.

![Plasma interleukin-1β concentration](image)

Fig. 6. Plasma interleukin-1β concentration during reperfusion after pancreatic damage induced by ischemia. Mean ± S.E.M. of 8—10 observations. *P < 0.05 compared with control.

Significant increase in plasma IL-10 was observed between 24 h and tenth day of reperfusion with maximal value at second day (Fig. 7). From fourteen day after ischemia, the plasma level of IL-10 returned to control.
The present study confirms and extends previous findings (28, 31) that the pancreas is an organ highly susceptible to ischemic damage and ischemia followed by reperfusion causes acute pancreatitis. Typically, as in models used by Hoffmann et al. (28) or Menger et al. (31), the ischemia of pancreatic tissue is induced by clamping of all arteries supplying the pancreas. They have clamped gastroduodenal, splenic and gastric left arteries, as well as, gastric short arteries (31). This procedure causes total ischemia and does not resemble any clinical circumstance related to pancreatitis (32). In our present study we prepared an experimental model of pancreatitis in which pancreatic damage was evoked by partial reduction of blood flow in the inferior splenic artery, followed by reperfusion. This procedure caused pancreatic hypoperfusion (reduction of pancreatic blood flow in the splenic region of pancreas by 80%) but not total ischemia what more accurately resembles clinical condition. Our study provided evidence that partial ischemia is also a sufficient factor for development of acute hemorrhagic and necrotizing pancreatitis.

In the present study we observed aggravation of pancreatic damage during first 24 h of reperfusion. This finding is in agreement with several lines of
evidences from experimental and clinical studies that reperfusion can paradoxically injure ischemic tissue (33). Reperfusion injury has been documented in the stomach, (34) intestine (35), cardiac (36) and skeletal muscle (37) or lungs (38). The tissue damage evoked by reperfusion is produced by reactive oxygen species, activation of leukocytes and formation of pro-inflammatory cytokines (33, 39). In our present study reperfusion resulted in a leukocyte infiltration accompanied by an increase in plasma IL-1β concentration. In acute pancreatitis, leukocytes intensively adhere to the endothelium of veins forming plagues and contribute to injury by reducing blood flow via occlusion of microvessels (40), as well as by releasing mediators of tissue damage (41). Norman et al. (42) have shown that activation of leukocytes is associated with rapid production of cytokines such as IL-1, IL-6 and TNF-α within the pancreas and systematically. The pancreatic tissue level of IL-1 and IL-6 rose faster and achieved higher value than serum level indicating that the pancreas itself is a major source of cytokine cascade induction in pancreatitis (42). IL-1 is a well known mediator in the disease and in the production of systemic acute phase responses (43). IL-1 stimulates own gene expression and synthesis, and plays a crucial role in induction of the release of other members of the pro-inflammatory cytokine cascade (43). The study performed by Noman et al. (44) has shown that blockade of IL-1 by use of naturally occurring receptor antagonist, almost completely attenuates the rise in serum IL-6 and TNF-α level and decreases severity of experimental acute pancreatitis.

In contrast to IL-1β, IL-10 has been found to be a major anti-inflammatory cytokine. It inhibits the production of pro-inflammatory cytokines (45) and reactive oxygen species (46) by macrophages and reduces activation of macrophages (46). The study performed by Van Laethem et al. (47) has shown that administration of IL-10 before and during induction of acute pancreatitis decreases the severity of pancreatitis, mainly by inhibiting the development of acinar cell necrosis. Van Laethem et al. have suggested that this effect, at least in part, is related to a decrease in tumor necrosis factor secretion (47). In our present study, IL-10 plasma concentration rose after development of acute pancreatitis reaching maximal value at the second day of observation, whereas maximal concentration of IL-1β has was observed one day earlier. This shift in the time between maximal IL-1β and IL-10 plasma concentration suggests that an increase in IL-10 is a result of an increase in IL-1β concentration, and IL-10 plays an important role in self-limitation of acute pancreatitis. The additional argument for this hypothesis is the fact that the maximal concentration of IL-10 corresponds with the beginning of pancreatic regeneration.

After the acute phase of pancreatitis, from the second day of reperfusion, we have observed reduction of pancreatic damage and initiation of pancreatic repair. It was expressed by the decrease in plasma amylase and IL-1β, as well as, the increase in pancreatic blood flow and DNA synthesis. Histological
examination also has shown the reduction in pancreatic damage. In acute edematous pancreatitis evoked by caerulein, the total structural and functional recovery is achieved nine to twelve days after induction of pancreatitis (48—50). In contrast to caerulein, ischemia-reperfusion causes hemorrhagic and necrotizing pancreatitis, and for this reason pancreatic regeneration after ischemia-reperfusion induced pancreatitis needs at least four weeks.

During pancreatic recovery between second and tenth day of reperfusion, we observed the chronic pancreatitis-like alterations such as fibrosis, necrosis of acinar cells and appearance of tubular complexes. Fourteen days after ischemia pancreatitis-like alterations were absent. These chronic pancreatitis-like alterations were also described by others in the course of experimental (51—54) and human (55) acute pancreatitis during the postacute period and disappeared in relatively short time. Regeneration involves acinar cells and tubular complexes, as evidenced by mitotic figures observed in these structures (52). Cells of tubular complexes are supposed to originate from acinar cells by dedifferentiation and structures very similar to tubular complexes were observed in embryonic pancreas, which suggest that the cells forming tubular complexes may have recovered pluripotency, what is essential in pancreatic development and repair (52). This concept is supported by findings that acinar (52), ductular (56) cells, as well as, endocrine islets cells (57, 58) can derive from cells of tubular complexes.

In summary, the present study demonstrated that temporary partial pancreatic ischemia followed by reperfusion is sufficient to induce acute hemorrhagic and necrotizing pancreatitis. Pancreatic damage is followed by subsequent pancreatic repair with transitory forming of tubular complexes. Pancreatic regeneration is completed within four weeks after ischemia.

REFERENCES


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Author’s address: Artur Dembinski, Department of Physiology, Jagiellonian University School of Medicine, ul. Grzegórzecka 16, 31—531 Kraków, Poland phone +48-12-421-10-06, fax +48-12-421-15-78, e-mail mpdembin@cyf-kr.edu.pl