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PROTECTIVE AND THERAPEUTIC EFFECT OF HEPARIN IN ACUTE PANCREATITIS

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The initiation and progression of acute pancreatitis is associated with disturbances in pancreatic microcirculatory. Microcirculatory disorders contribute to multiorgan dysfunction syndrome in the course of acute pancreatitis. The aim of this study was to determine the influence of heparin administration on the development and the course of ischemia/reperfusion-induced pancreatitis. Methods: Acute pancreatitis was induced in rats by pancreatic ischemia followed by reperfusion. In the first series of studies, heparin was administered 0.5 h before induction of acute pancreatitis and the severity of acute pancreatitis was assessed after 6-h reperfusion. In the second series of studies, heparin was administered twice a day, starting 24 h after the initiation of reperfusion. In both series of studies, heparin was administered subcutaneously at the dose of 150 U/kg. Results: Treatment with heparin, before induction of pancreatitis, inhibits the development of morphological signs of acute pancreatitis and reduced the pancreatitis-evoked increase in plasma level of pancreatic enzymes and pro-inflammatory interleukin-1β. These effects have been accompanied with improvement of pancreatic blood flow, pancreatic DNA synthesis and reduction in plasma concentration of D-dimer. Administration of heparin after induction of acute pancreatitis accelerates normalization of pancreatic histology, and reduces biochemical markers of the severity of acute pancreatitis. These effects have been accompanied with the improvement of pancreatic circulation, increase in APTT and reduction in plasma D-dimer level. Conclusions: Treatment with heparin inhibits the development of ischemia/reperfusion-induced pancreatitis and accelerates pancreatic regeneration in the course of this disease.

Key words: *ischemia/reperfusion-induced pancreatitis, pancreatic damage, interleukin-1β, plasma lipase activity, activated partial thromboplastin time*

INTRODUCTION

Coagulative disorders are known to occur in acute pancreatitis and are related to the severity of this disease. (1, 2). Acute pancreatitis activates the hemostatic system with formation of thrombi within blood vessels and coagulative disorders may range from scattered intravascular thrombosis to severe disseminated intravascular coagulation (DIC) (3). Inflammation and coagulation are closely linked processes (4) and inflammatory cytokines activate coagulation by increasing expression of tissue factor on monocytes and endothelium, leading to thrombin formation (5).

Heparin prevents coagulation after binding with a plasma α_2-globulin, antithrombin III. Antithrombin III is a protease inhibitor and complex heparin-antithrombin III inhibits activity of thrombin, as well as neutralizes active forms of factors IX, X, XI and XII (6). However, the most important action of heparin is not inhibition of thrombin activity, but inhibition of thrombin creation, especially through accelerating the neutralization of factor Xa. In large concentration, heparin combined with antithrombin III also inhibits platelet aggregation (6).

Beside anticoagulative properties related to direct or indirect inhibition of protease involved in cascade of coagulation, heparin inhibits also other proteases present in plasma and tissues, including pancreatic enzymes. Heparin alone or in complex with antithrombin III or heparin cofactor II reduces activity of trypsin (7, 8), chymotrypsin (9) and inhibits conversion of trypsinogen to trypsin (10, 11). On the other hand, premature activation of pancreatic enzymes within the pancreas plays a crucial role in the development of acute pancreatitis and treatment with protease inhibitors may reduce the mortality in patients with moderate and severe acute pancreatitis (12). These findings suggest that heparin, reducing activity of pancreatic enzymes, may prevent the development of acute pancreatitis.

In various experimental and clinical studies, heparin has been found to exhibit the anti-inflammatory activity (13). Heparin has shown protective and therapeutic effect in patients suffering from a range of inflammatory diseases, including rheumatoid arthritis (14), allergy (15) and ulcerative colitis (16). Heparin reduces the process of leukocyte recruitment into site of injury or presence of pro-inflammatory cytokines. Salas *et al.* (17) have reported that heparin down-regulates tumor necrosis factor-α (TNF-α)-induced leukocyte rolling, adhesion and migration into gut tissues without affecting vascular permeability. These data are in agreement with studies showing that heparin decreases leukocyte adhesion to vascular endothelial cells (18) and recruitment of leukocyte into tissues during inflammation (19). Moreover, heparin reduces pro-inflammatory effect of leukocyte stimulation. Heparin inhibits activity and release of leukocyte enzymes, especially elastase (20, 21). Also, in some condition, heparin inhibits activity of neutrophil-derived cathepsin G (22) and catepsin G-induced activation of platelets (23).

There are experimental and clinical studies showing beneficial effect of heparin administration in acute pancreatitis. Experimental studies have been performed using two models of acute pancreatitis, edematous acute pancreatitis induced by cerulein and severe lethal taurocholate or bile-induced acute pancreatitis. In cerulein-induced acute pancreatitis in rats, Dobosz *et al.* have shown that pretreatment with heparin inhibits the development of pancreatic damage (24), reduces the pancreatitis-induced increase in serum level of pro-inflammatory interleukin-6 and improves blood flow through visceral organs and skeletal muscle (25). Unfortunately, they did not examine the influence of heparin on established markers of acute pancreatitis severity such as plasma level of lipase or amylase. Other problem is a short time of observation. They have terminated experiment 5 h after the first injection of cerulein. For this reason their observation are limited to initial period of acute pancreatitis.

In bile-induced severe acute pancreatitis in dogs, Gabryelewicz *et al.* have found that pretreatment with heparin reduces pancreatic damage and serum amylase activity, as well as increases survival rate of animals (26). Similar effects have been observed by Qiu *et al.* (27). They have shown that low molecular weight heparin, administered 4 h after application of taurocholate, reduces serum level of amylase, TNF-α and endothelin-1, improves pancreatic morphology and pancreatic circulation, and increases survival rate of rats with taurocholate-induced pancreatitis. However, in both studies, examination of the severity of acute pancreatitis was performed 24 h after induction of acute pancreatitis, showing effect of heparin administration only on initial phase of this disease.

Clinical data concerning the influence of heparin administration on acute pancreatitis are limited to two groups of studies. The first group of studies has reported the effect of pretreatment with heparin on the development of acute pancreatitis after endoscopic retrograde cholangiopancreatography (ERCP). Reports are not conclusive, because some of them have indicate that pretreatment with heparin reduces frequency of post-ERCP pancreatitis (28), whereas other studies have not shown any protective effect of low dose of unfractionated heparin (29) or low molecular weight heparin (30) against ERCP-induced pancreatitis. However, it must be pointed out that both studies with negative results contain a small number of patients and heparin has been used in low doses. A multicenter trail with a large number of patients and appropriate higher doses of heparin are needed to reach conclusive data.

The second group of clinical studies has been performed to determine the influence of heparin on the course of hyperlipidemia-induced pancreatitis. Heparin exhibits an anti-lipidemic action due to liberation of a lipoprotein lipase from tissues into blood (31), and has been used in the treatment of hyperlipidemia. Numerous clinical studies have shown therapeutic effect of heparin given together with insulin in hyperlipidemic acute pancreatitis (32-34) and this procedure is recognized as a standard treatment in this disease.

Above mentioned data suggest that heparin may be useful in the treatment of acute pancreatitis. The present study was designed to determine the influence of unfractionated heparin on the development and course of acute ischemia/reperfusion-induced pancreatitis.

MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 180-200g 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. Experiments were carried out in three separate series.

The first series of studies were performed to evaluate the influence of unfractionated heparin (Heparinum, Polfa, Warszawa, Poland) on activated partial thromboplastin time (APTT). Heparin was given subcutaneously at the dose of 25, 50, 100, 150 or 200 U/kg in intact rats. Heparin at the dose of 150 U/kg caused two-fold increase in APTT. A fixed therapeutic range for APTT of 1.5 to 2.5 times the control value is widely accepted (35). For this reason, heparin at the dose of 150 U/kg was used in next series of studies.

The second series of studies were performed to assess the influence of pretreatment with heparin on the development of acute pancreatitis. In this part of studies, heparin at the dose of 150 U/kg was administrated 0.5 h before induction of acute pancreatitis and the severity of pancreatitis was assessed after 6-h reperfusion.

The third series of studies was performed to evaluate the influence of heparin administration oh the course of acute pancreatitis. In this part of study, heparin at the dose of 150 U/kg was administered twice a day, starting 24 h after the initiation of reperfusion. The severity of acute pancreatitis was assessed between the 2nd and 21st day of reperfusion.

Before induction of acute pancreatitis, rats were fasted for 24 h with free access to water. Animals were anesthetized with ketamine (50 mg/kg i.p., Bioketan, Vetoquinol Biowet, Gorzów Wielkopolski, Poland) and acute pancreatitis was induced by severe pancreatic ischemia followed by reperfusion as described previously in detail (36). Briefly, the splenic inferior artery was occluded for 30 min and after them microvascular clips were removed to obtain pancreatic reperfusion. The abdominal cavity for time of reperfusion was closed by suture. In sham-operated control rats, longitudinal laparotomy and mobilization of the celiac artery without clamping was performed. Control rats and rats without administration of heparin were treated subcutaneously with saline at the same time as heparin-treated rats.

Determination of pancreatic blood flow

At the end of studies, rats were anesthetized with ketamine and the abdominal cavity was opened. Pancreases were 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 (37). The pancreatic blood flow was presented as percent change from control value obtained in sham-operated rats.

Biochemical analysis of plasma

After the measurement of pancreatic blood flow, arterial blood was taken from the abdominal aorta, anticoagulated with 3.8% sodium citrate and plasma was collected. APTT was determined in

fresh plasma, using Plastelin LS (Organon Teknika Corporation, Dirham, NC, USA). Plasma D-Dimer concentration was determined using a latex-enhanced immunoturbidimetric assay (D-dimer test, Roche Diagnostics). Plasma lipase and amylase activity was determined with a Kodak Ectachem DT II System analyzer (Eastman Kodak Company, Rochester, NY, USA) using Lipa and Amyl DT Slides (Vitros DT Chemistry System, Johnson & Johnson Clinical Diagnostic, Inc., Rochester, NY, USA). Plasma concentration of interleukin-1β (IL-1β) and interleukin-10 (IL-10) were measured using the BioSource Cytoscreen rat IL-1β and IL-10 kits (BioSource International, Camarillo, California, USA) based on ELISA.

Determination of pancreatic DNA synthesis

After the blood withdrawal, the pancreas was carefully dissected out from its attachment to the stomach, duodenum, and spleen. Fat and peripancreatic tissue were trimmed away. Samples of pancreatic tissue were taken for study of DNA synthesis and morphological examination. The rate of DNA synthesis was measured by incubation of minced pancreatic tissue at 37°C for 45 min in 2 ml of medium containing 8 μCi /ml of [^{3}H]thymidine ([6-^{3}H]-thymidine, 20-30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic), as described previously (38, 39). DNA synthesis was expressed as [^{3}H]thymidine disintegrations per minute per microgram DNA (dpm/μg DNA).

Histological examination of pancreatic damage

Morphological examination of pancreatic tissue was performed in hematoxilin and eosin stained slides as describe previously in detail (40). The histological grading of edema was made using a scale raging from 0 to 3 (0 = no edema, 1 = interlobular edema, 2 = interlobular and moderate intralobular edema, and 3 = interlobular edema and severe intralobular edema). Leukocytic infiltration was also graded from 0 to 3 (0 = absent, 1 = scarce perivascular infiltration, 2 = moderate perivascular and scarce diffuse infiltration, 3 = abundant diffuse infiltration). Grading of vacuolization was based on the appropriate percentage of acinar cells involved: 0 = absent, 1 = less than 25%, 2 = 25-50% and 3 = more than 50% of acinar cells. Hemorrhagia was graded: 0 = no hemorrhagia, 1 = 1-2 hemorrhagic foci per slide, 2 = 3-5 hemorrhagic foci per slide, 3 = more than 5 hemorrhagic foci per slide. Necrosis was graded: 0 = no necrosis, 1 = less than 15 % of pancreatic cells involved, 2 = 15-35 % of cells involved, 3 = more than 35 % of cells involved. Results of histological examination have been expressed as a predominant histological grading in each experimental group of animals.

Statistical analysis

Results, except histological data, have been expressed as means ± SEM. In all series of studies, we used eight rats in each experimental group and each time of observation. Statistical analysis was made by analysis of variance followed by Tukey's multiple comparison test. A difference with a p value of less than 0.05 was considered significant.

RESULTS

The first series of studies

Heparin administered subcutaneously in intact rats caused a dose-dependent increase in APTT (*Fig. 1*). Two-fold increase in APPT was reached after heparin at the dose of 150 U; and for this reason this dose was used in the next series of studies.

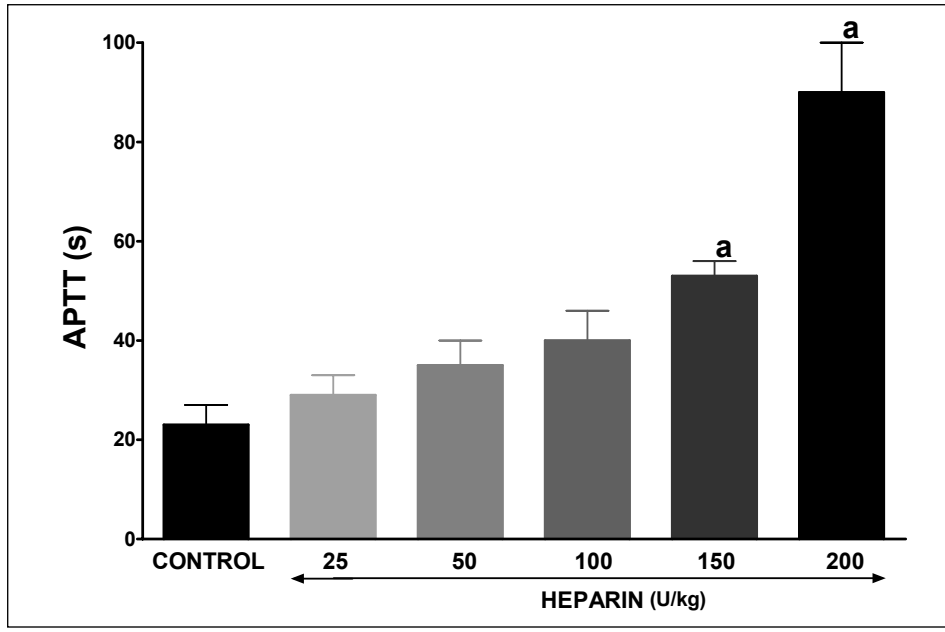

Fig. 1. Activated partial thromboplastin time (APTT) determined in rats 6 h after subcutaneous administration of heparin at the dose of 25, 50, 100, 150 or 200 U/kg. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control.

Table 1. Influence of ischemia/reperfusion (I/R)-induced pancreatitis and heparin applied alone or in combination (HEPARIN+I/R) on morphological signs of pancreatic damage.

	EDEMA (0-3)	INFLAMMATORY INFILTRATION (0-3)	VACUOLIZATION (0-3)	NECROSIS (0-3)	HEMORRHAGES (0-3)
CONTROL	0	0	0	0	0
I/R	2	2	0	1-2	1-2
HEPARIN	0	0	0	0	0
HEPARIN + I/R	1-2	1-2	0	1	1

Numbers represent the predominant histological grading in each group.

The second series of studies

Pancreatic ischemia followed by 6-h reperfusion induced acute hemorrhagic pancreatitis in all tested rats (*Table 1*). In histological examination, moderate inter- and intralobular edema was accompanied with moderate perivascular and scare diffuse leukocytic infiltration. Necrosis was observed in all cases of acute pancreatitis and involved from less than 15% up to 35% of acinar cells. Hemorrhage was limited to 1-5 foci per slide. Histological findings were

associated with biochemical signs of acute pancreatitis. Ischemia/reperfusion-induced pancreatitis caused 17-fold increase in plasma activity of lipase (*Fig. 2*) and 10-fold increase in plasma activity of amylase (*Fig. 2*). Plasma concentration of pro-inflammatory IL-1β was increased by around 300% when compared to control rats (*Fig. 3*); whereas pancreatic DNA syntheses and pancreatic blood flow were reduced by 57 and 70%, respectively (*Fig. 4*). Moreover, ischemia/reperfusion-induced pancreatitis strongly increased APTT and plasma D-Dimer concentration (*Fig. 5*). Plasma concentration of anti-inflammatory IL-10 was not affected by pancreatic ischemia followed by 6-h reperfusion (*Fig. 3*).

In rats without induction of acute pancreatitis, heparin did not affect pancreatic morphology (*Table 1*) or other parameters, apart from APTT. APTT was increased by around 150% (*Fig. 5*).

In rats with induction of acute pancreatitis, pretreatment with heparin attenuated the development of ischemia/reperfusion-induced pancreatitis, what was demonstrated in histological examination as the reduction in pancreatic edema, inflammatory infiltration, necrosis and hemorrhages (*Table 1*). Also, in these rats, heparin administration decreased plasma activity of lipase and amylase (*Fig. 2*), and lowered plasma concentration of pro-inflammatory IL-1β (*Fig. 3*); whereas the pancreatitis-evoked fall in pancreatic DNA synthesis and pancreatic blood flow was partly reversed (*Fig. 4*). Moreover, pretreatment with heparin partly reversed the pancreatitis-evoked increase in APTT and reduced plasma level of D-Dimer (*Fig. 5*). Plasma concentration of anti-inflammatory

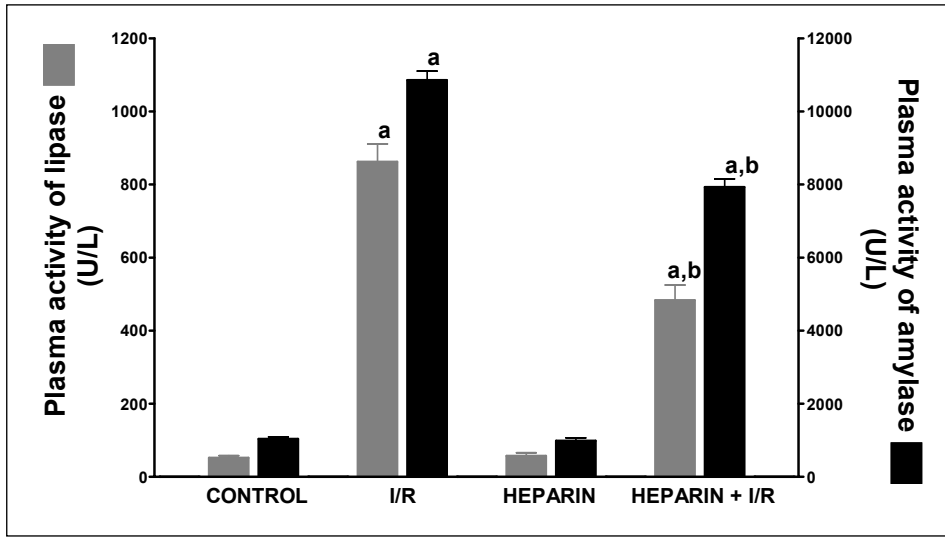

Fig. 2. Influence of ischemia/reperfusion (I/R)-induced pancreatitis and heparin applied alone or in combination (HEPARIN+I/R) on plasma activity of lipase and amylase. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control; [b]P<0.05 compared to I/R alone.

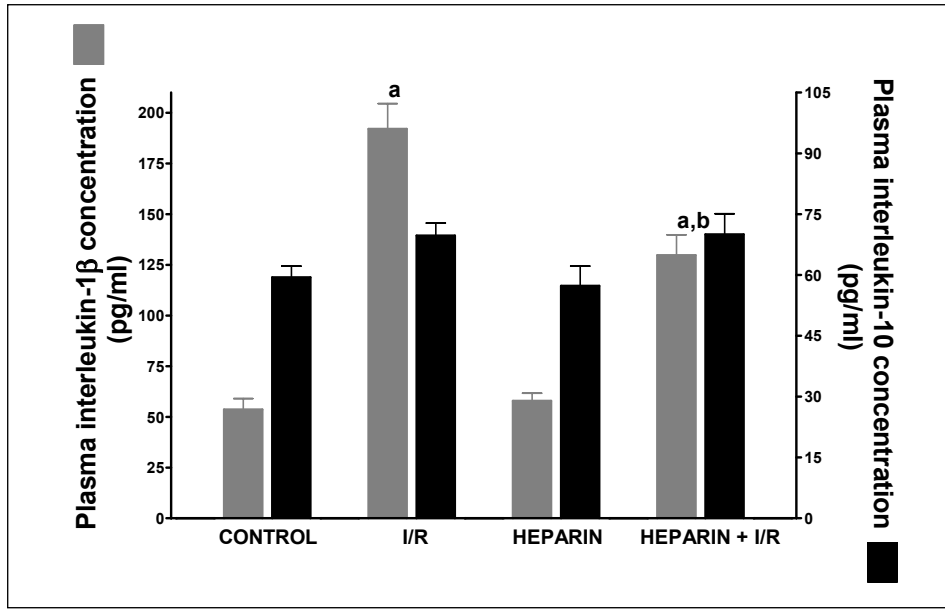

Fig. 3. Influence of ischemia/reperfusion (I/R)-induced pancreatitis and heparin applied alone or in combination (HEPARIN+I/R) on plasma concentration of pro-inflammatory interleukin-1β and anti-inflammatory interleukin-10. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control [b]P<0.05 compared to I/R alone.

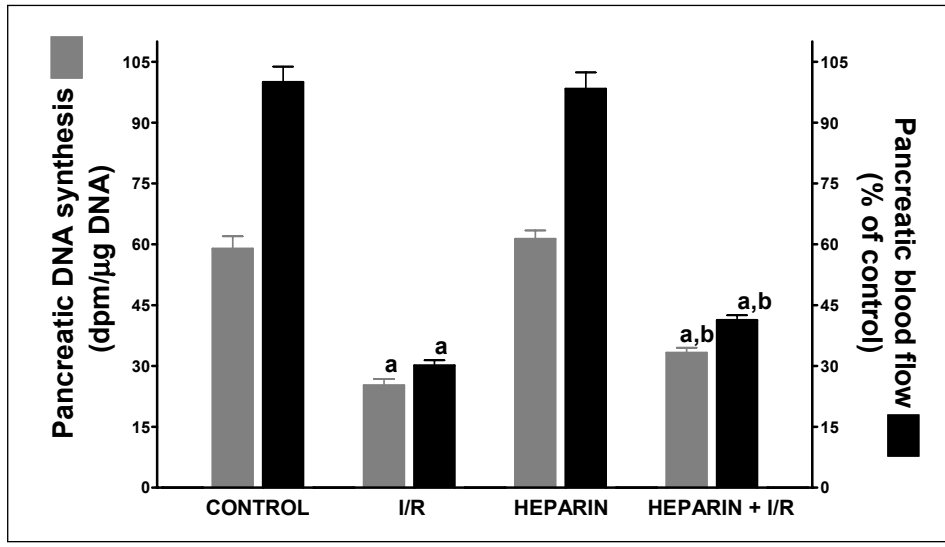

Fig. 4. Influence of ischemia/reperfusion (I/R)-induced pancreatitis and heparin applied alone or in combination (HEPARIN+I/R) on pancreatic DNA synthesis and pancreatic blood flow. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control; [b]P<0.05 compared to I/R alone.

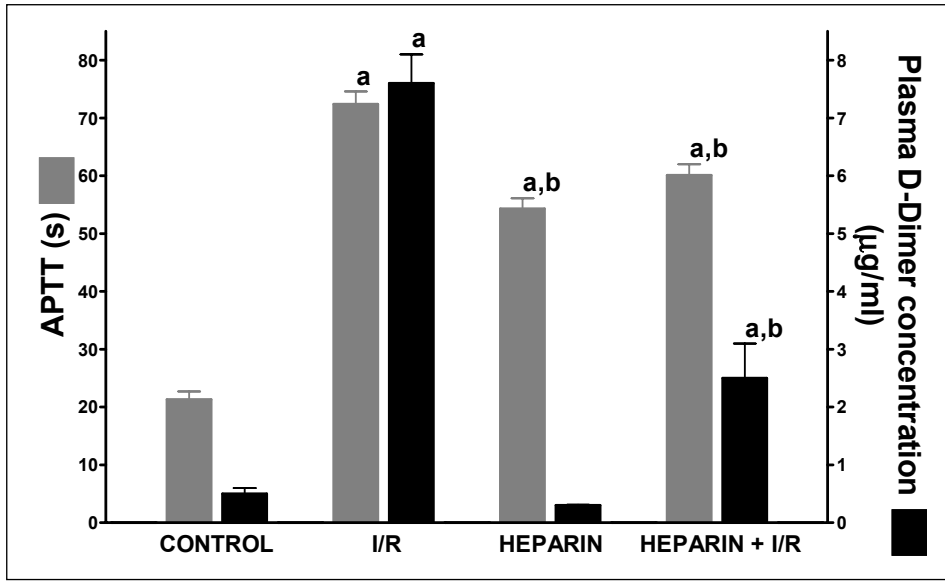

Fig. 5. Influence of ischemia/reperfusion (I/R)-induced pancreatitis and heparin applied alone or in combination (HEPARIN+I/R) on activated partial thromboplastin time (APTT) and plasma concentration of D-Dimer. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control; [b]P<0.05 compared to I/R alone.

IL-10 was not affected by heparin given in rats with induction of acute pancreatitis (*Fig. 3*).

The third series of studies

Exposure to pancreatic severe ischemia followed by reperfusion led to the development of acute hemorrhagic pancreatitis followed by spontaneous pancreatic regeneration. In histological examination, maximal intensity of pancreatic edema was observed between the 6th and 24th h of reperfusion (*Table 2*). Vacuolization reached maximal grade after 24-h reperfusion; whereas leukocytic inflammatory infiltration, necrosis and hemorrhage were maximally expressed between the 1st and 2nd day of reperfusion. From the 5th day of reperfusion, histological signs of pancreatic damage tended to reduce. Twenty one days after induction of acute pancreatitis, the morphological features showed almost normal pancreatic tissue, except slight interlobular edema in some cases (*Table 2*).

Histological findings were well correlated with biochemical signs of acute pancreatitis. After 6-h reperfusion, plasma lipase (*Fig. 6*) and amylase activity (*Fig. 7*), plasma concentration of pro-inflammatory interleukin-1β (*Fig. 8*), APTT (*Fig. 12*), plasma D-Dimer concentration (*Fig. 13*) were increased; whereas pancreatic DNA synthesis (*Fig. 10*) and pancreatic blood flow (*Fig. 11*) were

Table 2. Influence of heparin (H) administered after induction of acute pancreatitis on morphological signs of pancreatic damage in the course of ischemia/reperfusion (I/R)-induced pancreatitis.

	EDEMA (0-3)	INFLAMMATORY INFILTRATION (0-3)	VACUOLIZATION (0-3)	NECROSIS (0-3)	HEMORRHAGES (0-3)
CONTROL	0-1	0	0	0	0
I/R 6 h	3	1-2	1	1	1-2
I/R 1 day	3	3	1-2	1-2	2
I/R 2 days	2	3	1	1-2	2
I/R + HEPARIN 2 days	2	2-3	1	0-1	1-2
I/R 5 days	1-2	2	1	1	0-1
I/R + HEPARIN 5 days	1	1	0-1	0-1	0
I/R 9 days	1	2	0-1	0	0-1
I/R + HEPARIN 9 days	0-1	1	0	0	0
I/R 14 days	1	1	0-1	0	0
I/R + HEPARIN 14 days	0	0	0	0	0
I/R 21 days	0-1	0	0	0	0
I/R + HEPARIN 21 days	0-1	0	0	0	0

Numbers represent the predominant histological grading in each group.

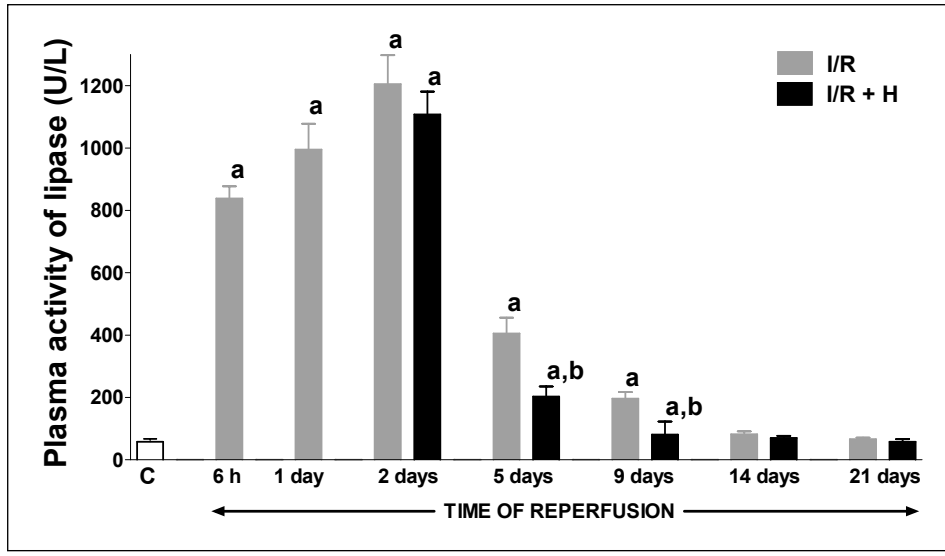

Fig. 6. Influence of heparin (H) administered after induction of acute pancreatitis on plasma activity of lipase in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

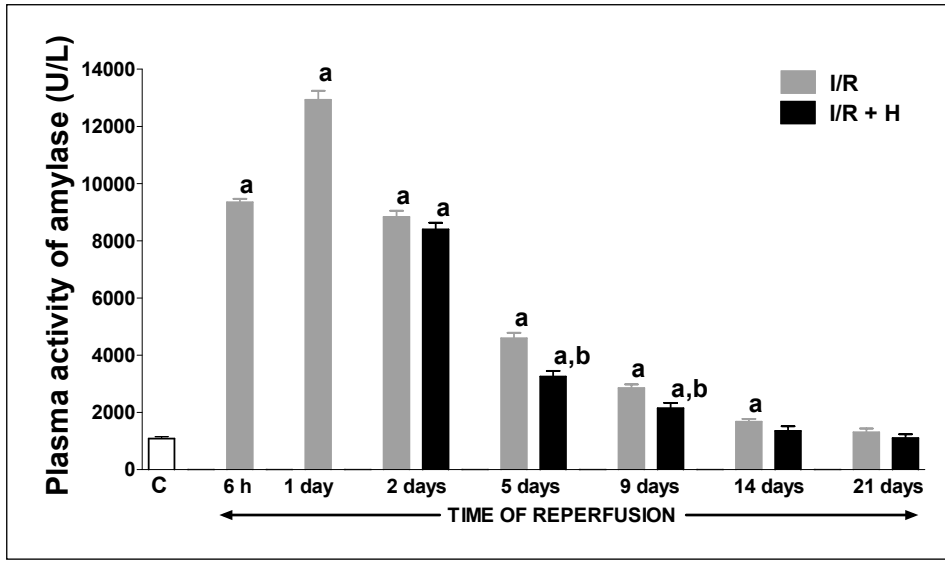

Fig. 7. Influence of heparin (H) administered after induction of acute pancreatitis on plasma activity of amylase in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

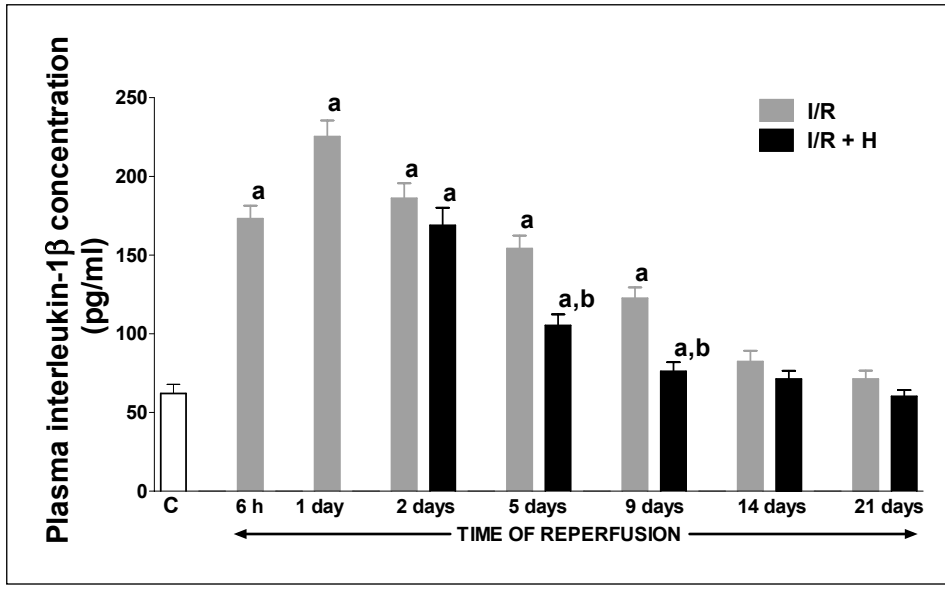

Fig. 8. Influence of heparin (H) administered after induction of acute pancreatitis on plasma concentration of pro-inflammatory interleukin-1β in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

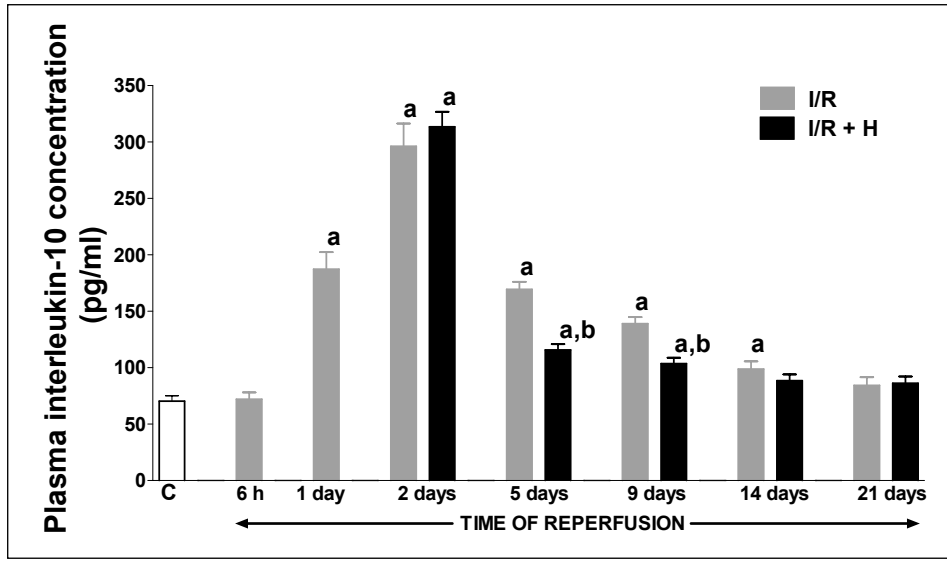

Fig. 9. Influence of heparin (H) administered after induction of acute pancreatitis on plasma concentration of anti-inflammatory interleukin-10 in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

reduced. In contrast to that, plasma concentration of anti-inflammatory IL-10 was not changed after 6-h reperfusion (*Fig. 9*). Maximal increase in plasma lipase activity (*Fig. 6*) was observed after 2-days reperfusion. At the 14th day of reperfusion, plasma lipase activity returned to control value. Plasma activity of amylase was significantly increased between the 6th h and 14th day of reperfusion and reached maximal value at the 1st day of reperfusion (*Fig. 7*). Plasma IL-1β concentration was increased significantly between the 6th h and 9th day of reperfusion (*Fig. 8*), and maximal plasma concentration of IL-1β was observed at the 1st day of reperfusion. Plasma concentration of anti-inflammatory IL-10 grew significantly starting from the 1st day of reperfusion, reaching maximal value at the 2nd day of reperfusion (*Fig. 9*). At the 21st day of reperfusion plasma concentration of IL-10 returned to control value. Pancreatic DNA synthesis (*Fig. 10*) and pancreatic blood flow (*Fig. 11*) were significantly reduced between the 6th h and the 14th day of reperfusion. Maximal decrease in pancreatic DNA synthesis was observed at the second day of reperfusion (*Fig. 10*); whereas pancreatic blood flow was maximally reduced at the 1st day of reperfusion (*Fig. 11*). Exposure to pancreatic severe ischemia followed by reperfusion led to the significant increase in APTT between the 6th h and 5th day of reperfusion (*Fig. 12*), the longest APTT was observed at the 1st day of reperfusion. Plasma concentration of D-Dimer was significantly increased between the 6th h and 14th

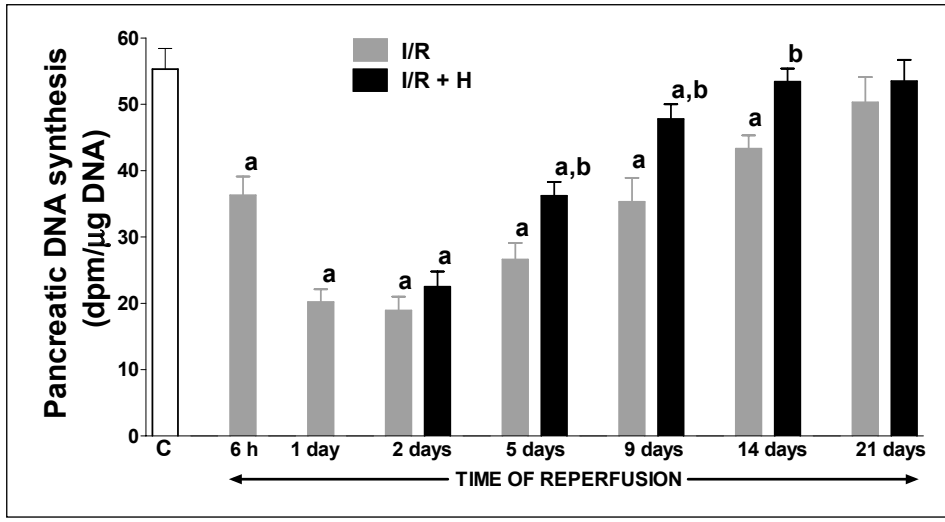

Fig. 10. Influence of heparin (H) administered after induction of acute pancreatitis on pancreatic DNA synthesis in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

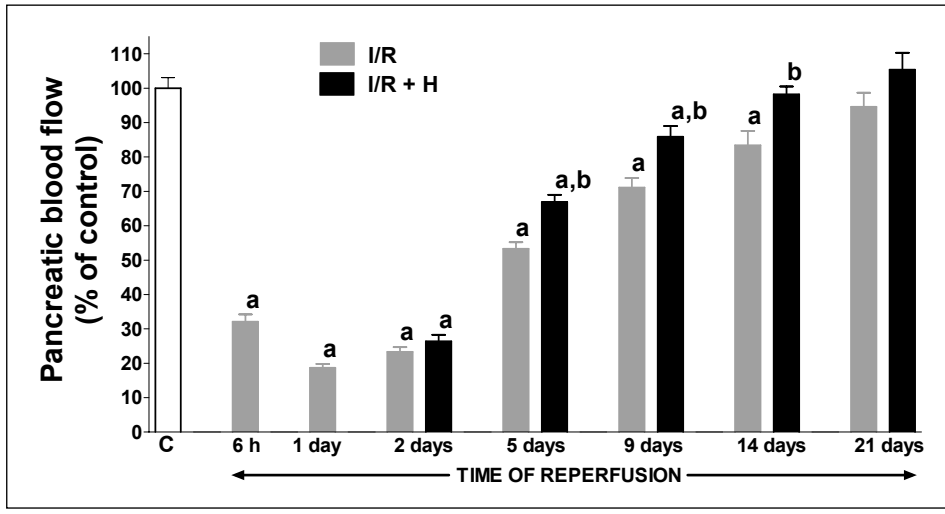

Fig. 11. Influence of heparin (H) administered after induction of acute pancreatitis on pancreatic blood flow in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

day of reperfusion, maximal value of plasma D-Dimer concentration was observed at the 1st day of reperfusion (*Fig. 13*).

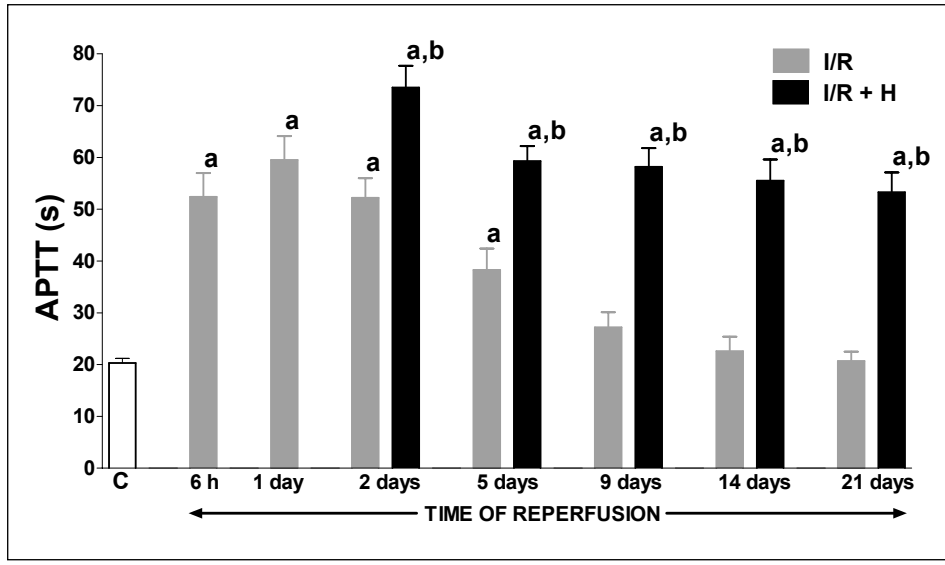

Fig. 12. Influence of heparin (H) administered after induction of acute pancreatitis on activated partial thromboplastin time (APTT) in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

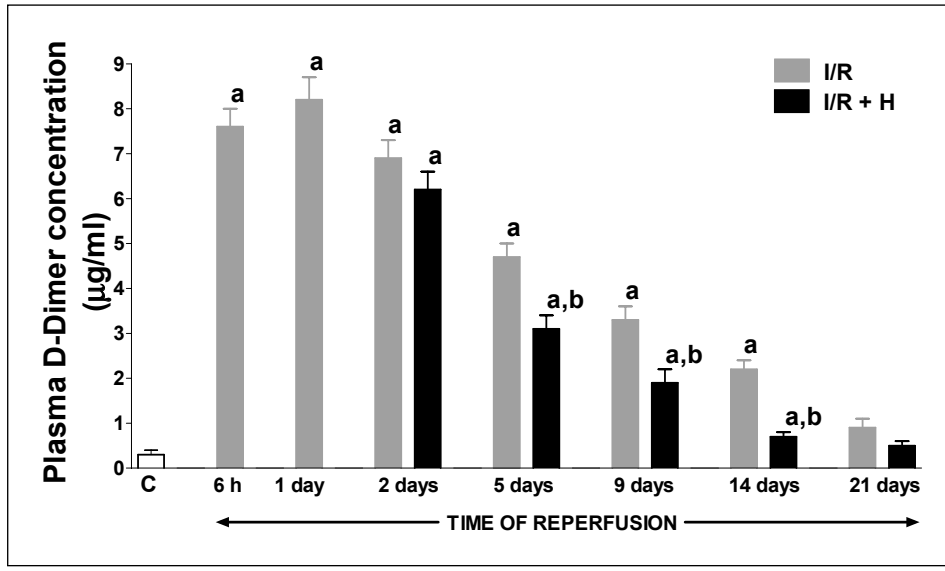

Fig. 13. Influence of heparin (H) administered after induction of acute pancreatitis on plasma concentration of D-Dimer in the course of ischemia/reperfusion (I/R)-induced pancreatitis. Mean ± SEM. N = 8 in each group of rats. [a]P<0.05 compared to control (C); [b]P<0.05 compared to I/R alone at the same time of observation.

Treatment with heparin after induction of acute pancreatitis reduced the severity of this disease and accelerates pancreatic regeneration. In histological examination, the reduction in pancreatic edema, inflammatory infiltration, vacuolization of acinar cells, necrosis and hemorrhages was observed (*Table 2*). Pancreases of animals treated with heparin after induction of acute pancreatitis recovered normal pancreatic morphology at the 14th day of reperfusion; whereas pancreases of animals without treatment with heparin did not reach normal morphology before the 21st day of reperfusion (*Table 2*). Also, treatment with heparin reduced biochemical indexes of the severity of acute pancreatitis. Treatment with heparin significantly reduced the pancreatitis-evoked increase in plasma activity of lipase (*Fig. 6*) and amylase (*Fig. 7*), and plasma concentration of pro-inflammatory IL-1β (*Fig. 8*). The pancreatitis-evoked fall in pancreatic DNA synthesis (*Fig. 10*) and pancreatic blood flow (*Fig. 11*) was partly reversed. Treatment with heparin after induction of acute pancreatitis prolonged APTT (*Fig. 12*) and this effect was associated with a reduction in plasma concentration of D-Dimer (*Fig. 13*). Treatment with heparin in animals with acute pancreatitis reduced plasma level of anti-inflammatory IL-10 (*Fig. 9*) and this effect was statistically significant between the 5th and 9th day of reperfusion.

DISCUSSION

Our present study has brought several important findings concerning the influence of heparin administration on the development and treatment of acute pancreatitis. First of all, we have found that pretreatment with heparin inhibits the development of ischemia/reperfusion-induced acute pancreatitis and reduces the severity of this disease. Protective effect of heparin on the pancreas was manifested by a reduction in histological and biochemical signs of pancreatic damage. Morphological features of pancreatic tissue has shown that pretreatment with heparin reduces pancreatic edema, necrosis, hemorrhage and leukocyte infiltration.

Activation of leukocytes and release of pro-inflammatory cytokines are responsible for local pancreatic damage and development of systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) in the course of acute pancreatitis (41). Norman *et al.* have shown that pro-inflammatory cytokines such as IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) are produced within pancreas and subsequently within distant organs, developing organ dysfunction in severe pancreatitis (42). The severity of acute pancreatitis is well correlated with production of pro-inflammatory cytokine (42) and IL-1β plays the essential role in the release other members of pro-inflammatory cytokine cascade and the induction of systemic acute phase response (43). The role of leukocyte activation and IL-1β release has been additionally evidenced by finding that administration of interleukin-1 receptor

antagonist prevents the rise in serum IL-6 and TNF-α, and decreases severity of experimental acute pancreatitis (44). These data are in agreement with our present observation and can explain, at least in part, the protective effect of heparin on the pancreas in acute ischemia/reperfusion-induced pancreatitis. In our present study, pretreatment with heparin has reduced leukocyte inflammatory infiltration of pancreatic tissue and decreased the pancreatitis-induced increase in plasma IL-1β concentration. This anti-inflammatory effect of heparin is in harmony with the previous observation. Already in 1984 Laghi Pasini *et al.*(45) have shown *in vitro* study that heparin inhibits granulocyte aggregation and degranulation stimulated by chemotactic factor FMLP (N-formyl-methionyl-leucyl-phenylalanine) or by zymosan-activated serum, and reduces the FMLP-dependent superoxide anion generation. Next studies have confirmed anti-inflammatory properties of heparin. Heparin inhibits neutrophil aggregation stimulated by FMLP or PAF (platelet-activating factor), as well as reduces elastase release by these cells (46). Moreover, Hochart *et al.* (47) have found that heparin downregulates the monocyte-mediated immune response. Pretreatment of human monocytes with unfractionated heparin or low-molecular weight heparin significantly attenuates lipopolysaccharide-induced production of pro-inflammatory TNF-α, IL-8, IL-6 and IL-1β, as well as nuclear translocation of the pro-inflammatory nuclear factor κB (NF-κB). Inhibition of NF-κB activation probably represents one of the most important mechanisms by which heparin exerts its anti-inflammatory effects. Also products of enzymatic degradation of heparin exhibit this anti-inflammatory effect (48).

The increase in serum activity of lipase and amylase is a well established index of acute pancreatitis severity with high sensitivity and specificity (49). In our present study, pretreatment with heparin has reduced the pancreatitis-evoked increase in serum activity of pancreatic digestive enzymes. This observation is a next evidence of protective effect of heparin on the pancreas. Study performed by Keck *et al.* (50) has shown that presence of active trypsin and elastase in the circulation up-regulates the expression of adhesion molecules on leukocytes and endothelial cells. This effect leads to increase in leukocyte-endothelial interaction promoting pancreatic microcirculatory failure. On the other hand, heparin reduces activity of trypsin (7, 8) and chymotrypsin (9), and inhibits conversion of trypsinogen to trypsin (11). For this reason, the heparin-evoked reduction in plasma activity of pancreatic enzymes in acute pancreatitis can be a result or/and cause of its protective effect on the pancreas.

Pancreatic ischemia plays an important role in the development of acute pancreatitis (51). In ischemia/reperfusion-induced pancreatitis, disturbance of pancreatic blood flow is a primary cause of acute pancreatitis. Our present study has shown that pretreatment with heparin improves pancreatic blood flow and inhibits the development of acute pancreatitis. Mechanism of this circulatory effect is probably mainly associated with a well-known anticoagulant activity of heparin. Induction of acute pancreatitis has reduced pancreatic blood flow and

this effect was connected with an increase in APTT and D-Dimer concentration. APTT is a general coagulation test. It cumulatively explores the activity of plasma coagulation factors of the intrinsic and common pathways of blood coagulation. D-dimer is a product of proteolytic action of plasmin on fibrin polymer and plasma level of D-dimer is a marker of fibrinolysis activation (52). In our present study, induction of acute pancreatitis by severe ischemia followed by reperfusion led to a three and half-fold elongation of APTT and fifteen-fold increase in D-Dimer concentration. These data indicate that development of acute pancreatitis is associated with formation of thrombi within pancreatic and systemic circulation. Changes in APTT seem to be a result of consumption of factors involved in coagulation, whereas the increase in D-Dimer concentration indicates a subsequent activation of coagulation. Administration of heparin, without induction of acute pancreatitis, has prolonged APPT, but this effect was significantly smaller then that observed in animals with acute pancreatitis. Heparin given alone was without effect on plasma D-Dimer concentration. On the other hand, pretreatment with heparin before induction of acute pancreatitis, reduced the pancreatitis-induced increase in APTT and plasma D-Dimer concentration. This observation indicates that pretreatment with heparin prevents activation of coagulation and for this reason reduces consumption of coagulation factors and creation of products of fibrinolysis.

Initial series of our studies have shown that pretreatment with heparin exhibits protective effect in the pancreas inhibiting the development of ischemia/reperfusion-induced pancreatitis. This effect may be useful in the prevention of acute pancreatitis. However, clinically patients are usually seen several hours after the onset of acute pancreatitis and therapy is started at this period. For this reason, more important is answer to the question whether treatment with heparin after induction of acute pancreatitis can affect the course of acute pancreatitis. This subject has been undertaken in the third series of our study. We have found that administration of heparin exhibits therapeutic effect in ischemia/reperfusion-induced pancreatitis. It was manifested as a reduction in the severity of acute pancreatitis and a faster normalization of pancreatic morphology and biochemical markers of inflammation.

Very interesting observation of our study is the influence of heparin on pancreatic DNA synthesis. Pancreatic DNA synthesis is an index of cell proliferation in the pancreas and the reduction in pancreatic DNA synthesis is well-correlated with pancreatic damage in acute pancreatitis (53-55). In our present study, pretreatment with heparin before induction of acute pancreatitis, has attenuated the pancreatitis-evoked fall in pancreatic DNA synthesis. This finding indicates the better vitality of pancreatic cells and is an additional evidence of protective effect of heparin in ischemia/reperfusion-induced pancreatitis. On the other hand, treatment with heparin, after induction of acute pancreatitis, has increased pancreatic DNA synthesis. This observation indicates that heparin may stimulate pancreatic cells proliferation, leading to inhibition of

inflammation and acceleration of pancreatic regeneration. The mechanism of this phenomenon is unclear. There are studies indicating that heparin may stimulate the proliferation of intestinal epithelial cells in primary culture (56). These findings could suggest direct action of heparin on the pancreatic cell proliferation. However, it is most likely that growth promoting effect of heparin on the pancreas in the course of acute pancreatitis is indirect effect related to interaction of heparin with growth factors. Several growth factors have been characterized, which form tight complexes with heparin, including members of the fibroblast growth factor (FGF) family (57, 58), vascular endothelial growth factor (VEGF) (59), heparin-binding epidermal growth factor-like growth factor (HB-EGF) (60, 61), hepatocyte growth factor (HGF) or granulocyte colony-stimulating factor (G-CSF) (62). A number of studies have indicated that these growth factors, especially FGF-1 and FGF-2, cannot bind their receptor or activate signal transduction unless heparin or other glycosaminoglycan, heparan sulfate are present (59, 63-65). Also, presence of heparin or heparan sulfate protects growth factors from proteolytic degradation (66).

Growth factors are involved in pancreatic development, growth and regeneration (67). Pancreatic overexpression of mRNA transcript for growth factors such as FGF-1, FGF-2, insulin-like growth factor-1 (IGF-1), (HGF) and transforming growth factor-α (TGF-α), have been detected in the course of acute experimental and clinical pancreatitis (68-71). Also experimental studies have shown that administration of growth factors such as EGF (72-74), FGF-2 (75), HGF (76-77), IGF-1 (78) growth hormone (79) or ghrelin (80, 81) attenuates the pancreatic damage and accelerates pancreatic recovery. This findings taken together suggest that therapeutic effect of heparin in the course of acute pancreatitis is partly mediated be growth factors.

Acute pancreatitis ranges from a mild, self-limiting edematous pancreatitis to the life-threatening severe pancreatitis with multiple organ failure. Severe acute pancreatitis reveals its progress into two phases. Overall mortality rate of severe acute pancreatitis has been reported to range between 10 to 30% (82-84). In severe acute pancreatitis, early deaths occur in half of patients within 14 days after the onset disease due to systemic inflammatory response syndrome (SIRS) with secondary multi-organ failure (MOF) owing to the release of large amount of pro-inflammatory cytokines (83, 84). The remainder half of deaths occur later from complications secondary to the infection of pancreatic and peripancreatic necrosis and secondary MOF (83, 84). Increased risk of pancreatic infection in the second phase of severe acute pancreatitis is a result of compensatory anti-inflammatory response syndrome (SIRS) (85), which is a consequence of previous SIRS. The extensive release of anti-inflammatory cytokines from CARS effector cells and impaired leukocyte antigen-DR expression lead to potent immunosuppression with development of severe infection (85). In our present study, treatment with heparin has decreased plasma level of proinflammatory IL-1β, reducing the probability of development of SIRS, as well as has reduced

plasma concentration of anti-inflammatory IL-10 in the second phase of acute pancreatitis, preventing agaist the development of CARS. These data indicate the next mechanism of therapeutic effect of heparin, which reduces the possibility of development of two serious complications of acute pancreatitis, SIRS and CARS.

In conclusion, we can say that heparin, a safe and well-known medicine, exhibits strong protective and therapeutic effect in the course of acute pancreatitis. Also, our data strongly suggest that heparin may be useful in routine clinical management of this disease.

Conflict of interest statement: None declared.

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