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ORIGINAL ARTICLE

Ischemia–reperfusion rat model of acute pancreatitis: protein carbonyl as a putative early biomarker of pancreatic injury

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Abstract Acute pancreatitis (AP) is an inflammatory disorder that can affect adjacent and/or remote organs. Some evidence indicates that the production of reactive oxygen species is able to induce AP. Protein carbonyl (PC) derivatives, which can also be generated through oxidative cleavage mechanisms, have been implicated in several diseases, but there is little or no information on this biomarker in AP. We investigated the association between some inflammatory mediators and PC, with the severity of ischemia–reperfusion AP. Wistar rats ($n = 56$) were randomly assigned in the following groups : control; sham, 15- or 180-min clamping of splenic artery, with 24 or 72 h of follow-up. The relationships between serum level of PC and thiobarbituric acid reactive species (TBARS) to myeloperoxidase (MPO) activity in tissue homogenates and to cytokines in culture supernatants of pancreatic samples were analyzed. MPO activity was related to the histology scores and increased in all clamping groups. Tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin-6 were higher in the 180-min groups. Significant correlations were found between MPO activity and the concentrations of TNF-α and IL-1β. PC levels increased in the 15-min to 24-h group. TBARS levels were not altered substantially. MPO activity and TNF-α and IL-1β concentrations in pancreatic tissue are correlated with AP severity. Serum levels of PC appear to begin to rise early in the course of the ischemia–reperfusion AP and are no longer detected at later stages in the absence of severe pancreatitis. These data suggest that PC can be an efficient tool for the diagnosis of early stages of AP.

Keywords Protein carbonylation · Thiobarbituric acid reactive substance · Reactive oxygen species · Myeloperoxidase · Cytokines

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Introduction

Acute pancreatitis (AP) has an annual incidence ranging from 5 to 80 cases per 100,000 population [1]. Despite the array of new diagnostic and therapeutic tools, acute pancreatitis remains a critical condition with high rates of morbidity and mortality. The clinical spectrum of AP is widely variable, from mild, self-limiting localized disease to widespread multi-organ failure, with a mortality rate ranging from 14 to 30 % [2].

The time from the onset of clinical manifestations to hospital admission and the establishment of the correct diagnosis can vary widely. Therefore, most evaluation routines use one or more scoring systems for the prognostic evaluation, which is obtained at admission, usually within the first 24 to 48 h, or daily thereafter [3]. A large number of clinical features and laboratory tests have been used in order to attempt to predict the severity of AP, and new data are continuously added [3]; however, a gold standard has not yet been established.

In the course of AP, activation of leukocytes and release of pro-inflammatory cytokines are responsible not only for local pancreatic damage but also for the development of the systemic inflammatory response syndrome (SIRS) and multiple organ failure. The production of pro-inflammatory cytokines is correlated with AP severity; in particular, interleukin 1 beta (IL-1β) appears to play a crucial role in the induction of the systemic acute-phase response and in the pro-inflammatory cytokine cascade [4]. Experimental studies, both in vivo and in vitro, have suggested that tumor necrosis factor-alpha (TNF-α) also plays an essential role in AP, triggering not only the development of pancreatic tissue injury but also further damage in extra-pancreatic organs. Moreover, TNF-α can induce the release of several cytokines such as IL-1β, interleukin-6 (IL-6), and IL-8 [5].

Prediction of severity in AP and any additional information, especially early in the course of the disease, may be helpful to improve the patient's treatment and outcome [6]. In such circumstances, markers of the inflammatory response have been investigated. The levels of IL-8 and IL-6 are regarded as predictive, but are technically difficult to measure; C-reactive protein (CRP) is well known as a marker of severity, but there is often a delay of several days from the onset of symptoms before CRP levels rise [7]. Recently, procalcitonin has been proposed as a marker of severity, but it seems that this marker is most closely related to the presence rather than the severity of the lesion [3, 7]. Myeloperoxidase (MPO) is usually considered to be a marker of neutrophil activity, causing the formation of reactive oxygen metabolites that damage pancreatic acinar cells. However, only one study has already evaluated MPO blood levels in relation to the severity of AP and its time course [8].

Little attention has been given to ischemia and the reperfusion mechanism in the pathogenesis of AP. Oxidative stress has a primary role in the injury to the pancreas and occurs in various clinical settings including cardiac surgery, hemorrhagic shock, hypothermia, and pancreas transplantation [9]. Reliable evidence indicates that the production of reactive oxygen species (ROS) such as superoxide ($\cdot O_2$), hydrogen peroxide (HOOH), and hydroxyl ($\cdot$OH) radicals is able to induce AP, causing inflammatory cell infiltration, interstitial edema, enzymatic elevation, and activation of pro-inflammatory cytokines, overwhelming the antioxidant defenses [10–12]. Moreover, oxidant chain reaction increases the permeability of the microcirculation of the pancreas leading not only to microvascular leakage but also to lack of perfusion due to intravascular no-reflow phenomenon caused by endothelial cell swelling, adhesion of neutrophils, thromboxane A2, and others vasoactive mediators. Such impairment of pancreatic microcirculation in the early phase of acute pancreatitis plays an important role in the pathogenesis of this disease [13, 14].

ROS damage cell membranes directly by the process of lipid peroxidation. As a result, toxic products such as malondialdehyde (MDA) are released, which in turn may increase cellular injury or act as a chemoattractant to trigger the systemic inflammatory response syndrome (SIRS). This raises the possibility that MDA could play a role as a marker of severe pancreatitis [6].

Protein carbonyl (PC) groups have been implicated in several human diseases, including respiratory distress syndrome, Alzheimer's disease, atherosclerosis, diabetes, sepsis, chronic renal failure, rheumatoid arthritis, and Parkinson's, among others [15]. Nevertheless, investigators do not mention any relationship between protein carbonylation and AP caused by ischemia and the reperfusion mechanism.

Despite some improvements over time, the reporting of important aspects of prognostic studies in AP remains suboptimal. The main focus of this study was to investigate in the presence of pancreatic ischemia and reperfusion, the association between some pro-inflammatory mediators and PC in the early diagnosis of AP.

Methods

The present study was approved by the Ethics Committee for Laboratory Animal Use in Research of the Federal University of Rio de Janeiro (UFRJ), Project Number 78/09, in accordance with the International Guiding Principles for Biomedical Research Involving Animals, recommended by the World Health Organization.

The rats were maintained at the Center of Experimental Surgery of the Department of Surgery, School of Medicine of UFRJ. Before the experiment, the rats were fed with

standard rat chow and water ad libitum and housed in a caging system with controlled temperature (24 °C) and 12-h light/dark cycles.

Animals and experimental design

Fifty-six female Wistar rats (*Rattus norvegicus*), free of specific pathogenic species (SPF) and weighing from 180 to 250 g, were randomly assigned to seven experimental groups with eight animals each: group 1, control **(C)**—without undergoing any surgical procedure. Only blood and pancreas samples were collected. Group 2, sham 24 h **(S/24 h)**—laparotomy with the isolation of the splenic artery, kept alive for 24 h. Group 3, sham 72 h **(S/72 h)**—laparotomy with the isolation of the splenic artery, kept alive for 72 h. Group 4, ischemia/reperfusion (**15′/24 h)**—clamping of the splenic artery for 15 min, kept alive for 24 h. Group 5, ischemia/reperfusion (**15′/72 h)**—clamping of the splenic artery for 15 min, kept alive for 72 h. Group 6, ischemia/reperfusion (**180′/24 h)**—clamping of the splenic artery for 180 min, kept alive for 24 h. (**180′/72 h)**—clamping of the splenic artery for 180 min, kept alive for 72 h.

The anesthetic procedure consisted of the intraperitoneal injection of ketamine (25 mg/kg) combined with xylazine (3 mg/kg). Immediately after anesthetic administration, the animals were immobilized in dorsal decubitus, the surgical site was shaved, and the skin was cleaned with an antiseptic surgical-scrub solution. Laparotomy was performed through a midline incision. A microvascular clip positioned just after the polar branch of the spleen was used to clamp the splenic artery. The abdomen was closed in two layers. In the first two postoperative days, animals received analgesia with dipyrone (1 mg/mL), in the drinking water. At 24 or 72 h, according to the group, all rats were killed without pain with an anesthetic overdose (four times the regular one).

Blood samples were obtained from the inferior cava vein at 24 and 72 h of follow-up, for the determination of malondialdehyde (MDA) levels by TBA reaction, PC group levels by slot blotting, and measurement of C-reactive protein (CRP) and amylase levels. After euthanasia, three samples were excised from the pancreas for histological assessment, determination of myeloperoxidase activity (MPO) in tissue homogenates, and measurement of pro-inflammatory cytokines in 24-h pancreas culture supernatants, by ELISA.

Tissue preparation and histological evaluation

Histological evaluation of the pancreatic sections was performed by the same pathologist, who was unaware of the experimental data. Specimens were immersion-fixed overnight at 4 °C in freshly prepared 4 % paraformaldehyde buffered with 0.1 M sodium phosphate (pH 7.4). Paraffin sections of 4 μm were placed on slides and stored at room temperature until further processing. The sections were stained with hematoxylin and eosin (H&E) and observed under light microscopy at 40×, 100×, and 400× magnifications.

The following histopathological parameters were evaluated based on the classification described by Spormann: mucosal edema, inflammatory infiltration, and zymogenic degranulation [11, 16]. Each parameter was scored from 0 to 3, according to the intensity of the damage (no lesion, mild, moderate, and severe, respectively). The presence or absence (score 0 or 1) of fat necrosis, parenchymal necrosis, and hemorrhage was also evaluated. A total score was obtained by summing all parameters for each animal.

Myeloperoxidase activity

The pancreatic tissue samples were frozen at −80 °C until protein and MPO extraction. Briefly, samples were homogenized in potassium phosphate buffer (pH 6.0), frozen and thawed twice, homogenized again in potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyltrimethyl-ammonium bromide (Sigma Chemical Co., St. Louis, MO, USA), and centrifuged at 40,000*g* for 30 min (4 °C). The supernatants were discarded, and the insoluble pellets were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyltrimethyl-ammonium bromide. Ten microliters of each supernatant was added to a 1-cm light path cuvette containing 290 μL of 50 mM potassium PBS (pH 6.0), 3 μL of substrate solution, containing 20 mg/mL of o-dianisidine (Sigma Chemical Co., St. Louis, MO, USA), and 3 μL of H_2O_2 (20 mM). The cuvette components were rapidly mixed, and the absorbance was determined at 460 nm for 1 min with a spectrophotometer. MPO activity was measured by a standard curve of the samples in units of MPO/mg of pancreatic sample. The total protein content of the biopsy specimens was estimated by the Pierce® BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and used for normalizing the results.

Organ culture and cytokine measurements

Pancreatic explants were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum (Life Technologies, USA), 10 mM HEPES (Promega, USA), penicillin (100 KU/L), and streptomycin (100 μg/mL) (Sigma, USA) for 24 h at 37 °C in a 5 % CO_2 humidified incubator. After incubation for 24 h, the supernatant was collected and stored at −20 °C. Samples were centrifuged, and the supernatants were used for measuring the concentration of cytokines TNF-α, IL-1β, and IL-6, by a commercial sensitive enzyme-linked immunosorbent assay (ELISA) method (R&D Systems, MN, USA), according to the

manufacturer's instructions: interleukin-1 beta (IL-1β—PeproTech Inc., NJ, USA), interleukin-6 (IL-6—Bender MedSystems, Vienna, Austria), TNF-alpha (TNF-α—PeproTech Inc., NJ, USA). The total protein content of the explants was estimated by the BCA method, and the values were used to normalize the ELISA results. The minimum detectable concentrations of rat TNF-α, IL-1β, and IL-6 were typically <5.0 ng/mL.

Oxidative stress markers

Thiobarbituric acid reactive species: TBARS (lipid peroxidation levels)

Aliquots of the plasma were precipitated with TCA (10 %; 2:1 vv), and the supernatants were centrifuged (2,000*g* for 6 min), added to 1 % thiobarbituric acid and 0.1 N EDTA, maintained in boiling water for 15 min, cooled to room temperature, and then measured using a Hitachi U-100 spectrophotometer at 532 nm. The results were expressed as μM MDA/g protein.

Protein carbonyl

First, an aliquot of the plasma sample (1 μL) was slot-blotted onto a polyvinylidene difluoride (PVDF) membrane using a Slot blot device (Bio-Dot SF apparatus). The PVDF membrane was then incubated with 0.1 mg/mL DNPH in 2 N HCL for 5 min, washed extensively in 2 N HCL (5 min), and 100 % methanol (5 min) was added to remove free unreacted DNPH. Then, the membrane was treated with a primary anti-DNP antibody (Sigma-Aldrich, USA) at a concentration of 2 μL for 2 h, washed with PBS, and finally treated with a peroxidase-conjugated secondary antibody (Sigma-Aldrich, USA). Slot blot was detected by means of the chromogenic method. Image analysis of the plasma was carried out by using Quantity One 4.5.0 1-D Analysis Software, and the results were expressed as optical density (OD) [10].

Serum amylase activity and C-reactive protein (CRP)

Commercially available assays were used to quantify amylase (Labtest, Santa Luzia, Brazil) and the CRP (ultra-sensitive turb CRP, EBRAM, São Paulo, Brazil).

Statistical analysis

Data were analyzed with the aid of a computerized statistical program (version 10.0.1, SPSS Inc., 1989–1999, USA). The results of different groups were compared by *t* test or one-way ANOVA, in which pairwise multiple comparisons were carried out using Dunnett's T3 post hoc test. Correlations between the histological scores and the concentrations of amylase, CRP, TBARS, PC, and cytokines were assessed using the Spearman rank correlation coefficient. Statistical significance was considered for $P < 0.05$.

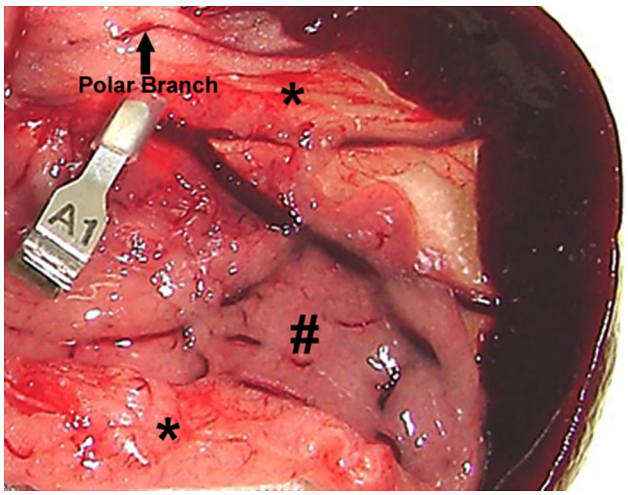

Fig. 1 Atraumatic occlusion of the splenic artery branch of the rat. The microclamp was placed selectively in the splenic artery just after the emergence of the polar branch (*arrow*). It was noticed the presence of *bluish color* in the occluded segment of splenic artery and a congested tail of the pancreas (*number sign*) compared with nonischemic lobes of the same organ (*asterisk*)

Results

Macroscopy

The effect of a selective blockage by clamping the splenic artery immediately after the emergence of the polar splenic branch was demonstrated by the ischemia at the tail of the pancreas (Fig. 1).

Histopathology

Both sham groups showed a mild enhancement of the nuclei/zymogen proportion. Groups 4 and 6 (15 min ischemia) showed an increase in the lobules, with multiplication of the spaces between the lobules and with a thin stroma, characterized by moderate edema and inflammatory cell infiltration. Groups 5 and 7 (180 min ischemia) showed a marked increase in the nuclei/zymogen proportion, with severe edema and inflammatory cell infiltration. Parenchymal necrosis was found in 3 animals, 2 from group 7 (180′/72 h) and one from group 6 (180′/24 h) (Fig. 2a). Higher scores were found in both clamping groups, in particular, the 180-min ones (Fig. 2b). No histopathological difference was found

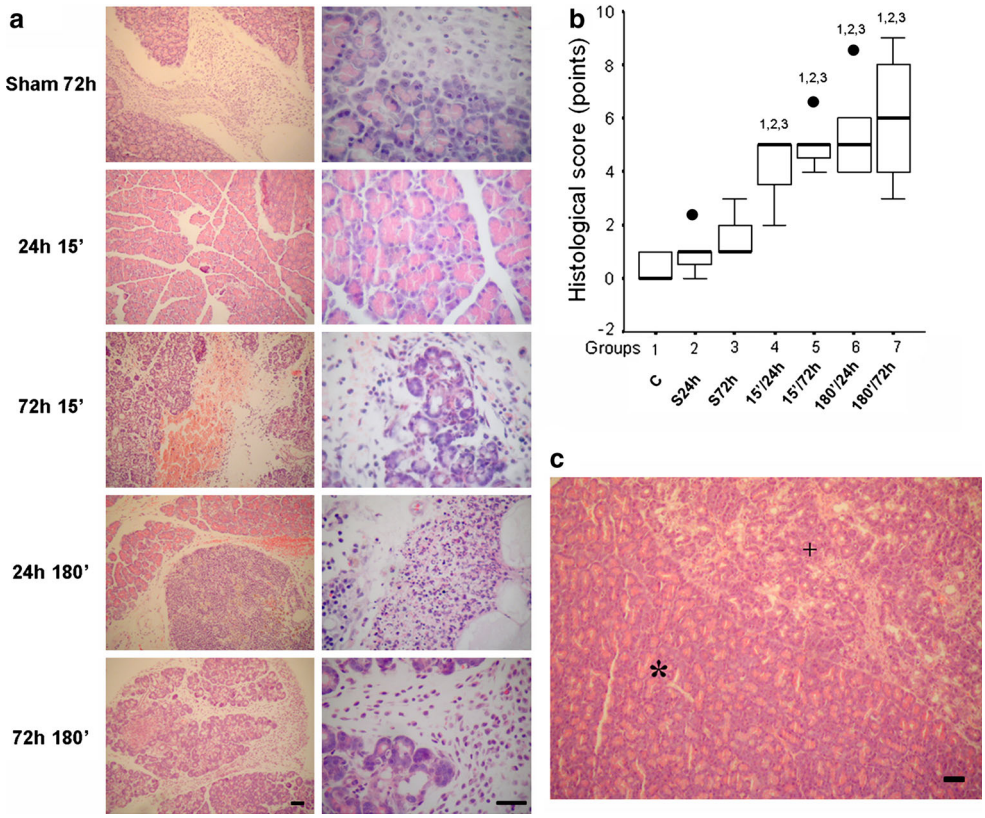

Fig. 2 Hematoxylin-eosin-stained slides of pancreatic samples obtained at different time points. Images were captured at ×100 and ×400 magnifications, respectively. Sham groups showed mild enhancement of the nuclei/zymogen proportion. The groups of 15-min ischemia showed an increase in the lobules, with the multiplication of the spaces between the lobules and with a thin stroma, characterized by moderate edema and inflammatory cell infiltration. The groups of 180-min ischemia displayed increased nuclei/zymogen ratio, with severe edema and inflammatory cell infiltration, with apoptotic bodies, and eventually parenchymal necrosis (**a**). Higher histological scores were found in both clamping groups, in particular, the 180-min ones. No difference was found between the 24- and 72-h groups with the same time of ischemia (**b**). Detail of a sample from group 7 (180′/72 h) showing normal parenchyma (*), clearly separated by areas of acute pancreatitis (+) with tubular formation, mild interstitial infiltrate, severe degranulation, and the presence of apoptotic bodies (**c**). *Scale bars* = 50 μm in (**a**, **c**)

between the 24- and 72-h groups with the same time of ischemia. Acinar cells with condensed small nuclei, separated from surrounding intact cells by a clear halo and compatible with apoptotic bodies, were detected in 5 of 8 rats in the 15′/72 h group, one of 8 rats in the 180′/24 h group, and 3 of 8 rats in the 180′/72 h group (Fig. 2c).

Myeloperoxidase activity

The MPO activity from the pancreas sample was related to the histology scores and increased in both the 15- ($P < 0.02$) and 180-min ($P < 0.003$) groups at 72 h. At 24 h, only the 180-min clamping group showed higher MPO activity (group 6, $P < 0.034$) (Fig. 2). A significant correlation was found between the histological scores and the MPO activity ($r = 0.68$, $P < 0.001$) (Fig. 2b compare to Fig. 3).

Amylase and C-reactive protein

Amylase levels significantly increased in the 180′/24 h group in comparison with the control and sham groups. The ischemia time of 15′ was not sufficient to cause hyperamylasemia that differed statistically from the control group. Nevertheless, at 72 h of ischemia, amylase declined to levels similar to those detected in the control and sham groups (Fig. 4a).

CRP levels increased significantly in the 15′/72 h (group 5) and 180′/24 h (group 6) groups compared with the sham and control groups and also in the 180′/72 h group (group 7) compared with the control group ($P < 0.05$). Notably, a significant correlation existed between the levels of amylase and the histological score ($r = 0.41$; $P < 0.005$) and also the CRP levels ($r = 0.29$, $P < 0.04$) (Fig. 2b compare to Fig. 4a, b).

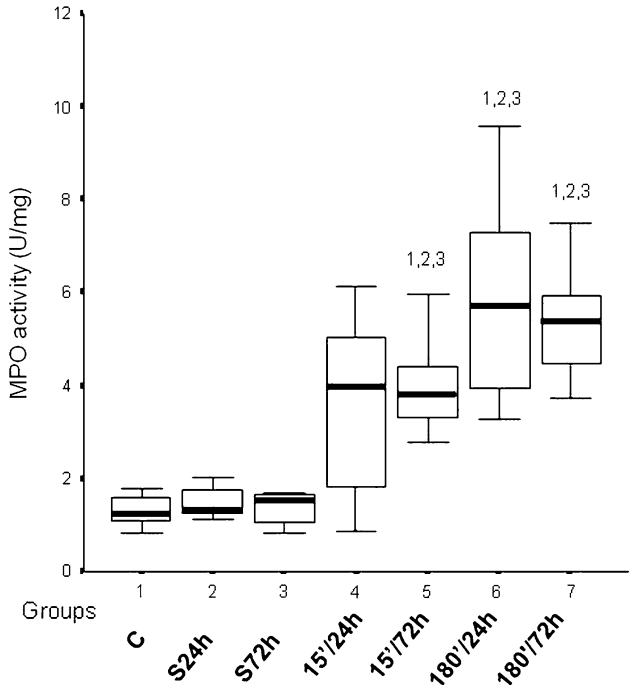

Fig. 3 Myeloperoxidase activity in pancreatic samples. Myeloperoxidase activity was higher in groups 5, 6, and 7 (15′/72, 180′/24 and 180′/72 h) compared with control and sham groups ($P < 0.05$). *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentiles, and *vertical bars* represent ranges. $N = 6$ animals in each group

Measurement of serum TBARS and protein carbonyl

TBARS blood levels showed a significant increase ($P = 0.043$) in group 6 (180′/24 h) compared with the control group (Fig. 5a). PC levels increased significantly in the 15′/24 h group (group 4) compared with the control ($P = 0.007$) and sham 24/72 h ($P = 0.008/P = 0.049$) groups (Fig. 5b). Neither TBARS nor PC showed a significant correlation with any other test variables.

Cytokine production by the pancreatic samples

Concentrations of TNF-α ($P < 0.04$) and IL-1β ($P < 0.04$) in pancreatic culture supernatants were higher in both the 15- and 180-min groups, especially when assessed 72 h after the arterial clamping (Fig. 6a, b).

IL-6 concentrations increased only in the 180-min ischemia group and were significantly higher in groups 6 ($P = 0.03$) and 7 ($P = 0.023$) compared with the control group (Fig. 6c). Significant correlations were detected between the concentrations of pancreas supernatant cultures of TNF-α and the histological score ($r = 0.71$; $P < 0.001$), MPO activity ($r = 0.75$; $P < 0.001$), IL-1β ($r = 0.84$; $P < 0.001$), and IL-6 ($r = 0.70$; $P < 0.001$) (Fig. 2b compare to Fig. 3 and Fig. 6b, c). In regard to IL-1β, significant correlations were found with the histological score ($r = 0.63$; $P < 0.001$), MPO activity ($r = 0.73$; $P < 0.001$), and IL-6 ($r = 0.74$; $P < 0.001$) (Fig. 2b compare to Fig. 3 and Fig. 6a, b). Finally, IL-6 correlated significantly with the histological score ($r = 0.79$; $P < 0.001$) and with MPO activity ($r = 0.62$; $P < 0.001$) (Fig. 2b compare to Fig. 3 and c).

Discussion

Reperfusion is an important pathophysiological mechanism in AP development, but such condition is not well known.

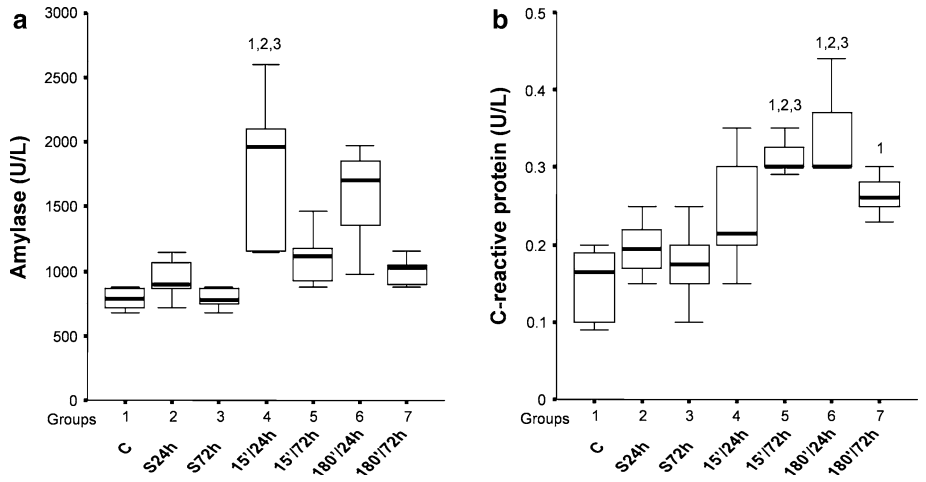

Fig. 4 Levels of amylase and C-reactive protein. Amylase levels significantly increased in the 180′/24 h group compared with control and sham groups. At 72 h of ischemia, amylase declined to levels similar to those in control and sham groups (**a**). CRP levels were significantly higher in the 15′/72 h (group 5) and 180′/24 h (group 6) groups compared with the sham and control groups and also in the 180′/72 h group (group 7) compared with the control group ($P < 0.05$) (**b**). *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentiles, and *vertical bars* represent ranges. $N = 6$ animals in each group

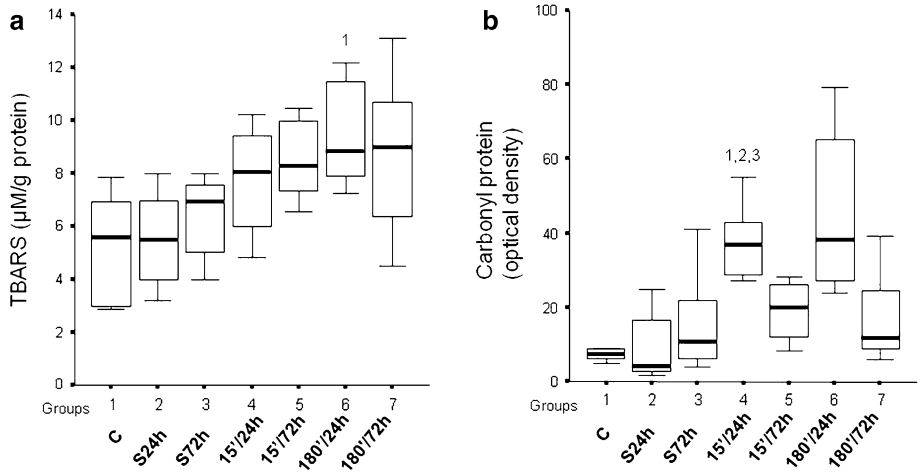

Fig. 5 TBARS and PC evaluation. Group 6 (180′/24 h) showed significantly higher levels of TBARS compared with control ($P = 0.043$) (**a**). Significantly higher values of PC were found in group 4 (15′/24 h) compared with control ($P = 0.007$) and sham 24/72 h ($P = 0.008$/$P = 0.049$) groups. Higher values in group 6 did not reach statistical significance (**b**). *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentiles, and *vertical bars* represent ranges. $N = 6$ animals in each group

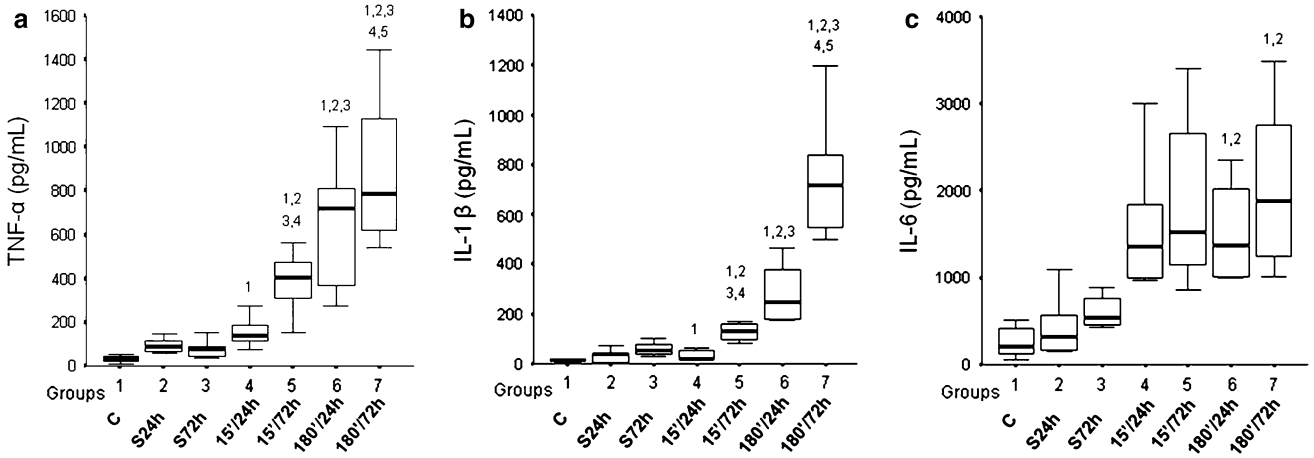

Fig. 6 Cytokine production by the pancreas. Concentration of cytokines in 24-h organ cultures was measured by ELISA and expressed as picogram per milliliter of culture supernatant, normalized by the protein content of tissues. TNF-α concentrations were higher in groups 4, 5, 6, and 7 compared with the control group ($P < 0.05$). Group 7 (180′/72 h) had higher values ($P = 0.047$) than group 5 (15′/72 h). Levels in the 15-min ischemia groups were higher in group 5 (kept alive for 72 h) than in group 4 (24 h) ($P = 0.019$) (**a**). Concentrations of IL-1β in group 7 were higher than in all other groups, except group 6 ($P = 0.914$). Among groups with 15 min ischemia time, group 5 had higher levels than group 4 ($P = 0.019$). In 72-h groups, levels of IL-1β were higher at 180 min than at 15 min (groups 7 vs. 5, $P = 0.047$). No difference was found between the 24-h groups (groups 6 vs. 4, $P = 0.068$) (**b**). Concentrations of IL-6 in groups 6 and 7 (180 min ischemia) were higher compared with control groups 1 and 2 ($P < 0.03$) (**c**). *Horizontal bars* represent medians, *boxes* represent the 25th and 75th percentiles, and *vertical bars* represent ranges. Significant values are shown. $N = 6$ animals in each group

One common and very early event in the evolution of pancreatitis is the inflammatory process. At the onset of pancreatitis, neutrophil recruitment produces oxygen free radicals, increasing the acinar cell injury. Vascular injury with vasoconstriction, capillary stasis, and ischemia, followed by microcirculatory vasodilation during the reoxygenation phase, increase the edematous and interstitial lesions seen in pancreatitis. Several vasoactive mediators are also implicated in these deleterious effects. Oxidative stress plays a central role in pancreatic injury and causes lipid peroxidation of the cellular membranes. Another main ultrastructural change that characterizes oxidative injury is the formation of protein-bound carbonyl groups. Plasma measurement of PC is not currently used as a biological marker of the inflammatory cascade in response to early acute pancreatitis, but may be a useful tool for the diagnosis.

The most widely used scoring systems for acute pancreatitis (Ranson, Apache) although useful are often

difficult to apply in the early assessment of the clinical evolution of such disease. In rats, no translational study was found validating both scores. Large volume of intravenous blood sample may cause interference with normal physiology of the rats, and the authors chose to evaluate serum level of protein carbonyl and thiobarbituric acid reactive species (TBARS) related to oxidative stress. Besides, measurement of C-reactive protein (CRP) and amylase levels was made because they are usually obtained in normal clinical practice and both are still considered as reliable biomarkers.

Several investigators have demonstrated that an ischemia–reperfusion injury might contribute to the pathogenesis of AP [16, 17]. Different ischemia periods could produce AP, but because of individual variations in the pancreatic blood supply and ischemia tolerance, experimental models have a variable outcome [11]. Clamping the splenic artery near the celiac trunk may produce ischemia, not only of the whole spleen, but also in the stomach and small intestine [12, 16, 17]. In the current study, the rat model of pancreatic ischemia–reperfusion triggered an acute inflammatory process at the tail of the pancreas.

In a study using a selective ischemic–reperfusion model in rats (60 min of ischemia), apoptotic acinar cells were identified 48 h after reperfusion [2]. The histological changes were attenuated at 72 h after reperfusion, and there was little evidence of necrosis in those specimens [2]. In our study, we found only three cases of necrosis. One possible interpretation of the underlying mechanisms of this process is that massive cell damage can be triggered by oxygen-derived free radicals and cytokines such as TNF-α and IL-1β, which can contribute to reperfusion injury. Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are produced during pancreatitis and ischemia, and the reperfusion mechanism triggers this effect [18].

It is well known that the decrease in pancreatic microcirculation leads to the formation of thrombi in capillaries, with activation of leukocytes and release of proteolytic enzymes and pro-inflammatory cytokines. However, it is noteworthy that the main tissue damage is induced by reperfusion with ROS production [19].

At the beginning of the pancreatic inflammation, some proteases cause the cleavage of xanthine dehydrogenase into the xanthine oxidase form. This enzyme converts hypoxanthine (an ATP breakdown product) into xanthine and produces superoxide radical, an oxygen-derived free radical [20]. Thus, a chain reaction occurs, with the synthesis of new ROS followed by lipid peroxidation. With the oxidative process, the organic enzymatic antioxidant reserves are rapidly exhausted, overwhelmed by the burst of production of oxygen-derived free radicals and their subproducts. Unstable oxidizing species have cytotoxic effects on virtually all cellular components, not only disrupting membranes and the cytoskeleton, but also compromising other important organelles such as lysosomes, mitochondria, and even nucleic acids [16].

Malondialdehyde (MDA) is the main product of polyunsaturated fatty acid peroxidation, and it is usually considered an ordinary marker of lipid peroxidation. One study found that in the early clinical course of AP, there is evidence of a significantly greater MDA release than in the mild form of the disease [18]. This is a potential marker of severity used after admission to hospital, but it is likely to disappear in the first hours [6, 21]. In fact, there were no significant differences in MDA/TBARS values among all the groups studied here. Similar results were obtained by other authors who used an alcoholic model of AP [22], probably due to a much earlier peak of lipid peroxidation or lack of sensitivity for an early detection. However, several authors have described increased levels of MDA 24 and 72 h after the initial stimulus [19, 23, 24]. Oxidative changes involving amino acids give rise to a carbonyl group that can be detected in tissue or blood samples. An increasing number of studies have used noninvasive measurements of the PC group in several human diseases [10], but we were unable to find any reference concerning to AP reperfusion conditions. In fact, measurement of PC in plasma has not been used in the diagnosis of pancreatitis. The present study did not show a relationship of PC to the severity of acute pancreatitis, but did reveal an early inflammatory process in the pancreas. A correspondence with early increases in levels of the other markers studied was also found. Kiziler and colleagues, studying alcoholic AP in rats, found higher levels of PC after 24 and 72 h, with no difference between the groups [21]. We found an increase in the 24-h groups, but not at 72 h. The difference between these two experimental models emphasizes that PC detection can be effective in early and reversible stages of the pancreatic damage, in contrast to the alcoholic AP model, where possibly more extensive and irreparable damage may already have occurred.

MPO activity is considered to be a marker of local neutrophilic activity and causes tissue damage in various inflammatory diseases. Most frequently, the MPO tissue concentration is measured in patients with severe AP and respiratory complications. One study showed a clear dependence of MPO blood level on the severity of AP and on cytokine blood levels [8]. In our study, we found a similar correspondence. IL-1β and TNF-α levels were positively correlated with MPO activity. MPO also contributes to the production of reactive oxygen metabolites such as TBARS and PC [8]. In a study using an ischemia–reperfusion model, MPO activity showed a time-dependent increase, reaching its maximum 48 h after reperfusion and subsequently decreasing [2]. No significant increase in serum amylase activity was observed, similarly to our findings, which

showed significant differences only in group 6 (180′/24 h group). It is possible that our findings may reflect the affected area of the whole pancreas in rats, following the clamping of the splenic artery [2]. In an alcoholic AP model in rats, levels of MPO increased in the 24 h after induction [22]. Another study, also using an alcoholic pancreatitis model, found higher levels 20 h after induction but not after 10 h [25]. Our findings showed that MPO levels were higher after 24 h and remained elevated until 72 h. Zhang and colleagues infused sodium taurocholate into the main pancreatic duct and described increased levels of MPO 24 h after the induction of AP [23].

Another research group, using a model of ischemia–reperfusion (30 min of ischemia), reported a maximum increase in plasma amylase and IL-1β activity 24 h after reperfusion [26]. A different ischemia–reperfusion study indicated that the expression of IL-1β and TNF-α matched with hyperamylasemia and the histopathological signs of pancreatitis, whereas the levels of IL-6 increased later in the course of AP [18]. These findings are consistent with our study, in which a progressive increase in IL-1β and TNF-α was related to the severity of pancreatitis, followed by later alterations in IL-6.

We chose to evaluate MPO and cytokines production in pancreatic tissue samples because of limitations imposed by the amount of peripheral blood required to perform in vivo measurements of TBARS and PC as well as amylase and reactive PC without adverse hemodynamic consequences. Indeed, it is well known that the pathophysiological mechanism of the oxidative stress on the pancreatic tissue can directly induce the release of pro-inflammatory cytokines, which can diffuse locally from the interstitial fluid to the plasma [18].

Conclusion

Our evaluation of several inflammatory mediators in an ischemic–reperfusion model of AP showed that levels of MPO, TNF-α, and IL-1β correlated with the detection of PC, in the early course of AP, can contribute to a better assessment of the severity of pancreatitis. Although PC proved to be, among all tested pro-inflammatory mediators, an efficient early biomarker of AP in the consequence of oxidative stress, further studies will be necessary in order to identify these chemically stable moieties as a promising prognostic marker of AP. The PC marker could form the basis of a standard laboratory test for use in clinical and surgical practice.

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Conflict of interest None.

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