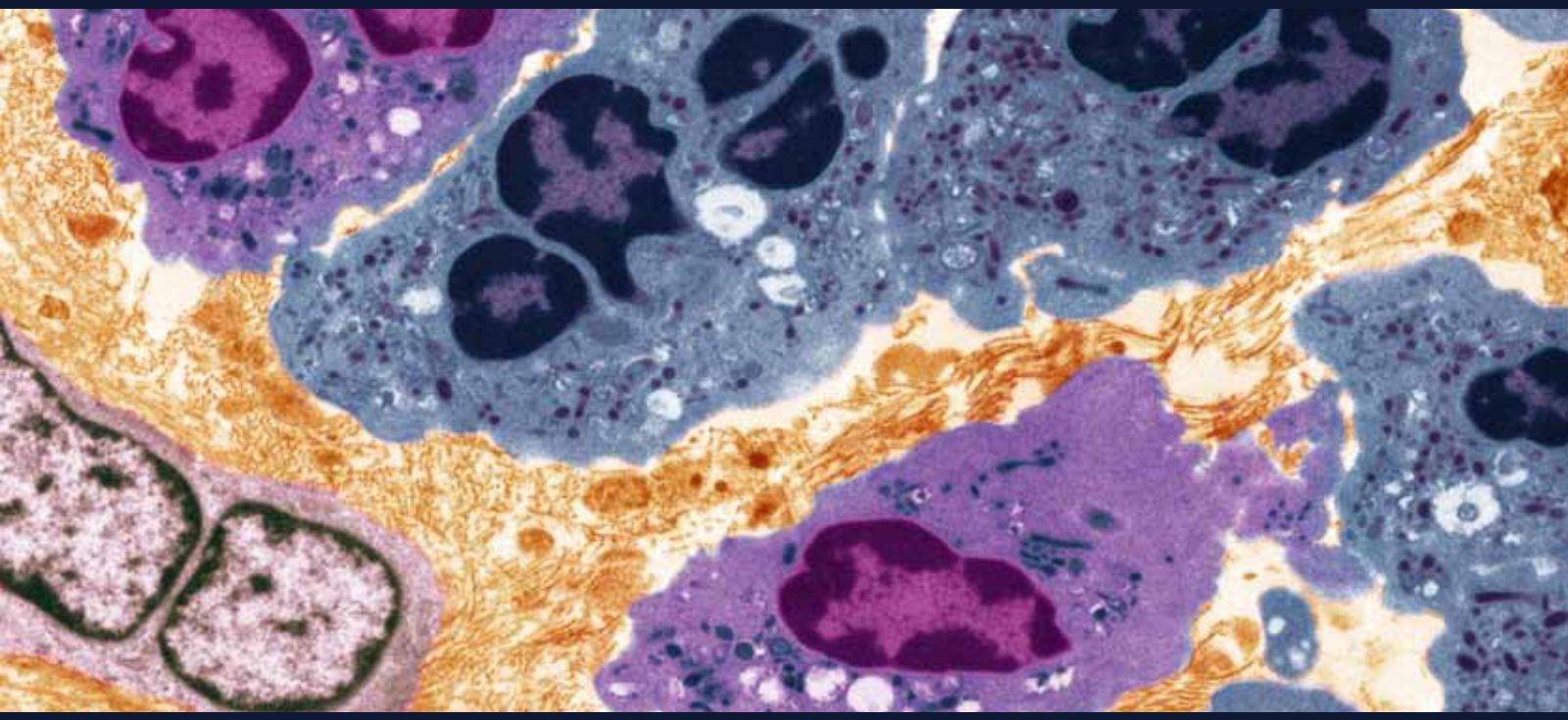
International Journal of Inflammation

# Acute and Chronic Pancreatic Inflammation

Guest Editors: Derek A. O'Reilly, Zoltan Rakonczay Jr., and Marja-Leena Kylänpää



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# Editorial Board

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*Editorial*

# Acute and Chronic Pancreatic Inflammation

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Acute and chronic pancreatitis result in considerable morbidity, are increasing in incidence, and have a high mortality rate. Due to the inaccessibility of the pancreas to study, our understanding of the pathophysiology of pancreatitis remains limited. Our current knowledge of the evolution of pancreatitis can be described as a progression from an initial injury to both the acinar and ductal components of the exocrine pancreas to local and systemic inflammatory responses [1]. If resolution fails to occur, infection or chronicity may supervene. In this special issue we present a series of review and original papers that shed light both on our evolving understanding of the basic mechanisms causing pancreatitis as well as current treatment controversies.

*Lessons about Basic Mechanisms.* Toll-like receptors (TLR) belong to the superfamily of interleukin-1 receptors and enable the innate immune system to recognize different groups of pathogens, while initiating appropriate and distinct immunological responses. TLR4 proteins, expressed on the cell surface, are receptors for the Gram-negative bacteria cell membrane component, lipopolysaccharide (LPS, endotoxin). The heat shock protein HSP60 may play an important role in the protection of the pancreas against damage and against early zymogen activation in acute pancreatitis. J. Bonior et al. report a series of experiments investigating the effects of endotoxemia induced in newborn rats on TLR4, HSP60 and proapoptotic Bax, caspase-9 and -3, or anti-apoptotic Bcl-2 protein expression in the pancreatic acinar cells of adult animals. Their results indicate that exposure of the infant rats to LPS promote the induction of HSP60 via TLR4 in their adult life and, in turn, activated Bax/Bcl-2 and caspase-9 and -3. It is likely that this process could play a part in the LPS-induced protection of the pancreatic tissue against acute damage in this experimental model.

Melatonin, a product of the pineal gland, is released from the gut mucosa in response to food ingestion and specific receptors for melatonin have been detected in many gastrointestinal tissues including the pancreas. Melatonin has been shown to attenuate the severity of acute pancreatitis. J. Jaworek et al. review the protective effects of melatonin on acute pancreatitis, reported in many experimental studies and supported by clinical observations. They conclude that the beneficial effects of melatonin are sufficient to warrant clinical evaluation as a supportive therapy in acute pancreatitis.

The causal relationship between tissue injury and pancreatitis and the mechanisms whereby tissue injury induces pancreatic inflammation is examined in a series of experiments reported by Nakumura et al. Although DNA has historically been believed to be immunologically inert, it is now appreciated that DNA can be released into the systemic circulation when cells undergo necrosis/apoptosis and recognized by the immune system. These authors hypothesised that cytosolic double-stranded DNA released by injured host cells may act as a "danger signal," which affects pancreatic stellate cells by increasing the expression of several inflammatory genes in the rat.

Research into the pathogenesis of pancreatitis is hampered by the inaccessibility and hazards of pancreatic tissue sampling during the course of the disease as well as by the ethical constraint that pancreatic tissue sampling is not a routine part of disease management. Infected pancreatic necrosis (and some cases of sterile necrosis) is an indication for intervention and I. Kovalska et al. have put together a

remarkable series of 224 operative and postmortem specimens subjected to histological analysis. This study underlines the importance of early pancreatic microcirculation and local coagulation disorders in the pathogenesis of acute necrotising pancreatitis and that, despite aggressive surrounding necrosis, the islets of Langerhans are preserved in 74.1% of cases, most probably due to fibrin capsule formation. This emphasises the importance of the movement in modern surgery to undertake removal of necrotic pancreas using minimally invasive and organ-preserving approaches [2].

Bridging the gap from bench to bedside, the editors of this special issue review the pro- and anti-inflammatory mediators that drive acute pancreatitis. This review examines the prospects for inflammatory mediators being identified as successful therapeutic targets. We explore the role of immunomodulation, monitoring the state of immune dysfunction by monocyte HLA-DR, signalling pathways of circulating leukocytes, and the narrow therapeutic window for intervention in acute pancreatitis.

*New Insights into Current Treatment.* The search for a specific therapy to treat pancreatitis remains the paramount goal of research in this field. Despite extensive efforts so far, no agent has proved successful. Further efforts to mount a therapeutic damage control strategy to contain an inappropriate inflammatory response are justified and remain ongoing. The APCAP (activated protein C in acute pancreatitis) trial randomised 32 patients with severe acute pancreatitis to receive either recombinant activated protein C (drotrecogin alfa activated) or placebo for 96 hours [3]. The results of the intervention and placebo groups were comparable. However, trials such as this also provide the opportunity to examine the systemic inflammatory response in great detail as it modulates with time and according to treatment. In this issue, L. Kyhälä et al. present the time course of the patients' plasma or serum levels of soluble markers (IL-8, IL-6, IL-10, IL-1ra, sE-selectin, PCT) and monocyte and neutrophil cell surface (CD11b, CD14, CD62L, HLA-DR) markers of systemic inflammatory response during the first 14 days after the randomization within APCAP.

With an increasing elderly population in many parts of the world, the effect of ageing on systemic inflammation, focusing on that induced by acute pancreatitis, has become more important than ever. Several reports have related an increased susceptibility to a proinflammatory status called inflammaging, which decreases the capacity of the immunological system to respond to antigens. M.C.C. Machado et al. review the effects of aging on the systemic inflammation in pancreatitis. They discuss the effects of cellular senescence and how this probably contributes to the chronic inflammatory state related to ageing.

State-of-the-art imaging remains a cornerstone of current management in acute pancreatitis. Early diagnosis and staging of complications are important clinical objectives. In this special issue, the method by which perfusion to an organ is measured by CT, and its clinical utility is reviewed by Y. Tsuji et al. They make the case that perfusion CT is a promising technique for diagnosis of local and systemic complications of severe acute pancreatitis at an early stage. Discriminating between focal chronic pancreatitis and pancreatic cancer remains challenging. Contrast-enhanced endoscopic ultrasound using Doppler techniques can reveal different vascularisation patterns in pancreatic tissue altered by chronic inflammatory processes and pancreatic cancer. M. Hocke et al. review the basics of contrast-enhanced high and low mechanical index endoscopic ultrasound and explain the pathophysiological differences behind the vascularisation of chronic pancreatitis and pancreatic carcinoma and how to use these techniques in daily clinical practice.

Pancreatic pseudocyst is a complication that develops in both acute and chronic pancreatitis. It may remain asymptomatic or develop life-threatening complications. A. K. Khanna et al. address the therapeutic dilemma of whether or not to treat a patient with a pancreatic pseudocyst, as well as when and with which technique.

*Conclusion: From Early Accounts to Future Hopes.* An early account of acute pancreatitis may have been furnished by the death of Alexander the Great (Alexander III of Macedon (20/21 July 356–10/11 June 323 BCE)). Alexander ascended to the throne and was to become the most successful of Greek generals, extending Hellenic influence throughout the known world. However, a case for acute pancreatitis being retrospectively applied to Alexander's death certificate can be made: a rich meal and heavy alcohol consumption preceded the onset of the disease and were probably the precipitating factors; the course of the disease is typical of acute pancreatitis in its onset, severity and irreversibility; fever and the subsequent systemic effects point towards acute necrotising pancreatitis with sepsis and multiple-organ failure [4]. If the cause of death was indeed acute pancreatitis, then perhaps the chief lesson to be learnt from this account is to reinforce the fact of our lack of progress in our understanding and particularly, in treating this condition. It should be remembered that (despite recent advances in critical care medicine) no specific treatment for acute pancreatitis has, as yet, proved superior to that employed by Alexander's generals; a forlorn plea for divine intervention, to the gods of the ancient Greeks.

We hope, however, that ongoing research, such as that contained within this special issue of the International Journal of Inflammation, provides the insight and inspiration necessary to make progress in our endeavours to obtain sufficient understanding to discover an effective treatment for this disease.

## Acknowledgments

It only remains for us to thank the authors of these papers for submitting their work to this special issue and to the staff of Hindawi Publishing corporation for making these data freely accessible via open access publishing. We hope that you, the reader, will take something of value from it.

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*Zoltan Rakonczay Jr.*
*Marja-Leena Kylänpää*

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*Review Article*

# The Clinical Course of Acute Pancreatitis and the Inflammatory Mediators That Drive It

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Acute pancreatitis (AP) is a common emergency condition. In the majority of cases, it presents in a mild and self-limited form. However, about 20% of patients develop severe disease with local pancreatic complications (including necrosis, abscess, or pseudocysts), systemic organ dysfunction, or both. A modern classification of AP severity has recently been proposed based on the factors that are causally associated with severity of AP. These factors are both local (peripancreatic necrosis) and systemic (organ failure). In AP, inflammation is initiated by intracellular activation of pancreatic proenzymes and/or nuclear factor-$\kappa$B. Activated leukocytes infiltrate into and around the pancreas and play a central role in determining AP severity. Inflammatory reaction is first local, but may amplify leading to systemic overwhelming production of inflammatory mediators and early organ failure. Concomitantly, anti-inflammatory cytokines and specific cytokine inhibitors are produced. This anti-inflammatory reaction may overcompensate and inhibit the immune response, rendering the host at risk for systemic infection. Currently, there is no specific treatment for AP. However, there are several early supportive treatments and interventions which are beneficial. Also, increasing the understanding of the pathogenesis of systemic inflammation and the development of organ dysfunction may provide us with future treatment modalities.

## 1. Introduction

Acute pancreatitis (AP) is a disease of varied etiology, yet each produces a similar pattern of disease, indicating that they all converge at a common point, to initiate a cascade of events resulting in AP [1, 2]. The overwhelming evidence indicates that this common event involves the premature activation and retention of proteases within the acini which causes cellular injury [3]. In parallel or alternatively to these events, the proinflammatory transcription factor nuclear factor-$\kappa$B (NF-$\kappa$B) is activated resulting in the upregulation in expression of cytokines and chemokines [4]. Consequently, recruitment of inflammatory cells, such as neutrophils and macrophages, takes place [5]. This further amplifies the inflammatory reaction and the extent of pancreatic injury. The degree to which these mediators escape into the circulation determines the nature of the systemic inflammatory response. Finally, if the resolution fails to occur, pancreatic infection may supervene.

This paper outlines the clinical course of AP, especially the systemic inflammation and the key mediators that underpin it. We detail the importance of organ failure to outcome. Finally, we speculate upon the future prospects for immunomodulating treatments to act as therapeutic damage-control agents.

## 2. The Clinical Course of Acute Pancreatitis

According to the Atlanta classification, severe AP is defined by the presence of local complications and/or organ failure (shock, pulmonary insufficiency, and renal failure) [6]. The Atlanta classification has been criticized because it failed to discriminate between patient subgroups with different

outcomes; for example, it categorized patients with a local complication and a favorable outcome as severe [7]. Hence, a determinant-based classification of AP severity has recently been proposed [8]. This classification is principally based on the factors that are causally associated with severity of AP. These factors are called "determinants" and they are both local and systemic. The local determinant of severity is necrosis of the pancreas and/or peripancreatic tissue termed *(peri)pancreatic necrosis.* The systemic determinant of severity is covered by the term *organ failure. Organ failure* is defined for 3 organ systems (cardiovascular, renal, and respiratory) using the SOFA (Sepsis-Related Organ Failure Assessment) score [9] or when the relevant threshold is breached, as follows:

(i) *cardiovascular*: need for inotropic agent,

(ii) *renal*: creatinine $\geq 171\,\mu$mol/L ($\geq 2.0$ mg/dL),

(iii) *respiratory*: $PaO_2/FiO_2 \leq 300$ mmHg (40 kPa).

Organ failure is further characterised as transient, if evident for less than 48 hours or persistent, if longer. Thus, four categories of severity may be derived.

(i) *Mild acute pancreatitis* is characterized by the absence of both (peri)pancreatic necrosis and organ failure.

(ii) *Moderate acute pancreatitis* is characterized by the presence of sterile (peri)pancreatic necrosis and/or transient organ failure.

(iii) *Severe acute pancreatitis* is characterized by the presence of either infected (peri)pancreatic necrosis or persistent organ failure.

(iv) *Critical acute pancreatitis* is characterized by the presence of infected (peri)pancreatic necrosis and persistent organ failure.

The classification of AP severity will continue to evolve and further modifications will be required in the future, driven by clinical experience and evaluation of the proposed new system.

Organ failure develops often early in the course of AP. About half of the patients who will develop organ failure will have it at admission or within 24 hours after admission [10–12]. The most common organ failure in severe AP is respiratory failure. In the presence of a single organ failure, mortality is less than 10%, whereas in multiorgan failure the mortality rate is 35–50% [1]. Organ failure may occur in the renal, hepatic, cardiovascular, digestive, neurologic, coagulation, endocrine, or immunologic system [13]. Also, failure of different organs has differing effects on prognosis [14]. If organ failure is already present on admission, this progresses to multiorgan failure in most of the patients and the mortality rate is high [11]. Indeed, half of the mortality takes place during the first week of the disease and is related to severe multiorgan failure [15]. The second peak of mortality occurs much later and is related to organ failure due to infectious complications and sepsis [15]. The duration of organ failure is also critical. If organ failure is determined as transient (<48 h), the patient will have a favourable outcome. In a case with persistent (>48 h) organ failure, the risk of morbidity and mortality is increased [16, 17]. Nevertheless, early identification of patients who develop a severe AP with organ failure would be essential to improve prognosis by earlier intervention with appropriate resuscitation in specialized hospitals.

At present, no specific medical treatment of AP exists. Treatment of the disease is mainly supportive and targeted to prevent and treat systemic complications. It is evident that delayed admission to intensive care unit worsens prognosis in patients with severe AP and early organ failure [18]. Indeed, prognosis of severe AP has improved due to early and aggressive conservative treatment in intensive care units. Early endoscopic retrograde cholangiopancreatography (ERCP) is recommended in the management of biliary AP with biliary obstruction [19]. Enteral feeding is considered to be a preferred method of delivering nutrition in severe AP and results in reduction of infectious complications and the need for surgery and lowers mortality rate [20, 21]. Later in the course of AP, infection complications are the major cause of morbidity and mortality. Therefore, prophylactic antibiotics have been used. However, serious concerns exist about a policy of antibiotic prophylaxis [22, 23]. In a study by Beger et al. carried out before antibiotic prophylaxis became widely used, organisms cultured from infected pancreatic necrosis were predominantly of gastrointestinal origin (*Escherichia coli* and *Bacteroides* spp.) [24]. The microbiology results of a later study, comparing perfloxacin and imipenem in pancreatic necrosis, were dominated by methicillin resistant *Staphylococcus aureus* and *Candida* spp. [25]. This development is important because evidence exists that indicates that infection with fungi and drug resistant organisms is associated with a significantly increased mortality [26]. Furthermore, results from two further randomised controlled trials fail to show a benefit for prophylaxis with antibiotics [27, 28]. The largest and most recent study of antibiotic prophylaxis was a multicenter, prospective, double-blind, and placebo-controlled randomized study set in 32 centres within North America and Europe [28] enrolled 100 patients with clinically severe, confirmed necrotizing pancreatitis: 50 received meropenem and 50 received placebo. This study demonstrated no statistically significant difference between the treatment groups for pancreatic or peripancreatic infection, mortality, or requirement for surgical intervention and did not support early prophylactic antimicrobial use in patients with severe acute necrotizing pancreatitis.

The role of probiotic therapy was evaluated by the Dutch Acute Pancreatitis Study Group [29]. The PROPATRIA trial was a double-blind, placebo-controlled, and randomised multicentre trial that aimed to show a reduction in infectious complications by the enteral use of a multispecies probiotic preparation in patients with predicted severe AP. The rationale for this study was clearly established. Infection of pancreatic necrosis is the major cause of death in (AP). Bacterial translocation across the gastrointestinal mucosal barrier is the mechanism thought to be responsible for this complication. Antibiotic prophylaxis has failed to reduce infectious complications. In contrast, probiotics are non-pathogenic bacteria that, on delivery to the host's intestinal tract, may reduce bacterial translocation by preventing

bacterial overgrowth of pathogens, maintaining the gastrointestinal mucosal barrier, and by exerting local and systemic immunomodulatory effects. Thus, this was an eminently suitable topic for a randomised controlled trial. The finding of 15 excess deaths in this study in the probiotic group was unexpected and, indeed, shocking. Nine patients in the probiotics group developed bowel ischaemia (eight with fatal outcome), compared with none in the placebo group. However, the scientific evaluation of this new treatment may have saved many more lives; as it has prevented the *ad hoc* and widespread adoption of probiotic therapy, based on anecdote and personal bias, which would otherwise almost inevitably have occurred. This is precisely why randomised controlled clinical trials are performed. The mechanism of bowel ischaemia in the probiotics group remains a matter of further investigation. The administration of probiotic bacteria on top of enteral nutrition might have increased local oxygen demand, with a combined deleterious effect on an already critically reduced blood flow. A second possible explanation could be that the presence of probiotics caused local inflammation at the mucosal level. However, in view of the fatal nature of these complications, the administration of any type of probiotic in this category of patients must strongly be advised against.

Diagnosis of pancreatic necrosis is based on findings in dynamic contrast-enhanced computed tomography [30]. While determination of pancreatic necrosis requires contrast enhanced computed tomography, it may be inadvisable in a clinical emergency setting due to renal insufficiency and hypovolemia. Nowadays, it is recognized that, in terms of morbidity and mortality, organ failure is the most important factor [31, 32], regardless of the presence or absence of pancreatic necrosis, which develops later [33] and therefore the timing of contrast enhanced tomography may be delayed. The full extent of pancreatic necrosis cannot be appreciated until at least 3 days after symptom onset. It is recommended that patients with persisting organ failure, signs of sepsis, or clinical deterioration occurring after an initial improvement undergo CT scanning, which should be performed according to a pancreas protocol and all patients should receive oral and intravenous contrast [34].

Differentiation between sterile and infected necrosis is essential for those with >30% necrosis on CT and persistent symptoms or those with any degree of necrosis and signs of sepsis. This is achieved by fine needle aspiration for bacteriology of pancreatic or peripancreatic necrosis or the presence of retroperitoneal gas on CT [34, 35]. Patients with sterile necrosis should usually continue to be managed conservatively. The diagnosis of infected necrosis is an indication for radiological or surgical intervention.

Although good outcomes have been reported in patients with infected pancreatic necrosis managed by radiologically placed percutaneous drains, standard treatment remains surgical necrosectomy [34]. Novel minimal access approaches to necrosectomy have been described with particularly encouraging results obtained by a retroperitoneoscopic approach, combined with postoperative continuous irrigation [36]. A recent randomised controlled trial provided support for a "step-up" approach rather than primary open necrosectomy. This approach attempts to control sepsis with a radiologically placed drain and only if this is unsuccessful does the patient proceed to a necrosectomy. A minimally invasive approach is tried first, progressing to an open approach if sepsis does not fully resolve [37].

Generally, it is agreed that surgery should be postponed for as long as possible in AP. There exist, however, cases when intra-abdominal hypertension necessitates surgical decompression in an early phase of the disease [38].

## 3. Local and Systemic Inflammation

Irrespective of the etiological factor, triggering events of AP leads to a premature activation of pancreatic proteases as a result of intracellular colocalization of the digestive and lysosomal enzymes [2, 3]. Intracellular activation of pancreatic proenzymes leads to destruction of the parenchyma and autodigestion of the pancreas [2, 39–41]. It has recently been shown that autophagy (the principal cellular degradative pathway) in AP is activated but also impaired due to the defective function of lysosomes [42, 43]. Consequently, acinar cells become more prone to the deleterious effects of activated zymogens which will eventually lead to necrosis and inflammation [43, 44]. Also, there is an emerging body of evidence which suggests that the ubiquitous inducible transcription factor NF-$\kappa$B plays an important role in various stages of AP by mediating the expression of numerous genes involved in inflammation [4]. Since AP also affects extrapancreatic tissues, it was not surprising that NF-$\kappa$B activation could also be found outside of the pancreas. Although a link between pancreatic NF-$\kappa$B and trypsinogen activation in AP has been a matter of debate, recent results suggest that these processes may be unrelated and both can induce inflammation [4]. The earlier events may be mediated by intracellular $Ca^{2+}$ signaling and reactive oxygen species [45].

Proinflammatory mediators in AP include various cytokines (e.g., tumour necrosis factor $\alpha$ (TNF-$\alpha$), interleukin (IL)-1$\beta$, IL-2, IL-6, and IL-18), chemokines (e.g., IL-8, monocyte chemoattractant protein-1, macrophage inflammatory protein-1, monocyte chemoattractant protein-1, and growth-related oncogene-$\alpha$), adhesion molecules, platelet-activating factor, and reactive-oxygen and reactive-nitrogen species [45–49]. In mild AP, local inflammation is controlled by the host's inflammatory response with localization of proinflammatory mediators in the affected area. In other severe cases, injury and inflammation in the pancreas can proceed to systemic inflammation causing systemic inflammatory response syndrome (SIRS) (Table 1) [50]. In some cases, this response is overwhelming and disseminated, when proinflammatory mediators, such as TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-8, are released into the circulation [51, 52]. In the liver, IL-6 is a potent inducer of synthesis of acute phase proteins, that is, C-reactive protein and procalcitonin [53]. Also, circulating neutrophils and monocytes become activated and express adhesion molecules (i.e., CD11b) and release their proteolytic enzymes and oxygen radicals, which damage vascular endothelial cells and organ parenchymal cells. Vascular endothelium is activated in the whole

Table 1: Definitions of SIRS, sepsis, and MODS. Modified from American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis, 1992.

| |
|---|
| *Systemic Inflammatory Response Syndrome (SIRS)*: this response is manifested by two or more of the following conditions: |
| Temperature >38°C or <36°C |
| Heart rate >90 beats/min |
| Respiratory rate >20 breaths/min or $PaCO_2$ <4.3 kPa. |
| White blood cell count <4,000 or >12,000 cells/$mm^3$, or >10% immature (band) forms. |
| *Sepsis*: this response is manifested when two or more of the above conditions occur as a result of infection. |

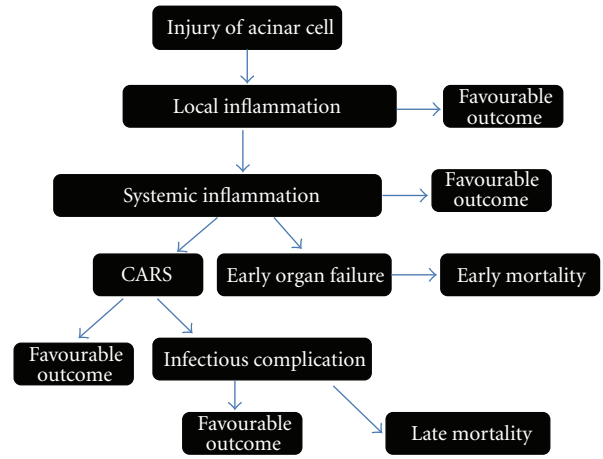


Figure 1: Inflammatory response in acute pancreatitis.

body and the expression of cellular adhesion molecules is upregulated which results in neutrophil extravasation and activation [54]. Endothelial permeability is enhanced leading to large amounts of tissue fluid (edema). This together with microvascular disturbances (i.e., vasoconstriction, inadequate perfusion, and increased blood viscosity) leads to lack of oxygen, which results in dysfunction and injury of vital organs [55, 56]. It has been demonstrated in experimental severe AP that microcirculatory disorders are present not only in the pancreas but also in the colon, liver, kidneys, and lungs [56]. In fact, lungs and kidneys are commonly injured organs in AP as they have an extensive capillary bed. Markers of hypovolemia (hemoconcentration, tachycardia, oliguria, and hypotension) are often seen in severe AP on admission.

The coagulation system is an integral part of the inflammatory response. It has been shown that coagulative disorders occur in severe AP [57–59] and they are related to severity of the disease and development of organ failure [60]. Systemic coagulation activation results in thrombosis in small and middle-sized vessels in many organs, which is called disseminated intravascular coagulation (DIC). Thrombocytopenia is a common sign of severe AP and is caused by excessive consumption of platelets in DIC. D-dimer is also a marker of DIC and has been shown to be high in severe AP [61]. Protein C is a natural anticoagulant in blood and has an essential role in the regulation of the coagulation cascade in inflammation. Protein C is activated by thrombin-thrombomodulin complex at the endothelial surface [62]. Activated protein C (APC) inactivates factor *V* and factor VIII and inhibits thrombin generation. APC also has anti-inflammatory effects in experimental studies [63, 64].

## 4. Compensatory Anti-Inflammatory Response Syndrome (CARS) and Immunosuppression

With the release of proinflammatory mediators, anti-inflammatory cytokines are concomitantly produced leading to a compensatory response syndrome (CARS) [65, 66]. High circulating concentrations of the anti-inflammatory mediators such as TNF-$\alpha$ receptors, IL-10, IL-11, and IL-1ra have been documented in AP [67–71]. If the anti-inflammatory response is adequate, the patient recovers. In a case of insufficient control, a proinflammatory burst leads to distant organ dysfunction. Anti-inflammatory reaction may also overcompensate and inhibit the immune response excessively rendering the patient susceptible to immunosuppression and infectious complications. Even though CARS happens in the early phase of severe AP, infectious complications are a result, at least partly, of impaired cellular immunity and occur in a later stage of the disease [24] (Figure 1).

Monitoring of HLA-DR expression is a useful marker for identifying monocyte function and is closely correlated with the clinical course in AP. In immunosuppression, defective host defence mechanisms include functional disturbances in monocytes which are characterized by a markedly reduced human leukocyte antigen (HLA)DR expression, and a diminished synthesis of proinflammatory cytokines, for example, TNF-$\alpha$ [72, 73]. IL-10, the most important anti-inflammatory cytokine, downregulates a number of proinflammatory cytokines [74]. In addition, it is able to decrease monocyte HLA-DR expression [75]. Monocytes with low HLA-DR density show impaired antigen presentation capacity [76]. IL-1ra is a specific antagonist to IL-1$\beta$, binds competitively to the IL-1 receptor, and blocks IL-1 mediated responses [77].

As early as 1989, Garcia-Sabrido et al. showed a correlation between poor outcome and anergy to delayed-type hypersensitivity testing as a marker of altered cellular immune function in AP [78]. Although IL-10 is an anti-inflammatory mediator, plasma IL-10 concentration is high in the very early phase of severe AP and is even a promising predictive marker of organ failure [70, 71]. Monocyte HLA-DR expression decreases at the early stage of severe AP [12, 79, 80]. Decreased monocyte HLA-DR expression predicts development of organ failure [12], development of secondary infections [66], and fatal outcome [80] in AP. There is a significant negative correlation of high plasma concentrations of IL-6 and IL-10 with HLA-DR expression in AP [66]. At present, the level of immunosuppression

can be measured by laboratory means but this is not yet widespread in clinical practice. For example, chemiluminescent immunoassays are available for IL-10 and IL-6 but for monocyte HLA-DR measurement, flow cytometry is needed.

Li et al. recently investigated the expression of sphingosine kinase 1 (SphK1)/sphingosine 1-phosphate (S1P) in immune-effector cells, including neutrophils, monocytes, and lymphocytes, of 22 patients with severe AP in an effort to identify the role for SphK1/S1P in modulating the inflammatory response [81]. The expression of SphK1 and SphK activity was markedly increased in peripheral immune cells in the early stage of SAP and then reduced in the restoration stage in the patients. Moreover, they found that the level of $S1P_3$ mRNA in peripheral neutrophils and lymphocytes of SAP patients was significantly elevated in the early stage as compared with the healthy volunteers, and it reduced in the restoration period. SphK1 expression on human peripheral neutrophils, monocytes, and $CD4^+$ T lymphocytes were positively correlated with the APACHE II and levels of serum proinflammatory cytokines including TNF-($\alpha$), IL-1 ($\beta$), and IL-6. These observations show a possible immunomodulating role for SphK1/S1P signaling in inflammatory response in SAP, suggesting that regulation of SphK1/S1P pathway may represent novel targets in the treatment of SAP [81].

## 5. Immune-Modulation Therapy

At present, there is no specific medical therapy for AP. Patients with mild AP recover without intervention and novel treatment strategies should focus on patients with severe AP and a risk of organ failure. There is increasing evidence that in the early phase of AP, excessive leukocyte activation and inflammatory cytokine burst are critical for development of early organ failure and increased risk of MODS [82–85]. New therapeutic strategies attempting to prevent the activity of proinflammatory mediators or to block their synthesis have been evaluated as therapeutic options.

Progress in AP research is hampered by the inaccessibility of the human pancreas to observation, the lack of safe pancreatic biopsy techniques, difficulty in distinguishing initiating events from the concomitant inflammatory response, and the self-destructive nature of the disease process itself. Consequently, most of our knowledge about AP is derived from animal models of the disease, but these suffer from a lack of translational impact when applied to the human condition [86, 87]. Much remaining information results from studies of circulating inflammatory mediators and cells. Unsurprisingly, progress in the understanding and treatment of AP has been slow. The key to future advances must lie in obtaining data upon humans who have developed this disease, in comparison with meaningful controls.

One of the main interests has been TNF-$\alpha$, which is the key regulator of other proinflammatory cytokines and a priming activator of immune cells [88]. In clinical studies, accurate evaluation of the role of TNF-$\alpha$ is problematic. Reasons explaining this general lack of correlation of this key inflammatory mediator with disease severity have concentrated on its short half-life, phasic release, the masking effects of circulating inhibitors, and its mainly paracrine level of function. It is important to appreciate that mainly tissue levels, not serum concentrations, are responsible for the vast majority of the biological effects of cytokines [4].

When given prophylactically or soon after the induction of experimental AP, anti-TNF antibodies decreased the severity of the disease in a rat [89] and mouse [90] model of AP, but there are also discouraging results [91]. Also, the clinical trials with anti-TNF in sepsis have not been successful [92, 93]. Blockage of the cytokine cascade at the level of the IL-1 receptor with its naturally occurring specific antagonist (IL-1ra) decreases pancreatic damage in caerulein-induced AP in mice [94]. Further, anti-inflammatory therapy with IL-10 agonist decreases the severity in a caerulein-induced AP in mice [95] and diminishes acute lung injury in a rabbit model of AP [96]. Selective inhibition of IL-1$\beta$ in taurocholate-induced AP in rats [97] and inhibition of IL-8 in a rabbit model of AP [98] have shown beneficial effects. In experimental studies with mice, treatment with antibodies against adhesion molecules like intercellular adhesion molecule (ICAM)-1 has been effective [99–101]. Also, in a rat model of severe AP, endothelin receptor blockage was reported to reduce capillary leakage and improve microcirculation [102, 103]. In human studies, the use of a platelet-activating factor antagonist initially seemed promising in AP [104, 105], but later trials could not confirm the beneficial effects [10]. However, treatment attempts at blocking various single proinflammatory responses seem to be a flawed strategy. In the complex network of inflammatory response, a combination therapy to inhibit several proinflammatory agents may be more useful [106, 107]. Clinical trials of anti-inflammatory therapy has been difficult to conduct, as many of the patients present at a late stage of the disease, when organ failure is about to develop or may already be present [10]. It seems that the therapeutic window for anti-inflammatory therapies is very limited in clinical practice (for more details, see Section 6) [10] as the patient may be already on his way to CARS or even in immunosuppression.

Systemic inflammation and organ failure in severe AP show the same characteristics as the mechanisms induced by sepsis, major surgery, trauma, or severe burn [108]. Thus, research results from these conditions should be relevant to severe AP. For example, in sepsis, decreased protein C level in blood correlates with poor prognosis [109]. In severe AP, protein C pathway defects have been shown to be associated with development of organ failure [60]. In meningococcemia patients with DIC, treatment with APC has prevented development of organ failure and decreased mortality [110]. In patients with severe sepsis, treatment with APC was safe and was thought to result in decreased mortality [111, 112]. However, a recent randomized, double-blind, placebo-controlled, and multicenter trial has demonstrated that APC failed to significantly reduce mortality at 28 or 90 days in patients with septic shock [113]. In fact, due to the latter results, recombinant human APC was withdrawn from the market to treat sepsis by Eli Lilly in 2011. In a rat model of severe AP, APC treatment reduced inflammation

in the pancreas and lungs and improved survival [114]. Recombinant human APC was also found to ameliorate cerulein-induced (mild) AP through apoptotic and NF-$\kappa$B pathways [115]. However, in a placebo-controlled clinical testing in 32 patients with severe AP, the APC treatment of severe AP did not bring clinical benefit for the patients [116].

Immunosuppression plays an important role in the development of secondary infections in the later course of AP. Treatment of patients with these late complications is a challenge with high mortality rates. Therefore, novel methods to diagnose and monitor the level of immunosuppression would be helpful. In clinical work therapeutic means to restore impaired host defence mechanisms would be helpful in patients with high risk of complications. Immunostimulation with interferon-gamma has proven to be beneficial in anergic septic patients [73]. In severe AP and sepsis, monocyte function is defective [73, 117]. Granulocyte-macrophage colony stimulating factor (GM-CSF) treatment was reported to increase monocyte HLA-DR expression and TNF-$\alpha$ production capacity and may also improve the clinical course in septic patients [118, 119]. *In vitro* study of monocytes taken from patients with AP showed that priming of cells by interferon-gamma and GM-CSF increases HLA-DR expression and restores lipopolysaccharide-induced TNF-$\alpha$ secretion [117]. These immunomodulatory therapies and the means to find the patients to benefit from them would be of utmost importance. However, further research must be done before optimal and individualized immunomodulatory treatment is possible.

The interventional window: the optimal timing for delivering damage-limiting interventions was described by Norman [120]. This "interventional window" exists between the time of patient presentation and the onset of the development of organ dysfunction. Typically, the former occurs at 12–18 hours after disease onset whilst, for the latter, the incidence rises rapidly on the second and third day, distinguishing those likely to have a complicated attack from those likely to have a mild attack. Cytokine production begins shortly after disease onset but does not peak until 36–48 hours later. This has been elegantly demonstrated using post-ERCP pancreatitis as a human model to examine the initial cytokine response after the initiation of the disease [121]. This scenario provides a potential therapeutic window of opportunity that begins at hospital presentation and may last for 2-3 days, during which inflammatory mediator antagonism could be employed, in an attempt to attenuate the development of MODS. However, the experience of clinical trials, such as the phase III lexipafant (PAF antagonist) trial, challenge this [10]. In this investigation, the primary hypothesis, upon which the power calculation was based, was invalidated by the unexpected finding that 44% of patients had organ failure on entry into the study; only 14% developed new organ failure.

## 6. Future Directions

Immunomodulation may represent a potential way to improve outcome in severe AP. However, it requires a thorough knowledge of underlying mechanisms and the patient's immunological state. At the moment we lack the essential information in order to modulate immune response, and more basic research is needed. Monitoring the state of immune dysfunction by monocyte HLA-DR expression during hospitalization of severe AP patients seems promising. Deeper understanding of the pathophysiology of AP is important to permit the design of effective interventions concerning the inflammatory response process. It is necessary to accurately identify patients with severe AP who are at risk of organ failure in order to transfer them urgently to an intensive care unit. Whether monitoring signaling pathways of circulating leukocytes, such as NF-$\kappa$B, signal transducers and activators of transcription (STATs), and members of mitogen activated protein kinase family helps us to find the patients at risk for secondary infections and, thus, late organ failure is at present under research [122–124]. Since multiple mediators are involved in the pathogenesis of AP, treatment strategies will probably focus on combination therapy in the future. Intuitively, it would seem helpful to depress the proinflammatory reaction in the patients at risk of excessive immune suppression so that inappropriate CARS would be prevented. However, it is evident that the window for anti-inflammatory therapy to suppress excessive activation of the inflammatory response is very limited. Finally, the analysis of signaling patterns of leukocytes may reveal novel therapeutic targets in severe AP.

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*Review Article*

# Vascularisation Pattern of Chronic Pancreatitis Compared with Pancreatic Carcinoma: Results from Contrast-Enhanced Endoscopic Ultrasound

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Discriminating between focal chronic pancreatitis and pancreatic cancer is always a challenge in clinical medicine. Contrast-enhanced endoscopic ultrasound using Doppler techniques can uniquely reveal different vascularisation patterns in pancreatic tissue alterated by chronic inflammatory processes and even allows a discrimination from pancreatic cancer. This paper will describe the basics of contrast-enhanced high mechanical index endoscopic ultrasound (CEHMI EUS) and contrast enhanced low mechanical index endoscopic ultrasound (CELMI EUS) and explain the pathophysiological differences of the vascularisation of chronic pancreatitis and pancreatic carcinoma. Furthermore it will discuss how to use these techniques in daily clinical practice.

## 1. Introduction

Adenocarcinoma of the pancreas is one of the solid carcinomas with the worst prognosis [1, 2]. The medium survival rate of an untreated pancreatic carcinoma is 6 months after the diagnosis is made [3]. Despite better diagnostic methods the detection of pancreatic cancer in an early stage is still a rare event [4]. This is due to the fact that the organ is not easy to investigate by percutaneous ultrasound and so far we do not have screening strategies even in high risk patients [5]. Even when diagnosed in time and treated by surgery the medium survival rate is 13.9 months [6]. The likely reason for the poor outcome of pancreatic cancer patients is the early micrometastatic spreading.

Unfortunately pancreatic carcinoma can mimic focal chronic pancreatitis. Because of the good resolution, modern diagnostic tools like endosonography, computed tomography (CT), or magnetic resonance imaging (MRI) can detect even small lesions in the pancreas down to 5 mm or less in size; however the discrimination of these lesions remains a challenge. This is due to the fact that even the imaging produced by contrast-enhanced CT [7] or MRI scanners [8] can be inconclusive. It is well known that adenocarcinoma of the pancreas normally shows less contrast enhancing effect than the surrounding pancreatic tissue and should be therefore possible to identify [9]. However in at least 10% of cases, these tumors have no visible contrast enhancing differences and remain hidden for CT and MRI scanners [7]. This effect can be even higher in patients with inflammatory pancreatic tissue. Chronic inflammation of the pancreas can lead to impaired contrast enhancing behavior of the normal tissue and can therefore hide the tumor. This is even more important because most adenocarcinomas (approx. 65%) are localized in the pancreatic head and lead to incomplete or complete pancreatic duct invasion with secondary chronic inflammation of the remaining pancreas [10]. In those cases the sensitivity and specificity of contrast-enhanced methods can go down to round about 70% (Table 1).

After introducing positron emissions tomography (PET) into clinical practice tumor diagnosis was supposed to be much more reliable. The advantage of using PET for diagnosis is the option of metabolic imaging of processes. However pancreatic carcinoma can have the same metabolic

Table 1: Discrimination of pancreatic lesions using contrast enhancement patterns in patients with chronic pancreatitis [11].

| | Number | Size of lesion (cm) | Hypervascularisation | Hypovascularisation |
|---|---|---|---|---|
| Chronic pancreatitis | 71 | 3.26 ± 0.75 [1.5–4.0] | 53 | 18 |
| Pancreatic carcinoma | 81 | 3.47 ± 1.04 [1.8–4.0] | 24 | 57 |

characteristics like chronic pancreatitis and therefore PET was also not able to produce reliable results [12, 13].

Histology is so far the only definitive diagnostic option for discrimination between pancreatic carcinoma and chronic pancreatitis. Because of sampling errors not even percutaneous biopsy of the pancreas is reliable and comes with the risk of cancer cell seeding [14]. This is the reason that preoperative biopsy of suspected pancreatic carcinoma is not recommended in recent guidelines like the German guideline for pancreatic carcinoma [15] or the international guideline for pancreatic cancer [16]. The guidelines recommend operative resection in every case of suspected pancreatic carcinoma which appears resectable. However this comes with an insignificant risk of morbidity and even mortality especially if the suspicion is not confirmed postoperatively and turns out to be a chronic pancreatitis [17, 18].

Intraoperative cytology seems to be an effective method to get the diagnosis [19] but still remains an invasive procedure. Endoscopic fine needle puncture of the lesion seems to be reliable in the absence of chronic pancreatitis [20]; however it cannot always provide reliable results in the presence of chronic pancreatitis again mostly because of sampling errors [21]. In addition even endoscopic fine needle cytology is not recommended per example by the German guidelines of pancreatic diseases because of the marginal risk of cell seeding and the catastrophic prognosis of the metastatic disease [22]. However there is increasing evidence that cell seeding is a rare phenomenon and due to the fact that the area of puncture will be removed by the operation is nearly neglectable [23], still it would be preferable to preselect patients for endoscopic fine needle puncture and furthermore the targeting area.

So far the problem of differential diagnosis of focal chronic pancreatitis and pancreatic carcinoma remains unsolved.

## 2. Contrast-Enhanced Ultrasound: A Step Forward in the Differential Diagnosis of Pancreatic Diseases

Contrast-enhanced ultrasound was already performed from 1982 mainly for echocardiographic reasons to enhance the echo signal [24]. In 1990 a first generation ultrasound contrast enhancer appeared for abdominal ultrasound [25]. After the first positive results in transcutanous ultrasound of the liver, contrast-enhanced ultrasound of the parenchymatous organs was born [26].

The gas bubbles of the contrast enhancers of the first generation were not stable enough for continuous ultrasound scanning. This meant that the sweep technique had to be developed. In this technique the contrast enhancer was injected and scanning was performed with high mechanical index after 2-3 minutes with a sweep over the suspected lesion. During the sweep the bubbles were destroyed and a contrast-enhanced image could be created normally with the help of the power Doppler mode. Especially for discrimination of liver lesions this method revealed astonishing results [27, 28].

The main disadvantage of this method was the scattered scanning of the lesion. Basically it was not different to CT and MRI scans with only one or two short chances to see the contrast enhancer effect and the lack of continuous scanning.

After a new contrast enhancer generation (SonoVue, Bracco) was introduced 2001/2002 [29] soon a new ultrasound technique was established. Ultrasound scanning with low mechanical index [30] could evolve [31]. These techniques allowed continuous ultrasound scanning of the contrast enhancer influx and distribution in the parenchymatous organs and therefore produced new insights into contrast enhancing dynamics.

The main advantage of the possibility of continuous scanning is the real-time viewing of the contrast enhancer effects.

Most studies were performed for liver lesions. This is due to the fact that the liver is fed by two different vessel systems (arterial blood and portal vein blood). Especially the portal vein system makes the differentiation of liver tissue-like lesions with portal veins inside (e.g., focal nodular hyperplasia) from metastatic tissue without portal veins inside (e.g., colonic cancer metastasis) easy [32, 33].

It has to be mentioned that the use of contrast-enhanced ultrasound is not approved for other parenchymatous organs than the liver. However lots of studies have already been done for basically all parenchymatous organs including the pancreas so that recently the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) published their guidelines for clinical practice [34].

Percutaneous contrast-enhanced ultrasound for pancreatic diseases is nowadays nearly as widespread in clinical practice as liver investigations [35–38]. Although percutaneous ultrasound has already an incredible resolution, it is sometimes hampered by overlying air or patient's physiognomy [39]. Using the contrast enhancing effect in endoscopic ultrasound was a logical progression, but the technique could not develop quickly because of the lack of low mechanical index high resolution ultrasound probes.

Some interesting studies however could show the feasibility of the contrast-enhanced endoscopic ultrasound in a color Doppler setting using high mechanical index ultrasound [40–42]. This was the beginning of a new understanding of the underlying processes which made it possible to give a new dimension to the differential diagnosis of chronic pancreatitis to pancreatic carcinoma.

## 3. Understanding the Neovascularisation of Chronic Pancreatitis and Pancreatic Carcinoma for Differential Diagnosis

Becker et al. could show that using ultrasound contrast enhancer for endoscopic ultrasound in a Doppler mode (Power Doppler mode) was able to reveal different enhancement patterns from chronic pancreatitis and pancreatic carcinoma [43]. In a preliminary study they assumed they had produced a contrast enhancing effect in the pancreatic tissue like the Levovist studies in the liver or contrast enhancing effects of CT or MRI scans. They were able to show a contrast enhancing effect in all patients studied with chronic pancreatitis and a lack of contrast enhancing effect in almost all patients with pancreatic carcinoma. The basic misunderstanding at that time was that using a Doppler technique in high mechanical index mode can only lead to a contrast enhancing effect in vessels but not in the pancreatic tissue. The tissue enhancing or nonenhancing effect was based on multiple microvessels combined with a blooming effect after contrast enhancer influx.

Understanding those basics led to the realisation that later on in the scanning process the underlying multiple microvessels could be imaged after the self-limitation of the blooming effect. Once the microvessels could be visualized two different vessel patterns appeared [44].

Typical for chronic pancreatitis is a netlike homogenous and rich microvessel system over the whole lesion. In contrast, pancreatic carcinoma shows an irregular and diminished microvessel system without a netlike appearance. It should be emphasized that those microvessel patterns could not be detected before the introduction of contrastenhanced endoscopic ultrasound. To visualize those vessels, a method with a high resolution has to be combined with a contrast technique of microvessels. CT and MRI scan as well as angiographic methods are not able to produce this kind of resolution. Doppler techniques alone even with high resolution ultrasound probes cannot provide the necessary effect to analyze those vessel systems either [45].

However, the knowledge of the different types of microvascularisation cannot discriminate chronic pancreatitis from pancreatic cancer in every case. It has to be taken into account that small cell adenocarcinomas of the pancreas with a rich vessel system and forms of chronic pancreatitis with abundant fibrous tissue and a diminished vessel system exist. Using the technique of contrast-enhanced endoscopic power Doppler ultrasound revealed another unique and more reliable display of the microvessel system. Whereas the neovascularisation of the chronic inflammatory process creates arterial and venous vessels without any signs of compression and basically in the same size, the neoplastic neovascularisation is characterized by just visible arterial microvessels without any venous microvessels visible. The method to discriminate between these kinds of vessels simply involves performing pw-Doppler scanning during the available contrast-enhancing effect of approximately 3 minutes. The fact that no venous vessels are visible in pancreatic carcinomas using contrast enhanced high mechanical Doppler endosonography means this method works even when the tumor shows an atypical rich vessel system [46].

Intraparenchymal pressure differences between pancreatic carcinoma and chronic pancreatitis might be a major cause of this unique phenomenon. This could also be indirectly shown by contrast enhanced endoscopic ultrasound. The comparison of the resistance index of arterial vessels in chronic pancreatitis to pancreatic carcinoma did show a relevant difference. In a high percentage the arterial microvessels of the pancreatic carcinoma showed a resistence index above 0.7, whereas the arterial microvessels of the areas with chronic pancreatitis showed a resistance index below 0.7. This means that neoplastic microvessels have a much higher intraluminal pressure than microvessels of the inflammatory neovascularisation [47]. It should be pointed out that the assumed difference of the intraparenchymal pressure is only a thesis which requires further studies to be backed up.

Histopathological investigations could confirm the basic pattern of microvascularisation of pancreatic carcinoma however it seems to be difficult to discriminate between arterial and venous vessels in histopathology and so no attention was given so far to this phenomenon [48].

Using the method of contrast enhanced high mechanical endoscopic ultrasound with pw-Doppler vessel analysis, pancreatic carcinoma can be discriminated from chronic pancreatitis with a sensitivity and specificity over 90 percent [49] (see Figures 1 and 2).

## 4. Perfusion Studies Using Contrast-Enhanced Low Mechanical Index Endoscopic Ultrasound

In 2010 contrast-enhanced low mechanical index endoscopic ultrasound evolved [50]. Because of the accuracy of the method for discriminating of liver lesions [51–54] and similarly good results for the discrimination of pancreatic lesions [55–58] in percutaneous ultrasound, there was hope that the method could increase the efficacy of endoscopic ultrasound for the discrimination of chronic pancreatitis from pancreatic carcinoma even further (see Figure 3). Initial experiences showed a reliable display of microvessel perfusions down to a size of a single contrast enhancer bubble [59–61]. However, Doppler analysis in combination with this method is not available so far and this makes the differentiation of arterial and venous microvessels impossible. Unfortunately, analyzing global perfusion behaviors of the lesions with this technique does not produce similar or better results than the method described above of contrast-enhanced high mechanical index Doppler endoscopic ultrasound [62]. This is mostly due to the fact that pancreatic lesions caused by chronic inflammatory processes often show impaired perfusion using this technique and cannot therefore be discriminated.

## 5. A Special Case: Autoimmune Pancreatitis

Autoimmune pancreatitis is a rare form of chronic pancreatitis and can involve the whole pancreatic organ as well as

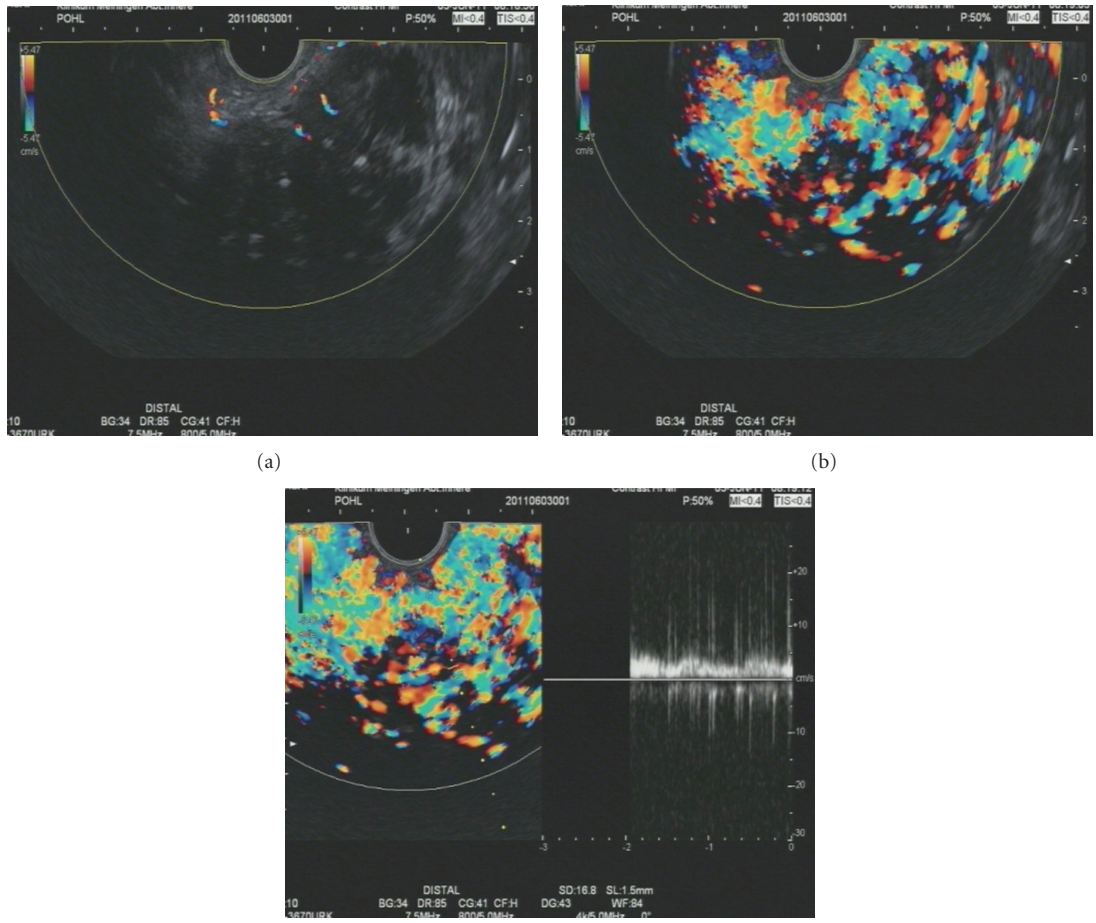



Figure 1: Contrast-enhanced high mechanical endoscopic ultrasound in a patient with chronic pancreatitis: (a) lesion before contrast enhancer injection the lesions is visible within the colour doppler window as a nearly black area; (b) Lesion after contrast enhancer influx (4.5 mL Sonovue) with a visible netlike vessel system the rich vessel system is visible mostly on the right side of the picture with different colours; (c) pw-Doppler analysis of the vessels with a clear venous signal; on the right half of the picture a laminar flow is displayed as a nearly flat white bark.

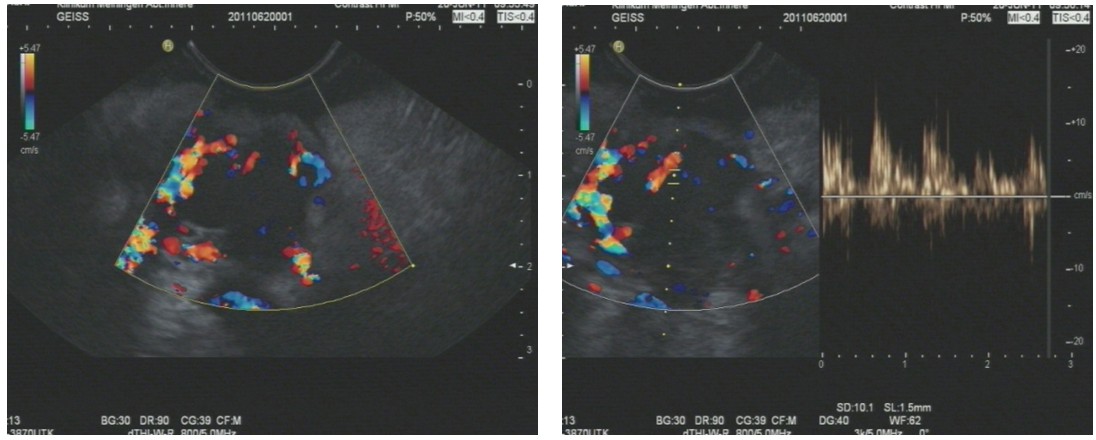
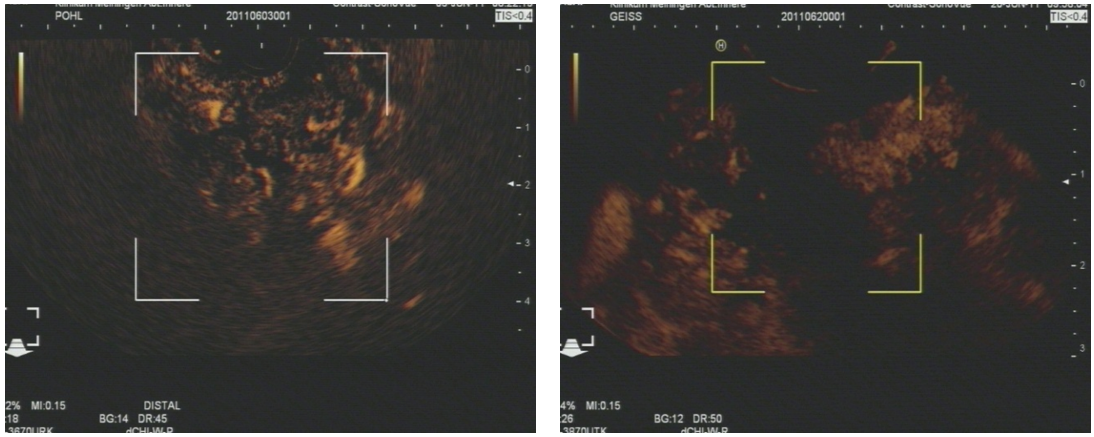
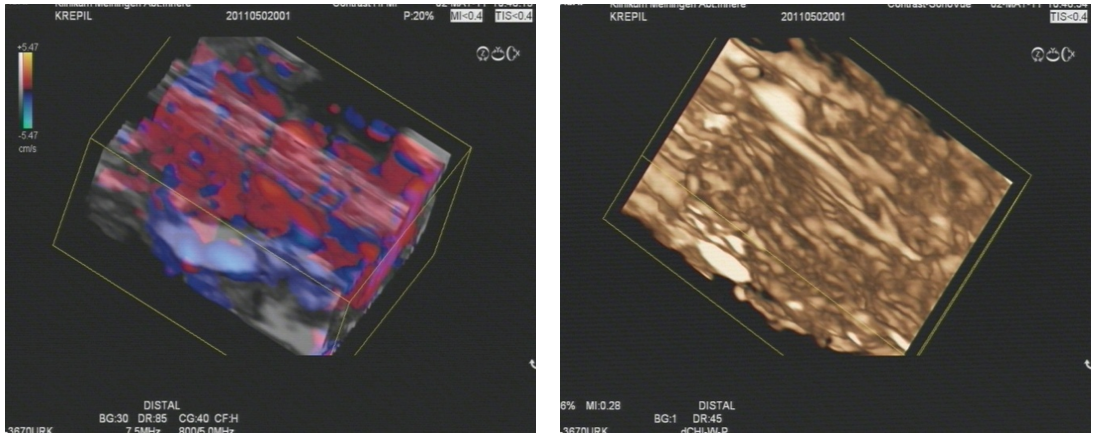



Figure 2: Contrast-enhanced high mechanical endoscopic ultrasound in a patient with pancreatic cancer (a) pancreatic cancer after influx of contrast enhancer (4.5 mL SonoVue); only a few vessels are visible, the lesion is visible within the colour Doppler window as the black area with the colour Doppler signals only on the edges; (b) pw-Doppler analysis reveals only arterial vessels; the atrial vessel signal appears in the right half of the picture in a pulsatile manner.




(a) (b)

Figure 3: Low mechanical index contrast enhanced endosonography: (a) chronic pancreatitis with a clear enhancement of the contrast enhancer; the lesion is visible inside the markers mostly in the right upper area; all the bright visible spots are contrast enhancer signals (b) pancreatic carcinoma with a lack of contrast enhancer in the lesion; the lesion is visible within the markers; there is a black area without any contrast enhancer signals.




(a) (b)

Figure 4: Contrast-enhanced high and low mechanical index endoscopic ultrasound with 3D reconstruction: (a) dense vessel involvement of the pancreas is impressively visible; all the red and blue spots are the Doppler colour signals from a section of the pancreas; (b) the influx of contrast enhancer is shown clearly in the low MI reconstruction to have spread homogenously through the organ.

focal areas. Because of the diagnostic difficulties mentioned before, most of the patients are diagnosed postoperatively. Lately some patients could be diagnosed before operation because gastroenterologists' understanding of the condition has developed in the last few years.

From the CT scans it is now well known that the typical appearance of diffuse autoimmune pancreatitis is a sausage-like form of the pancreas [63, 64]. The use of contrast-enhanced high mechanical index endoscopic ultrasound as well as low mechanical index endoscopic ultrasound reveals another typical behavior [65] (see Figure 4). In patients with autoimmune pancreatitis the whole pancreatic organ shows a strong hypervascularisation as well as hyperperfusion in most cases [66]. Consequently arterial and venous vessels can be discriminated in all patients. It has to be announced that these results are only based on case studies because of the rarity of this disease, however it supports the underlying theory of different kinds of neovascularisations in chronic inflammatory processes and cancer of the pancreatic organ.

## 6. Future Developments

Analyzing different kinds of neovascularisation of chronic pancreatitis and pancreatic carcinoma and using it for diagnostic purposes in clinical practice seems to be a step forward. To further improve our diagnostic possibilities, histopathological studies investigating those results would be of special interest. In addition improving the contrast-enhanced ultrasound technique even more to identify those differences more easily, for example by using automatic analyzing systems, might be helpful in future. This is especially interesting by using contrast-enhanced low mechanical

endosonography in context with perfusion studies with time-intensity-curve analysis which could be the next step to improve the technique.

As mentioned, there are different vascularisation patterns even within different pancreatic carcinomas as well as in chronic pancreatitis. Being able to relate those different types of neovascularisation to the treatment options could even improve our therapeutic options or allow us to draw prognostic conclusions [67].

## Glossary

| | |
|---|---|
| Blooming effect: | Overvisualization of the Doppler signal due to a strong signal. |
| Colour Doppler: | Kind of an ultrasound technique to display moving particles by Doppler technique. |
| Low mechanical index endosonography: | Pictures acquired with help of a special software of an ultrasound machine where the power of the ultrasound is reduced to a level, that the contrast enhancer bubbles remain intact and only the signals from the bubbles are displayed on the screen. |
| High mechanical index endosonography: | Use of the contrast enhancer as an increaser of the Colour Doppler signal. |
| Mechanical index: | Power of the ultrasound force used to create an image. |

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*Research Article*

# Long-Lasting Effect of Infant Rats Endotoxemia on Heat Shock Protein 60 in the Pancreatic Acinar Cells: Involvement of Toll-Like Receptor 4

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*Introduction*. Lipopolysaccharide endotoxin (LPS) is responsible for septic shock and multiorgan failure, but pretreatment of rats with low doses of LPS reduced pancreatic acute damage. *Aim*. We investigated the effects of the endotoxemia induced in the early period of life on Toll-like receptor 4 (TLR4), heat shock protein 60 (HSP60) and proapoptotic Bax, caspase-9 and -3 or antiapoptotic Bcl-2 protein expression in the pancreatic acinar cells of adult animals. *Material and Methods*. Newborn rats (25 g) were injected with endotoxin (*Escherichia coli*) for 5 consecutive days. Two months later, pancreatic acinar cells were isolated from all groups of animals and subjected to caerulein stimulation ($10^{-8}$ M). Protein expression was assessed employing Western blot. For detection of apoptosis we have employed DNA fragmentation ladder assay. *Results*. Preconditioning of newborn rats with LPS increased TLR4, Caspase-9 and -3 levels, but failed to affect basal expression of HSP60, Bax, and Bcl-2. Subsequent caerulein stimulation increased TLR4, Bcl-2, and caspases, but diminished HSP60 and Bax proteins in pancreatic acinar cells. Endotoxemia dose-dependently increased TLR4, Bax, HSP60, and both caspases protein signals in the pancreatic acini, further inhibiting antiapoptotic Bcl-2. *Conclusions*. Endotoxemia promoted the induction of HSP60 *via* TLR4 in the infant rats and participated in the LPS-dependent pancreatic tissue protection against acute damage.

## 1. Introduction

Lipopolysaccharide (LPS, endotoxin), which is a constituent of the outer membrane of gram-negative bacteria, plays a very important role in the pathogenesis of septic shock [1]. LPS is the pivotal stimulus for triggering an inflammatory cascade in macrophages *via* Toll-like receptor 4 (TLR4). LPS has also been identified as a ligand for TLR4 and takes a part in the pathophysiology of the sepsis syndrome [2–4]. Several pathways of endotoxin signal transduction have been suggested in case of endotoxin stimulation of the cell.

Toll-like receptors (TLRs), originally identified as homologues of *Drosophila* Toll, belong to the superfamily of interleukin-1 receptors [5]. TLRs are the most important family of pattern recognition receptors (PRRs) [6, 7]. The existence of several TLRs enables the innate immunity system to recognize different groups of pathogens while initiating appropriate and distinct immunological responses, according to the pathogen-associated molecular patterns (PAMPs) [8]. TLR4 proteins are expressed on the cell surface becoming the receptors for the Gram-negative bacteria cell membrane components, LPS. Stimulation of TLR4 by LPS is a complex process, which includes the participation of several molecules like LPS binding protein (LBP), CD14, and MD-2 [9, 10]. TLR4 might participate in the induction of both protective and harmful effects on the tissues. Beside TLR4, other ligands of TLR4, like hyaluronan, induce an immunological response initiating epithelia repair but in some cases TLR4 are involved in conveying of an endogenous

danger signals mobilizing high-mobility group box-1 protein or in response to free fatty acids what results in tissue damage [11–13]. Some reports point out the possibility of TRLR4 involvement in response to heat shock protein 60 (HSP60) as an endogenous ligand TLR4 [14].

HSP60 is involved in the protein folding, assembly, disassembly, and degradation under normal conditions. This protein, similar to other HSPs, is increased during cellular stress as an adaptive protection strategy [15]. Over the past decade investigators found that HSP60 and the pancreatic enzymes share a common location inside the pancreatic acinar cells, interacting intimately [16, 17]. Moreover, like the distributive characteristics of pancreatic enzymes, HSP60 showed an increasing gradient of collocation along the pancreatic secretory pathway from the rough endoplasmic reticulum and Golgi apparatus to zymogen granules in the acinar cells [16]. An increased transcription and production of HSP60 with protective action has been suggested in pancreatitis [18–20].

Acute pancreatitis (AP) is an emergent disease commonly seen in the clinical practice but its complicated pathogenesis is still incomprehensible. Scientists are in agreement that AP involves a cascade of events, and numerous reports have suggested that its initial step is the activation of trypsinogen inside the pancreatic acinar cells, resulting in damages evoked by the activated pancreatic enzyme [21, 22]. As to the mechanism of the abnormal enzyme activation, a number of theories have been considered, for instance: calcium overload or cathepsin B activation [23, 24]. A new theory advocates that HSP60 plays an important role in the protection of pancreatic tissues against damages and malfunctioning or weakening of HSP60 effect under physiological conditions is responsible for the early zymogen activation in AP [15, 24].

It has been shown that low doses of endotoxin (LPS) could protect the pancreas against caerulein-induced pancreatitis (CIP) [25–28]. Endotoxemia in the suckling rats attenuates acute pancreatitis and impairment of the exocrine function *in vitro* and *in vivo* models at adult age [29–32].

The aim of this study was to investigate in the pancreatic acinar cells isolated from adult animals, the effects of foregoing infant rats endotoxemia on TLR4, HSP60 and pro-apoptotic Bax, caspase-9 and -3 or antiapoptotic Bcl-2 protein expression.

## 2. Material and Methods

Studies were performed on male Wistar rats (weighing: newborn 25 g; adult: 170–200 g). Animals were housed in cages under standard conditions, on commercial pellet chow at water *ad libitum*, at room temperature with a 12-h light and dark cycle.

*2.1. Reagents.* Lipopolysaccharide from Sigma-Aldrich Co. (St. Louis, MO, USA) and caerulein (Takus) from Pharmacia GmbH, Erlangen, Germany, were used for the experiments.

*2.2. Experimental Protocol.* The experimental protocol was divided into two general parts: *in vivo* and *in vitro* researches.

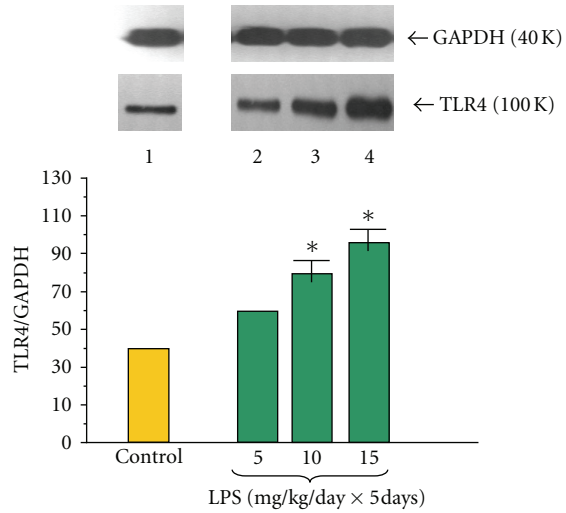


Figure 1: Western blot analysis of TLR4 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days (lane 2), 10 mg/kg/day × 5 days (lane 3), and 15 mg/kg/day × 5 days (lane 4) after 5 hours of incubation. Asterisk indicates significant ($P < 0.05$) change, as compared to the control group. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative for the observed phenomenon.

*2.3. In Vivo Experiments.* Newborn rats weighing 25 g were employed and divided into five main groups:

(1) control group: rats were injected with 200 $\mu$L of vehicle saline intraperitoneally (i.p.), once a day, during 5 consecutive days.

(2) LPS (*Escherichia coli*) group: rats were treated with LPS dissolved in 200 $\mu$L of vehicle saline, and animals were subjected to i.p. injection once a day, during 5 consecutive days. This rats were divided into three separate subgroups which were treated with a single dose of LPS:

2.1 group: 5 mg/kg/day × 5 days (total dose 25 mg/kg);
2.2 group: 10 mg/kg/day × 5 days (total dose 50 mg/kg);
2.3 group: 15 mg/kg/day × 5 days (total dose 75 mg/kg).

Each part of the study consists of several experimental groups of rats, 6–8 rats in each single group.

*2.4. In Vitro Experiments.* Two months following the injection of both vehicle saline or LPS solution, at adult age of animals, the pancreatic acinar cells were isolated by collagenase digestion as described previously [33, 34] and subjected to increasing concentration of caerulein ($10^{-12}$, $10^{-10}$ or $10^{-8}$ M). The cells were incubated in the presence of tested substance for: 0, 0.5, 1, 3, 5, or 7 h. Subsequently, $10^{-8}$ M concentrations of caerulein were found to be the most effective (data not shown) and selected for further experiments. Time-course experiments have shown that 5 h incubation time was the most effective and has been picked

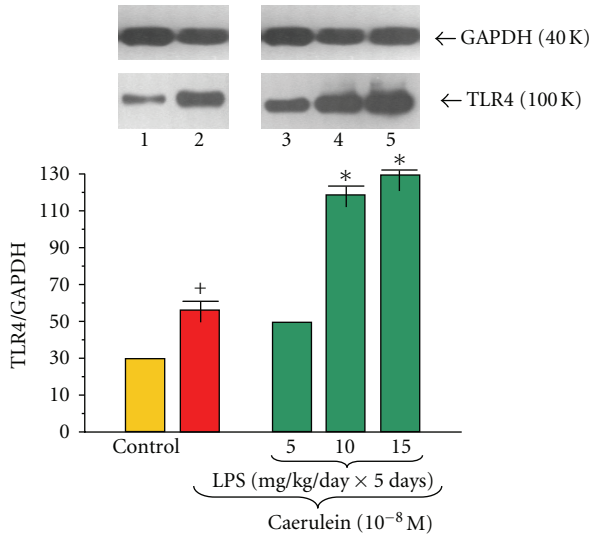


Figure 2: Western blot analysis of TLR4 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. Cross indicates significant ($P < 0.05$) change, as compared to the control group. Asterisk indicates significant ($P < 0.05$) change, as compared to the value obtained from the rats treated by increasing doses of LPS (10 or 15 mg/kg/day × 5 days) in combination with caerulein ($10^{-8}$ M), as compared to the caerulein ($10^{-8}$ M) alone stimulation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative for the observed phenomenon.

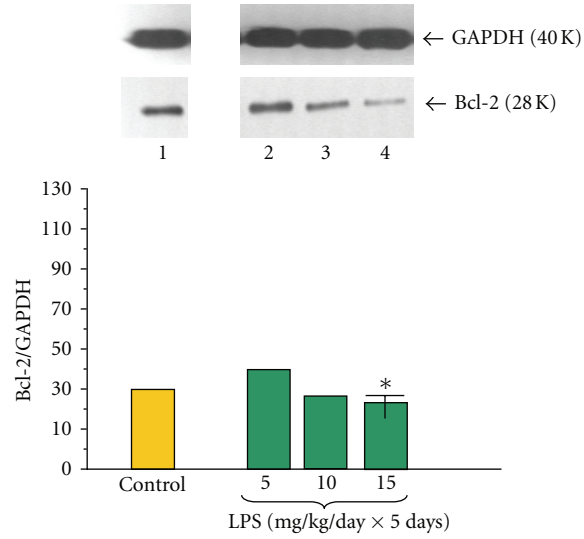


Figure 3: Western blot analysis of Bcl-2 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days (lane 2), 10 mg/kg/day × 5 days (lane 3), and 15 mg/kg/day × 5 days (lane 4) after 5 hours of incubation. Asterisk indicates significant ($P < 0.05$) change, as compared to the control group. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative for the observed phenomenon.

out for all further part of the study (data not shown). All the experiments were repeated at last three times. The results presented here were taken from the most representative experiments.

All experimental procedures performed in this study were approved by the Jagiellonian University Ethical Committee for Animals Experimentation.

*2.5. Western Blot.* The whole-cell extracts were prepared as described elsewhere [35]. Equal load of protein in each sample was assessed using QantiPro BCA Assay Kit (Sigma, USA). Protein samples were boiled with Western blot sample buffer and loaded on the 12% SDS-polyacrylamide gel. After electrophoresis and transfer of the samples, the PVDF membrane (BioRad, USA) was blocked with blocking buffer (5% non-fat dried milk in PBS) for 1 h in room temperature. Blocking procedure was followed with 1 h exposure to primary antibody diluted 1:1000 and secondary antibody diluted 1:1000 in blocking buffer.

After each antibody probing membrane was washed three times for 15 min. in TBST buffer (0,1 M Tris pH 8,0; 1,5 M NaCl; 0,5% TritonX-100). Detection of membrane bound proteins was performed using BM Chemiluminescence Blotting Substance (Boehringer, Mannheim, Germany). The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative for the observed phenomenon. The following items used in the Western blot reactions were purchased from Santa Cruz Biotechnology (Santa Cruz): antibodies mouse monoclonal anti-HSP60 $IgG_1$[sc-136291], mouse monoclonal anti-caspase 9 $IgG_1$ [sc-81663], mouse monoclonal anti-Bcl-2 $IgG_1$ [sc-7382], mouse monoclonal anti-Bax $IgG_1$ [sc-70408], mouse monoclonal anti-GADPH $IgG_1$ [sc-137179], goat polyclonal anti-caspase 3 IgG [sc-1225], rabbit polyclonal anti-TLR4 IgG [sc-30002], rabbit anti-goat IgG HRP [sc-2768], and goat anti-mouse $IgG_1$-HRP [sc-2060], goat anti-rabbit IgG-HRP [sc-2030].

*2.6. DNA Fragmentation.* To analyze DNA fragmentation due to induced apoptosis, cells ($5 \times 10^6$/sample) were lyzed with 150 $\mu$L hypotonic lysis buffer (edetic acid 10 mM, 0.5% Triton X-100, Tris-HCl, pH 7.4) for 15 min. on ice and were precipitated with 2.5% polyethylene glycol and 1 M NaCl for 15 min. at 4°C. After centrifugation at 13000 × g for 10 min. at room temperature, the supernatant was treated with proteinase K (0.3 g/L) at 37°C for 1 h and precipitated with isopropanol at 20°C. Centrifuged pellets were dissolved in 10 $\mu$L of Tris-EDTA (pH 7.6) and analyzed employing electrophoresis in a 1.5% agarose gel containing ethidium bromide. DNA pattern was visualized under ultraviolet light.

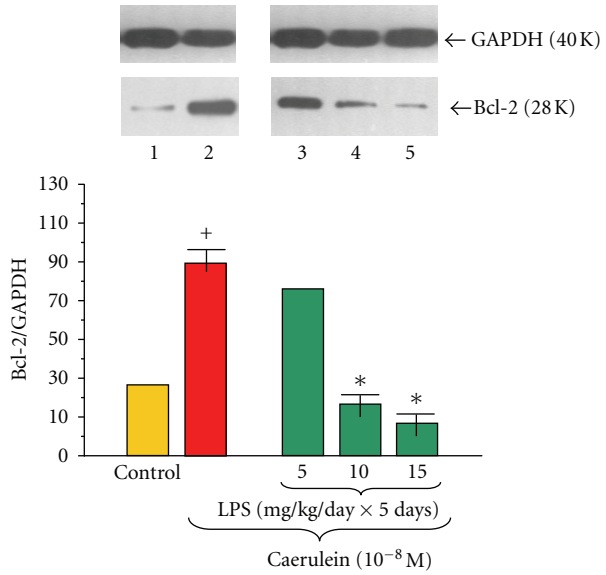


Figure 4: Western blot analysis of Bcl-2 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. Cross indicates significant ($P < 0.05$) change, as compared to the control group. Asterisk indicates significant ($P < 0.05$) change, as compared to the value obtained from the rats treated by increasing doses of LPS (10 or 15 mg/kg/day × 5 days) in combination with caerulein ($10^{-8}$ M), as compared to the caerulein ($10^{-8}$ M) alone stimulation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative for the observed phenomenon.

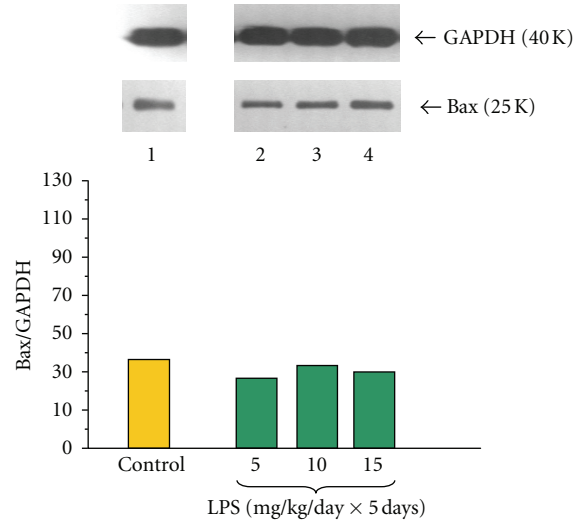


Figure 5: Western blot analysis of Bax protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days (lane 2), 10 mg/kg/day × 5 days (lane 3), and 15 mg/kg/day × 5 days (lane 4) after 5 hours of incubation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

*2.7. Statistical Analysis.* All experiments were performed in triplicates. Results are expressed as means ± SEM. Statistical analysis was performed using analysis on variance and two-way ANOVA test when appropriate. Differences with $P < 0.05$ were considered as significant.

## 3. Results

*The Study of the Effects of Lipopolysaccharide (Escherichia coli) and/or Caerulein on TLR4, Bcl-2, Bax, HSP60, Caspase-9, and Caspase-3 Protein Level and Apoptosis in the Pancreatic Acinar Cells.*

The amount of Toll-like receptor 4 (TLR4) proteins in the pancreatic acinar cells at adult rats was determined in all examined samples (Figures 1 and 2). The ratio of TLR4/GAPDH protein level in the control group was 40.0 ± 0.2 and significantly dose-dependently increased in the group of rats treated at early period of life with 5, 10 or 15 mg/kg/day doses of LPS for 5 consecutive days. The highest abundance of protein was detected in the cell samples from animals treated with LPS at doses of 10 or 15 mg/kg/day and the ratio of TLR4/GAPDH reached 79.0 ± 0.4 and 98.0 ± 0.4, respectively (Figure 1).

Application of caerulein ($10^{-8}$ M) to the acinar cells significantly upregulated TLR4 protein level, as compared to the control group with ratio of TLR4/GAPDH 58.0 ± 0.3 after 5 hours of incubation (Figure 2).

Endotoxemia in the newborn rats induced by increasing doses of LPS (5, 10 or 15 mg/kg/day × 5 days) resulted in significant and dose-dependent increase of TLR4 protein level in the acini incubated with caerulein ($10^{-8}$ M) as compared to the group subjected to caerulein alone. The most significant increase was detected in the cells isolated from rats treated with LPS at doses of 10 or 15 mg/kg/day. The ratio of TLR4/GAPDH in these groups extended to 119.0 ± 0.4 and 130.0 ± 0.4, respectively (Figure 2).

An antiapoptotic mitochondrial molecule Bcl-2 was detected in all examined samples of pancreatic acinar cells obtained from adult animals (Figures 3 and 4). The ratio of Bcl-2/GAPDH proteins in the control group was 29.00 ± 0.2 and significantly in dose-dependent manner decreased in the group of rats treated priorly with LPS at dose of 15 mg/kg/day × 5 days. The ratio of Bcl-2/GAPDH reached 22.0 ± 0.1 (Figure 3).

Application of caerulein ($10^{-8}$ M) to the acinar cells obtained from the control rats resulted in the significant upregulation of Bcl-2 protein level, as compared to the control, untreated with caerulein culture. The ratio of Bcl-2/GAPDH was 89.0 ± 0.3 after 5 hours of incubation (Figure 4).

Endotoxemia in the sucking animals caused by increasing doses of LPS (5, 10 or 15 mg/kg/day × 5 days) in significant and dose-dependent way downregulated Bcl-2 protein level in the pancreatic acini incubated with caerulein ($10^{-8}$ M)

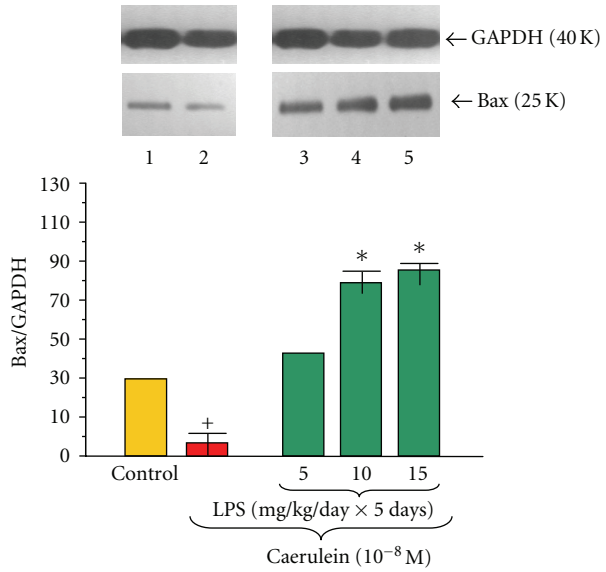


Figure 6: Western blot analysis of Bax protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. Cross indicates significant ($P < 0.05$) change, as compared to the control group. Asterisk indicates significant ($P < 0.05$) change, as compared to the value obtained from the rats treated by increasing doses of LPS (10 or 15 mg/kg/day × 5 days) in combination with caerulein ($10^{-8}$ M), as compared to the caerulein ($10^{-8}$ M) alone stimulation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

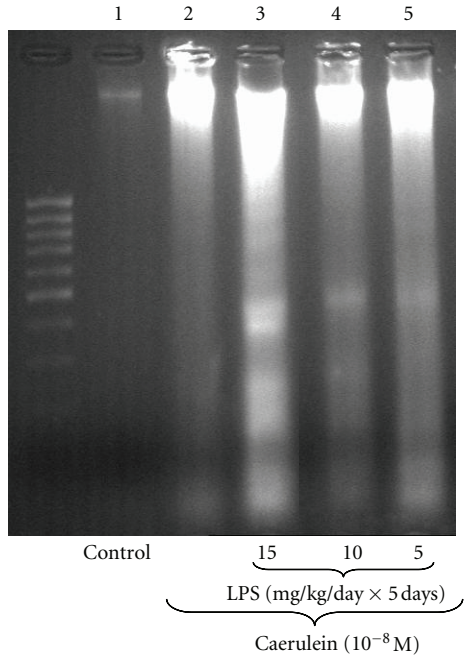


Figure 7: Analysis of DNA fragmentation pattern in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

as compared to the caerulein-treated group alone. The strongest signal was detected in the acini gained from animals treated with LPS at doses of 10 or 15 mg/kg/day. The ratio in these groups of Bcl-2/GAPDH reached 17.0 ± 0.1 and 08.0 ± 0.05 respectively (Figure 4).

The proapoptotic mitochondrial Bax protein level in pancreatic acinar cells obtained from adult animals was detected in all examined samples (Figures 5 and 6). The ratio of Bax/GAPDH protein level in the control group was 38.00 ± 0.2 and did not change in the group treated priorly with increasing doses of LPS 5, 10 or 15 mg/kg/day × 5 days (Figure 5).

Incubation of the pancreatic acinar cells with caerulein ($10^{-8}$ M) caused a significant decrease of Bax protein level, as compared to the untreated with caerulein control. The ratio of Bax/GAPDH protein in this group was 3.5 ± 0.05 after 5 hours of incubation (Figure 6).

Endotoxemia in the newborn rats due to increasing doses of LPS (5, 10 or 15 mg/kg/day × 5 days) caused significant and dose-dependent increase of Bax protein level in the acini incubated with caerulein ($10^{-8}$ M). The strongest signals were detected in the animals treated with 10 or 15 mg/kg/day of LPS. In these groups the ratio of Bax/GAPDH reached 80.0 ± 0.4 and 87.0 ± 0.4, respectively (Figure 6). The data obtained in DNA fragmentation ladder assay correspond with the results of the analysis of apoptosis-related proteins, revealing the strongest pattern of DNA apoptotic damage in the cultures of pancreatic acini isolated from animals preconditioned with highest doses of LPS and subjected to caerulein ($10^{-8}$ M) stimulation (Figure 7 lane 3). In the control cultures of pancreatic acini and those subjected to caerulein stimulation without foregoing preconditioning with LPS no apoptosis-related DNA damage pattern was observed (Figure 7 lanes 1, 2).

The HSP60 protein level was detected in all examined samples of pancreatic acinar cells obtained from adult animals (Figures 8 and 9). The ratio of HSP60/GAPDH protein level in the control group was 63.00 ± 0.3 and failed to change in the group treated at infancy by increasing doses of LPS 5, 10 or 15 mg/kg/day × 5 days (Figure 8).

Addition of caerulein ($10^{-8}$ M) to the pancreatic acinar cell culture obtained from untreated with LPS animals significantly decreased protein level of HSP60, as compared to the control group. The ratio of HSP60/GAPDH was 19.0 ± 0.03 after 5 hours of incubation (Figure 9).

To the contrary, endotoxemia in the suckling rats produced significant increase of HSP60 protein level detected in the acini culture incubated with caerulein ($10^{-8}$ M), as compared to the cells subjected to caerulein alone

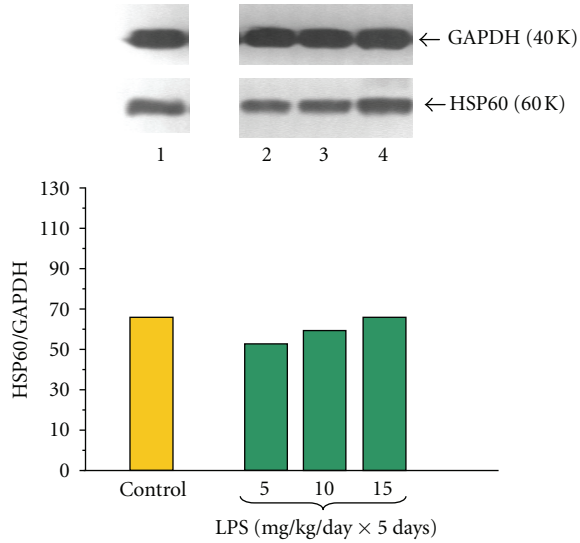


Figure 8: Western blot analysis of HSP60 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days (lane 2), 10 mg/kg/day × 5 days (lane 3) and 15 mg/kg/day × 5 days (lane 4) after 5 hours of incubation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

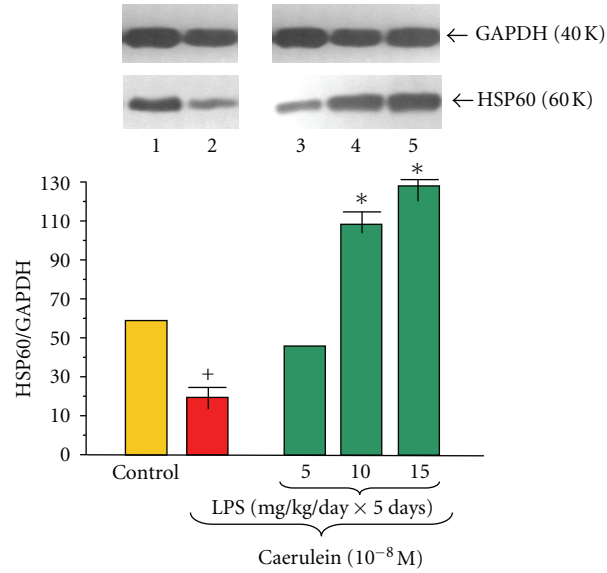


Figure 9: Western blot analysis of HSP60 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. Cross indicates significant ($P < 0.05$) change, as compared to the control group. Asterisk indicates significant ($P < 0.05$) change, as compared to the value obtained from the rats treated by increasing doses of LPS (10 or 15 mg/kg/day × 5 days) in combination with caerulein ($10^{-8}$ M), as compared to the caerulein ($10^{-8}$ M) alone stimulation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

(Figure 9). The most pronounced protein levels were detected in the cell culture obtained from animals treated with LPS at doses of 10 or 15 mg/kg. In these groups the distinct increase of HSP60/GAPDH ratio up to 109.0 ± 0.4 and 129.0 ± 0.5 was noticed, respectively (Figure 9).

The proapoptotic initiator caspase-9 protein level was not detected in pancreatic acinar cells obtained from adult animals in untreated control cultures (Figures 10 and 11). Endotoxemia in the newborn rats due to increasing doses of LPS (5, 10 or 15 mg/kg/day × 5 days) was stimulatory factor for caspase-9 expression. The ratio of caspase-9/GAPDH reached 37.0 ± 0.2 (Figure 10).

Incubation of the pancreatic acinar cells with caerulein ($10^{-8}$ M) caused a significant increase of caspase-9 protein level. The ratio of caspase-9/GAPDH protein in this group was 40.0 ± 0.2 after 5 hours of incubation (Figure 11).

Foregoing endotoxemia evoked by increasing doses of LPS (5, 10 or 15 mg/kg/day × 5 days) caused marked and dose-dependent upregulation of caspase-9 protein level in the pancreatic acini incubated with caerulein ($10^{-8}$ M) as compared to the caerulein-treated group alone with the highest expression values detected in the acini cultures from animals treated with LPS at doses of 10 or 15 mg/kg/day. The ratio in these groups for caspase-9/GAPDH reached 62.0 ± 0.3 and 70.0 ± 0.3, respectively (Figure 11).

Caspase-3 protein was not detected in pancreatic acinar cells obtained from adult animals in control samples (Figures 12 and 13). We have detected elevated level of caspase-3 protein in the acini isolated from animals subjected to the endotoxemia in the infancy due to increasing doses of LPS (5, 10 or 15 mg/kg/day × 5 days) with the ratio of caspase-3/GAPDH reaching 113.0 ± 0.4 (Figure 12).

Application of caerulein ($10^{-8}$ M) to the acinar cells significantly upregulated pro-apoptotic caspase-3 protein level. The ratio of caspase-3/GAPDH was at the level of 118.0 ± 0.4 after 5 hours of incubation (Figure 13).

Prior to LPS (5, 10 or 15 mg/kg/day × 5 days) endotoxemia resulted in significant and dose-dependent increase of caspase-3 protein level in the acini cultures incubated with caerulein ($10^{-8}$ M) as compared to the caerulein-treated group alone. The strongest signals were detected in the cell cultures obtained from the rats treated with LPS at the doses of 10 or 15 mg/kg/day. The ratio of caspase-3/GAPDH in these groups extended to 188.0 ± 0.4 and 209.0 ± 0.5, respectively (Figure 13).

## 4. Discussion

Acute pancreatitis (AP) is a pancreatic nonspecific inflammatory process resulting from the activation of many pathological mechanisms such as obstruction of pancreatic duct, acinar oversecretion, and pancreatic ischemia [21–24].

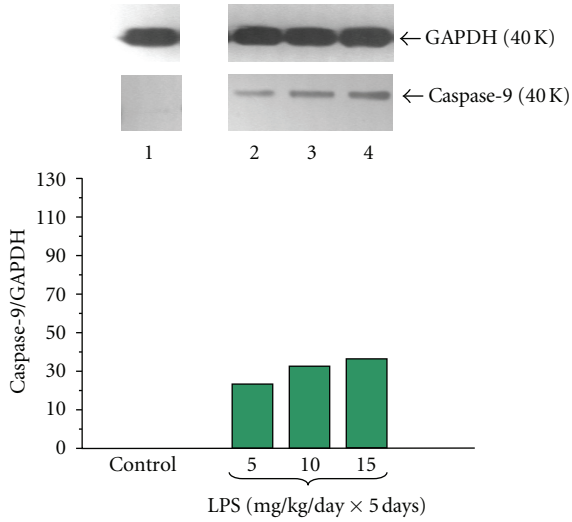


Figure 10: Western blot analysis of proapoptotic initiator caspase-9 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days (lane 2), 10 mg/kg/day × 5 days (lane 3), and 15 mg/kg/day × 5 days (lane 4) after 5 hours of incubation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

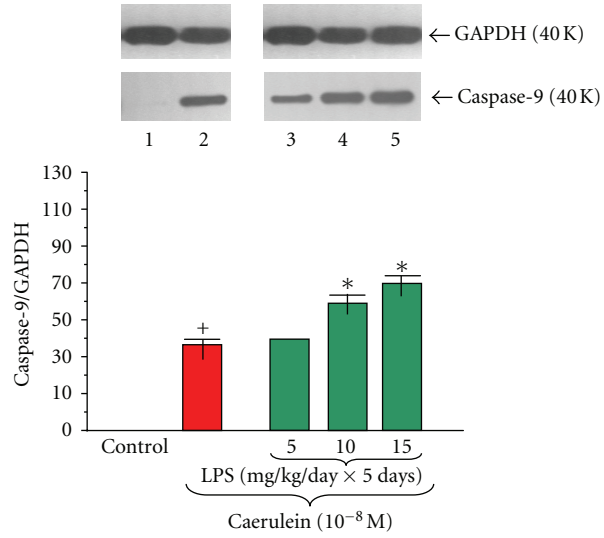


Figure 11: Western blot analysis of proapoptotic initiator caspase-9 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. Cross indicates significant ($P < 0.05$) change, as compared to the control group. Asterisk indicates significant ($P < 0.05$) change, as compared to the value obtained from the rats treated by increasing doses of LPS (10 or 15 mg/kg/day × 5 days) in combination with caerulein ($10^{-8}$ M), as compared to the caerulein ($10^{-8}$ M) alone stimulation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

As the result of above processes the innate immune system is involved in development of inflammatory cascade. Toll-like receptors (TLRs) are suspected to trigger this reaction. It is currently thought that TLRs play an important role in the recognition of endogenous or exogenous antigens and in the initiation of signal transduction for inflammatory reaction during AP [36–38]. Therefore, investigating the tissue-specific expression of these receptors in the pancreas and exploring their role could be important for clarifying the pathogenesis of AP.

In this study we demonstrate the presence of TLR4 on pancreatic acinar cells obtained from the adult rats. In the normal pancreas TLR4 are mainly localized in the epithelial (pancreatic duct epithelium) and endothelial tissue (arteries, veins, and microvascular endothelium) [39, 40]. Herein we have observed that TLR4 protein level in the pancreatic acini was dose-dependently increased in the animals, which have been treated in the early period of life with increasing doses of LPS (*Escherichia coli*). Since LPS has been identified as a ligand for TLR4, it is generally agreed that TLRs are upregulated under inflammatory conditions and downregulated by immunosuppression [2–4, 41–43]. In our study exposure of the pancreatic acinar cells to caerulein caused upregulation of TLR4 protein level. It was reported that these receptors have been rapidly upregulated during the early stage of rat caerulein-induced pancreatitis (CIP) and that might be associated with induction of apoptosis *via* the activation of both intrinsic and extrinsic apoptotic signaling pathways [39, 43, 44]. On the other hand, these receptors are downregulated in the late phase of severe acute pancreatitis (SAP) [45]. Moreover, TLR4 activity is associated with the increased apoptosis [46–51]. Treatment of acini with caerulein resulted in the dose-dependent increase of TLR4 protein level in the rats subjected to endotoxemia in the suckling period of life.

Acinar cell death and parenchymal necrosis is a major cause of severe complications and mortality in human pancreatitis [52, 53]. In AP acinar cells die through both necrosis and apoptosis. The severity of experimental pancreatitis correlates directly with the intensity of necrosis and, inversely, with apoptosis [53–55]. Bax and Bcl-2 family proteins are important regulators of cell apoptosis and their ratio determines the cell susceptibility to this process [56, 57]. Thus, elucidation of the mechanisms mediating acinar cells death in AP is important for understanding of the regulation of this disease and clinical relevance.

In present study we have demonstrate antiapoptotic mitochondrial molecule Bcl-2 in pancreatic acinar cells to be dose-dependently reduced in the group of rats treated at early period of life with highest dose of LPS. Application of caerulein to the acinar cells resulted in the upregulation of Bcl-2 and decrease of Bax protein levels, as compared to the control cells. AP has been shown to upregulate Bcl-2, whereas Bax mRNA was inhibited [58–60]. Our results revealed that in the pancreatic acinar cells obtained from

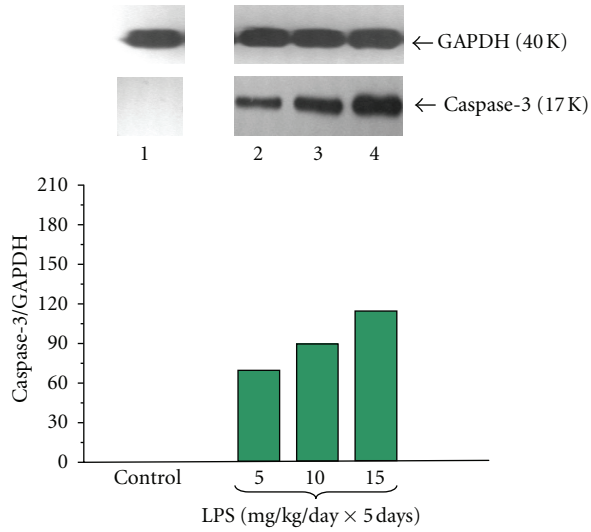


Figure 12: Western blot analysis of apoptosis executioner caspase-3 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days (lane 2), 10 mg/kg/day × 5 days (lane 3), and 15 mg/kg/day × 5 days (lane 4) after 5 hours of incubation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

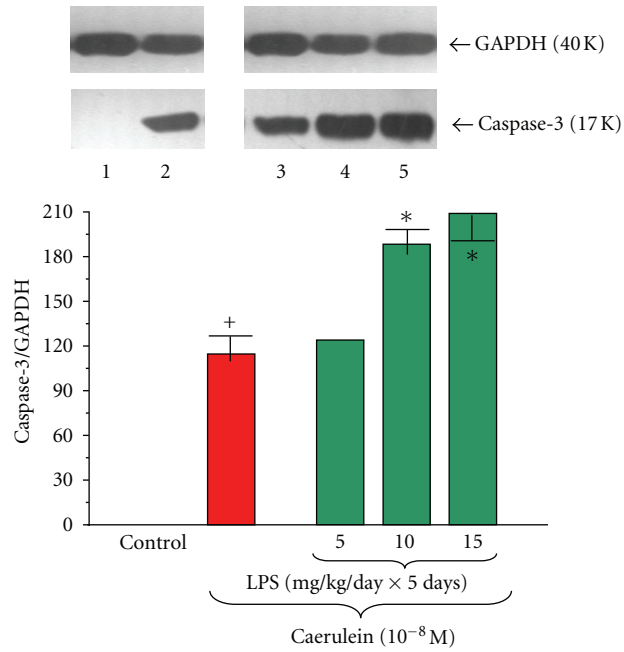


Figure 13: Western blot analysis of apoptosis executioner caspase-3 protein level in the adult rat pancreatic acinar cells under basal conditions (lane 1), stimulated *in vitro* by caerulein at the dose of $10^{-8}$ M (lane 2), treated in the infant animals by lipopolysaccharide (*Escherichia coli*) at the doses of 5 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 3), 10 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 4), and 15 mg/kg/day × 5 days + caerulein $10^{-8}$ M (lane 5) after 5 hours of incubation. Cross indicates significant ($P < 0.05$) change, as compared to the control group. Asterisk indicates significant ($P < 0.05$) change, as compared to the value obtained from the rats treated by increasing doses of LPS (10 or 15 mg/kg/day × 5 days) in combination with caerulein ($10^{-8}$ M), as compared to the caerulein ($10^{-8}$ M) alone stimulation. The blots were stripped and probed with GAPDH to document equal protein loading. All presented results were obtained in 4 consecutive experiments and are representative of the observed phenomenon.

the rats subjected in infancy to endotoxemia, caerulein caused dose-dependent: downregulation of antiapoptotic Bcl-2 and upregulation of pro-apoptotic Bax protein levels, as compared to the caerulein-treated cells alone, suggesting susceptibility of those cultures to apoptosis. Our assumption was confirmed with DNA fragmentation assay. Pancreatic acinar cells obtained from the rats subjected in infancy to endotoxemia and stimulated *in vitro* with caerulein manifested typical for apoptosis pattern of DNA damage.

We have found HSP60 protein in pancreatic acinar cells obtained from adult animals and this confirmed the previous observations concerning the presence of HSP60 protein in the pancreas and in the pancreatic cell line; AR42J [30, 31, 61–63]. This expression of HSP60 in pancreatic tissues has been decreased with prolonged stimulation with LPS [64]. Ohashi et al. [14] claimed that TLR4 mediates HSP60 signaling, as a putative endogenous ligand of the TLR4 complex. We have observed that exposition of the acinar cells in culture to caerulein decreased protein level of HSP60, as compared to the control group. This is in agreement with Rakonczay et al. [65] who have shown that repeated injections of supramaximal doses of CCK to the rat could reduce pancreatic HSP60. Our previous data also demonstrated that caerulein stimulation is able to reduce mRNA signal for HSP60 in the AR42J cells [62, 63]. It is interesting that in the rat pancreas caerulein in time- and dose-dependent manner increases mRNA but paradoxically reduces protein level of rat pancreatic HSP60 [64, 66]. Endotoxemia in the suckling rats resulted in the increase of HSP60 protein level in pancreatic acinar cells, obtained from adult animals, and subjected to caerulein overstimulation. This is in agreement with previous report showing upregulation of pancreatic HSP60 after treatment with combination of caerulein and LPS [61]. On the other hand, different researchers showed time- and dose-dependent increases of mRNA that were followed by paradoxical reduction of protein level of rat pancreatic HSP60 after application of caerulein and LPS [64]. Our previous studies have shown that endotoxemia induced in the early period of life limits AP and reduces the pancreatic exocrine function in response to caerulein both *in vivo* and *in vitro* models at adult age [29–32]. It is possible that pancreatoprotective effects, like cytokine modulation, superoxide dismutase (SOD) activity, and decrease of pancreatic enzyme secretion, are related to the upregulation of HSP60 protein level in the pancreatic acini. Otaka et al. [18] and Rakonczay et al. [19, 20, 65] showed that an increase of HSP60 transcription and production and/or in conjunction with the cytokine modulation and free radical scavenger enzymes (e.g., SOD) activities could be important of "adaptive cytoprotection" in the AP.

Le Gall and Bendayan [16], Li et al. [17] demonstrated hypothesis that the HSP60 would assist the proper folding and assembly of pancreatic secretory proteins and could also prevent their autoactivation before secretion and must be important for quality control and integrity of it.

In the present study we have not detected pro-apoptotic initiator caspase-9 and apoptosis executioner caspase-3 protein expression on pancreatic acinar cells obtained from the adult rats. However Gukovskaya et al. [67] and Mareninova et al. [68] have demonstrated the presence of active caspases-9 and -3 in the normal pancreatic tissue and pancreatic acini. We have shown that endotoxemia in the newborn rats, stimulated both caspases expression in acinar cells obtained from adult animals. This is in agreement with previous study showing that LPS treatment increased caspase-3 activity in the pancreas [69]. We have demonstrated that exposure of the acinar cells to caerulein ($10^{-8}$ M) upregulated pro-apoptotic caspase-9 and -3 protein level, as compared to the control group, what is in agreement with the observation of Gukovskaya et al. [67] and Mareninova et al. [68]. In experimental models of AP, acinar cells have been shown to die through necrosis and apoptosis [68]. We have found that endotoxemia in the sucking animals evoked by increasing doses of LPS caused dose-dependently upregulation of pro-apoptotic caspase-9 and executioner caspase-3 protein level in the pancreatic acini incubated with caerulein. Laine et al. [70] demonstrated that the LPS causes release of pancreatic phospholipase $A_2$ ($PLA_2$) into blood, its activation in pancreatic tissue and apoptosis of acinar cells. Kimura et al. [71] showed that LPS pretreatment increased remarkably the incidence of acinar cells apoptosis in AP. These results suggest that the pathological features of this disease might be modified by the presence of nonfatal endotoxemia through the induction of acinar cells apoptosis.

In conclusion, our data indicate that exposure of the infant rats to LPS promoted the induction of HSP60 *via* TLR4 in their adult life and, in turn, activated Bax/Bcl-2 and caspase-9 and -3. It is likely that this process could take a part in the LPS-induced protection of the pancreatic tissue against acute damage produced by caerulein overstimulation.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Acknowledgments

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*Research Article*

# Activated Protein C Does Not Alleviate the Course of Systemic Inflammation in the APCAP Trial

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The study aimed to determine the effect of the activated protein C on the course of systemic inflammation in the APCAP (activated protein C in acute pancreatitis) trial where we randomized 32 patients with severe acute pancreatitis to receive either recombinant activated protein C (drotrecogin alfa activated) ($n = 16$) or placebo ($n = 16$) for 96 hours. In the present study, we present the time course of the patients' plasma or serum levels of soluble markers (IL-8, IL-6, IL-10, IL-1ra, sE-selectin, PCT) and monocyte and neutrophil cell surface (CD11b, CD14, CD62L, HLA-DR) markers of systemic inflammatory response during the first 14 days after the randomization. The results of the intervention and placebo groups were comparable showing that recombinant APC treatment did not alter the course of systemic inflammation in severe acute pancreatitis. Our finding is in accordance with the clinical findings in the APCAP trial indicating that the intervention did not affect evolution of multiple organ dysfunctions.

## 1. Introduction

Acute pancreatitis (AP), a common cause of abdominal pain, is usually a mild, self-limited disease. However 25% of the patients suffer from severe AP (SAP) [1], and 20% of SAP patients die, [2], mostly due to the development of multiple organ dysfunction [3]. Systemic inflammation, typical of AP, is considered to contribute to the development of organ dysfunction. It is characterized by (i) an increase in circulating levels of proinflammatory cytokines [4, 5], anti-inflammatory cytokines [6–9], and soluble E-selectin (sE-selectin) [10–12], a marker of activation of the vascular endothelium, (ii) occurrence of activated phagocytes in the circulation [13, 14], and (iii) a decrease in HLA-DR expression on blood monocytes [9, 13, 15, 16], denoting the development of immune suppression.

Activated protein C (APC) is a plasma serine protease with effects on coagulation, apoptosis, and inflammation [17]. APC acts as an endogenous anticoagulant that promotes fibrinolysis and inhibits thrombosis. Protein C, an inactive precursor, is converted to activate protein C by thrombin-thrombomodulin complex on endothelium [18]. This process is accelerated in the presence of endothelial PC receptor (EPCR) [19]. APC inactivates the procoagulation factor Va and VIIIa shutting down the coagulation pathway. APC also inactivates plasminogen activator inhibitor, which results in increased fibrinolysis [18].

APC also has cytoprotective effects such as anti-inflammatory, antiapoptotic, and endothelial barrier protection effects [20].

Proinflammatory cytokines upregulate thrombin formation and downregulate the host's antithrombotic mechanisms, in particular the protein C (PC) pathway reviewed in [21]. Deficiency of PC and decreased generation of activated PC (APC), the major endogenous anticoagulant in man, associate with the development of organ dysfunction in AP

[22]. In patients with sepsis human recombinant APC shortened the duration of respiratory dysfunction and accelerated the reversal of shock [23]. We studied in a randomized trial patients with severe AP and found no differences in the evolution of multiorgan dysfunction between APC and placebo groups [24]. However, the effects of APC on inflammatory markers in patients with SAP have not been studied in a randomized controlled trial previously.

Accordingly, we aimed to determine the effects of the APC intervention on plasma levels of proinflammatory (IL-8), pro-/anti-inflammatory (IL-6) and anti-inflammatory (IL-10, IL-1ra) [25] cytokines and sE-selectin, on activation markers of blood monocytes (CD14, CD11b, CD62L) and neutrophils (CD11b, CD62L), on levels of monocyte cell-surface expression of HLA-DR, a marker of immune suppression, and on serum levels of procalcitonin (PCT), a marker of systemic inflammation used in clinical decision making.

## 2. Subjects and Methods

*2.1. Patients and Healthy Subjects.* We previously conducted a randomized study of APC in SAP patients [24]. In brief, this prospective randomized double-blind study included analysis of 32 patients with SAP in the tertiary care unit at the Helsinki University Central Hospital between June 2003 and August 2007. The inclusion criteria were (1) admitted to hospital <96 h from the onset of pain, (2) a 3-fold increase in serum amylase (IU/L) over normal upper range or/and verification of SAP in computer tomography, (3) at least one organ dysfunction (OD) defined as the Sequential Organ Failure Assessment (SOFA) of at least 3 of 4, and (4) <48 hours from the first OD. Patients were randomized to receive either APC (drotrecogin alfa activated) ($N = 16$) or 0.9% physiologic saline as placebo ($N = 16$). APC was administrated for 96 hours with a dose of 24 $\mu$g/kg/hour.

We obtained reference blood samples for the analyses of cell surface markers by flow cytometry from 65 healthy volunteers (137 samples) from the hospital and laboratory staff without medication and with no signs of infection. To monitor the level of fluorescence intensity, a blood sample from a healthy volunteer was studied according to the study protocol once a week. There were 58 women and 7 men in the reference group. In case of repeated sampling, mean of the data was used.

The study protocol was approved by the ethics committees of the Helsinki University Central Hospital. Informed consent was obtained from all patients or their next of kin. The study protocol was registered in ClinicalTrials.gov (NCT01017107).

*2.2. Blood Samples.* When a patient fulfilled the inclusion criteria we collected peripheral blood samples for determination of cell markers of inflammation by venipuncture for the first time. Then the patients were randomized. After that follow-up samples were collected in the morning of the third, fifth, seventh, and 14th day. Blood samples for flow cytometry and for plasma measurements were anticoagulated with pyrogen-free acid-citrate dextrose (ACD). Blood samples were immediately cooled in an ice-cold water bath and kept at 0°C until stained for flow cytometry. The plasma was separated by centrifugation at +4°C and stored at −70°C until concentrations of cytokines and sE-selectin were determined. Blood samples for determination of serum PCT were collected concurrently.

*2.3. Analysis of Soluble Markers.* The concentrations of IL-6, IL-8, IL-10, IL-1Ra, and E-selectin in plasma samples were determined by enzyme immunoassay (EIA) by using commercial reagents (IL-6 and IL-10: PeliPair ELISA, Sanquin, Amsterdam, the Netherlands; IL-8: Opt EIA, BD Biosciences, Erembodegem, Belgium; IL-1Ra: Duo Set ELISA, R&D Systems Europe Ltd, Abindgon, UK; E-Selectin: ELISA, HyCult Biotechnology, Uden, The Netherlands). The detection limits and intra-assay and interassay coefficients of variation (CV%) were as follows: IL-6: 0.3 pg/mL, 3.6% and 5.4%; IL-8: 1.6 pg/mL, 3.5%, 3.4%; IL-10: 0.3 pg/mL, 3.7%, 5.9%; IL-1Ra: 10 pg/mL, 4.2%, 5.2%; E-selectin 20.5 pg/mL, 3.5%, 6.9%.

Procalcitonin (PCT) was measured using ADVIA Centaur XP immunoassay system with ADVIA Centaur BRAHMS PCT assay. Assay is a sandwich chemiluminescent immunoassay using monoclonal antibody to fluorescein covalently linked to paramagnetic particles and two antibodies to procalcitonin labelled with fluorescein. According to the manufacturer, the within-run precision of the method is 4.3%, 1.5%, and 1.5% for PCT at 0.2, 0.97, and 65.9 $\mu$g/L, respectively. The between-run precision is 8.5%, 2.1%, and 7.2% for the respective concentrations. The limit of detection for the assay is 0.04 $\mu$g/L and dilution point 75 $\mu$g/L.

*2.4. Analysis of Cell Surface Markers*

*Monoclonal Antibodies and Flow Cytometry.* Monocyte expression of CD14, CD11b, CD62L and HLA-DR and neutrophil expression of CD62L and CD11b were determined using whole blood flow cytometry, as described previously [9, 13]. Monoclonal antibodies (mAbs) were as follows: phycoerythrin (PE) and fluorescein isothiocyanate (FITC) conjugates of anti-CD14 mAb (IgG2b, clone MFP9), PE conjugates of anti-HLA-DR mAb (IgG2a, clone L243), anti CD11b mAb (IgG2a, clone D12) and control mouse IgG2a, mAb, and FITC conjugate of anti-CD62L mAb (IgG2a, clone SK11). All reagents were purchased from Becton Dickinson (San Jose, CA, USA). Staining of aliquots of the whole blood sample at 0°C for flow cytometry was carried out as described previously [9, 13]. Data acquisition and analyses were done by a FACSCalibur flow cytometer and Cell Quest software (BD Sciences, San Jose, CA). Neutrophils were identified by the light scattering properties and monocytes by the clonal marker CD14. Monocyte HLA-DR expression was determined as the proportion of HLA-DR positive monocytes, as described earlier [13]. Fluorescence intensity is presented as relative fluorescence units (RFUs).

*2.5. Statistical Analysis.* The primary end point of the randomized study was the change in SOFA score. The sample

Table 1: Characteristics of patients.

| | Activated protein C | Placebo |
|---|---|---|
| No. of patients | 16 | 16 |
| Male/female | 16/0 | 15/1 |
| Etiology of SAP -Alcohol/biliary | 16/0 | 15/1 |
| Age (years) | 44 (34–36) | 47 (19–59) |
| SOFA score on admission | 8.0 (3–13) | 8.5 (3–15) |
| ICU stay (days) | 10.0 (2–43) | 11.0 (0–31) |

* Values are median (range).

size for the study was determined according to the primary end point: there would be three-point difference in change of SOFA score between the groups (with $P < 0.05$ and a power of 80%) [24]. Values are given as medians and ranges. Comparisons of marker levels between the two groups (the APC group and the placebo group) were performed by the Mann-Whitney $U$-test. In case of repeated sampling from the healthy volunteers mean data was used to get one value for each person and after that median was used. The Wilcoxon signed-rank test was used in comparisons of repeated measurements. A difference with a $P$-value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS19.0 statistical software (Chicago, Illinois).

## 3. Results

*3.1. Patients.* Characteristics of the 32 SAP patients are given in Table 1. All except one of the patients were admitted to the ICU. The time before patients were admitted to the ICU was 1,0 days (0–3 days) in APC group and 2,0 (1-2 days) in the placebo group ($P = 0.642$). In the APC group there were two nonsurvivors: one after receiving 13 hours of APC infusion and the other one having a laparotomy after 41 hours of APC infusion.

*3.2. Soluble Markers.* Plasma concentrations of proinflammatory cytokine IL-8 of all patients decreased during the first five days after the admission to hospital (day 0: 264 pg/mL versus day 5: 110 pg/mL, $P = 0.001$). The APC treatment had no significant effect on the changes in IL-8 concentrations during the follow-up period (Table 2).

Plasma concentrations of pro-/anti-inflammatory cytokine IL-6 (Figure 1), anti-inflammatory cytokines IL-10 and IL-1Ra of all patients decreased during the first five days of the follow-up time (IL-6 day 0: 670 pg/mL versus day 5: 215 pg/mL, $P = 0.001$; IL-10 day 0: 12.7 pg/mL versus day 5: 11.3 pg/mL, $P = 0.001$; IL-1Ra day 0: 2890 pg/mL versus day 5: 1250 pg/mL, $P = 0.007$). The APC treatment did not have any significant effect on the changes in IL-6, IL-10, and IL-1Ra concentrations during the first five or 14 days of follow-up time (Table 2).

Plasma concentrations of soluble E-selectin of all patients decreased along the course of the disease (day 0: 45.5 ng/mL versus day 5: 38.2 ng/mL, $P = 0.031$), but the APC treatment did not have any significant effect on the changes in concentrations of sE-selectin (Table 2).

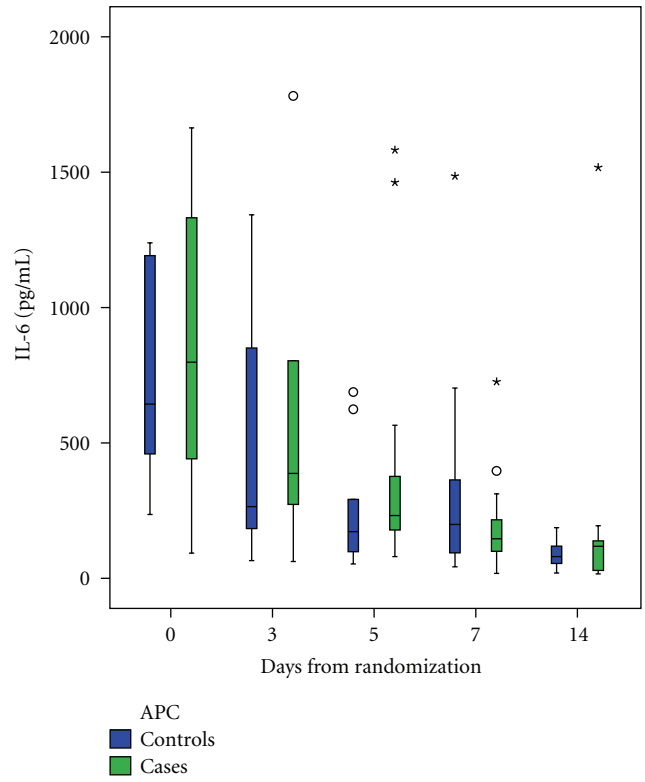


Figure 1: The changes in concentrations of IL-6 during the follow-up time. The APC did not have significant effect on the changes ($P = 0.288$). Box-Whisker plots show median, interquartile range (box) and highest and lowest values. Outliers (circles) and extreme values (asterisks) are shown separately.

There were no significant changes in serum concentrations of procalcitonin of all patients during the first five days (day 0: 0.97 ng/mL versus day 5: 0.66 ng/mL, $P = 0.487$), and administration of APC did not alter the changes in PCT concentrations (Table 2).

*3.3. Cell Surface Markers.* As a marker of immune suppression monocyte HLA-DR expression of all patients was not altered significantly during the first five days of follow-up period (day 0: 54% versus day 5: 58%, $P = 0.316$). Neither had the APC infusion any effect on the HLA-DR expression (Table 3).

The cell surface expressions of CD11b, CD14, and CD62L were measured as markers of activation of monocytes. The cell surface expression of CD11b, CD14, and CD62L on monocytes of all patients was downregulated during the first five days (MoCD11b day 0: 291 RFU versus day 5: 200 RFU, $P = 0.001$; MoCD14 day 0: 168 RFU versus day 5: 150 RFU, $P = 0.028$; MoCD62L day 0: 217 RFU versus day 5: 135 RFU, $P = 0.001$). The expression of CD11b, CD14, and CD62L did not differ significantly between the placebo and the APC-treatment group (Table 3).

Table 2: The changes in concentrations of soluble inflammation markers between days 0–5 and 0–14. Median (range).

| Marker | APC baseline $n = 16$ | Placebo baseline $n = 16$ | $P$-value | APC change 0–5 d $n = 14$ | Placebo change 0–5 d $n = 13$ | $P$-value | APC change 0–14 d $n = 11$ | Placebo change 0–14 d $n = 11$ | $P$-value |
|---|---|---|---|---|---|---|---|---|---|
| IL-8, pg/mL | 286 (56.4–1760) | 245 (41.5–1750) | 0.867 | −72.3 (−1630–60.8) | −39.5 (−275–470) | 0.159 | −84.9 (−266–263) | −132 (−1280–−50.4) | 0.270 |
| IL-6, pg/mL | 798 (93–7190) | 643 (235–41800) | 1.000 | −377 (−1350–−1090) | −517 (−41600–−182) | 0.159 | −724 (−1550–979)[5] | −884 (−41700–−388) | 0.288 |
| IL-1ra, pg/mL | 4480 (143–107000) | 2870 (656–12400) | 0.590 | −2210 (−20500–2160) | −1480 (−1150–−6380) | 0.627 | −1500 (−7850–3950) | −750 (−3890–2240) | 0.243 |
| IL-10, pg/mL | 13.7 (2.78–367) | 10.8 (4.17–145) | 0.724 | −5.51 (−28.9–6.04) | −6.72 (−137–28.1) | 0.438 | −6.70 (−33.2–72.1) | −6.20 (−139–77.0) | 0.898 |
| sE-selectin, ng/mL | 41.4 (25.3–195) | 86.1 (19.0–192) | 0.445 | 0.06 (−139–20.0) | −40.5 (−135–16.9) | 0.099 | 0.100 (−143–14.4) | −45.6 (−144–8.70) | 0.270 |
| PCT, ng/mL | 1.03 (0.12–8.33)[1] | 0.75 (0.18–3.96)[2] | 0.880 | 0.18 (−7.53–3.47)[3] | −0.21 (−1.91–1.62)[4] | 0.590 | 0.130 (−4.66–3.96) | −0.270 (−3.30–2.09)[6] | 0.918 |

[1] $n = 14$, [2] $n = 15$, [3] $n = 11$, [4] $n = 10$, [5] $n = 12$, [6] $n = 10$.

d: day; IL: interleukin; PCT: procalcitonin.

$P$-values are calculated for differences between the two groups in changes from baseline.

Table 3: The changes in concentrations of cell markers between days 0–5 and 0–14. Median (range).

| Marker median, range of healthy reference subjects, $n$ | APC baseline $n = 16$ | Placebo baseline $n = 15$ | $P$-value | APC change 0–5 d $n = 14$ | Placebo change 0–5 d $n = 12$ | $P$-value | APC change 0–14 d $n = 11$ | Placebo change 0–14 d $n = 10$ | $P$-value |
|---|---|---|---|---|---|---|---|---|---|
| MoHLA-DR, % 95.0 (42–99), $n = 64$ | 54.0 (13–73) | 44.0 (26–75) | 0.599 | 10.0 (−21–30) | 1.00 (−50–22) | 0.173 | 13.0 (−18–37) | 31.0 (−29–65) | 0.152 |
| MoCD11b, RFU 126 (72–297), $n = 63$ | 284 (118–770) | 337 (136–673) | 0.953 | −91.5 (−290–123) | −76.5 (−348–114) | 0.877 | −35.0 (−301–445) | −15.0 (−497–150) | 0.349 |
| MoCD14, RFU 206 (74–379), $n = 64$ | 161 (87–306) | 186 (96.0–294) | 0.626 | −28.5 (−115–68) | −21.5 (−142–58) | 0.797 | −5.00 (−75–19) | −59.5 (−119–60) | 0.173 |
| PMNCD11b, RFU 130 (75–466), $n = 63$ | 324 (172–791) | 325 (161–519) | 0.861 | −50.5 (−426–236) | −66.0 (−219–164) | 0.918 | −37.0 (−215–242) | −74.0 (−346–89) | 0.173 |
| MoCD62L, RFU 118 (63–279), $n = 64$ | 217 (133–313) | 216 (87–343) | 0.892 | −65.5 (−208–15) | −68.0 (−226–18) | 0.979 | −95.5 (−212–11) | −109 (−212–8) | 0.481 |
| PMNCD62L, RFU 131 (85–193) $n = 64$ | 123 (40–239) | 164 (78–227) | 0.264 | −46.0 (−212–43) | −67.5 (−123–25) | 0.898 | −76.0 (−165–69) | −80.0 (−165–65) | 0.251 |

Mo: monocyte; PMN: neutrophil; RFU: relative fluorescence units.

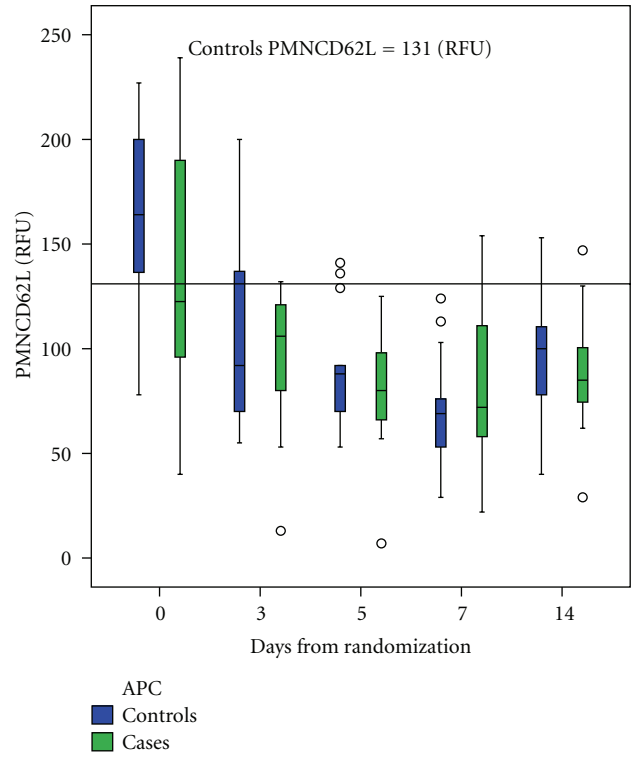


Figure 2: The changes in expression of CD62 on neutrophils during the follow-up time. The APC did not have significant effect on the changes ($P = 0.251$). Box-Whisker plots show median, interquartile range (box) and highest and lowest values. Outliers (circles) are shown separately.

The expressions of CD11b and CD62L were measured as markers of neutrophil activation. The expressions of CD11b and CD62L (Figure 2) of all patients were both downregulated during the first five days of follow-up period (PMNCD11b day 0: 325 RFU versus day 5: 259 RFU, $P = 0.001$; neutrCD62L day 0: 146 RFU versus day 5: 81 RFU, $P = 0.001$). The APC treatment did not have any effect on the changes in CD11b or CD62L expression on PMN cells between days 0 and 5 (Table 3).

## 4. Discussion

The results show that recombinant APC (drotrecogin alfa activated) treatment of patients with SAP did not alter the course of systemic inflammation, as determined using soluble and cellular markers of systemic inflammatory response. This is in accordance with the clinical findings of these patients, which indicated that SOFA score changes, organ-failure-free days, ICU or hospital stay time, ventilator-free days, renal replacement therapy-free days, vasopressor-free days, or days alive outside hospital (in 60 days) were comparable between APC and placebo groups [24]. The overall decreasing tendency of cytokines during the followup resembles earlier results, which show that both pro- and anti-inflammatory bursts are an early phenomenon in severe AP [15].

Several in vitro and animal studies show that APC has an anti-inflammatory activity. Administration of APC has been shown to downregulate the expression of inflammatory cytokines and chemokines. APC blocks cytokine production from Th2 lymphocytes [26]. APC has been shown to reduce production of endotoxemia-induced proinflammatory cytokines (IL-6, IL-8, IL-1beta, and TNF alfa) [27]. In vitro the production of IL-8 from monocytes is inhibited by APC (LPS-stimulated THP-1 cells) [28]. In vitro APC has been shown to inhibit chemotaxis and IL-6 release by human neutrophils [29]. APC inhibits TNF-alpha production by blocking nuclear factor (NF) kB transcription factor in monocytes [30]. APC has been shown to upregulate anti-inflammatory mediators, like IL-10 in blood monocytes in patients with severe sepsis [31]. APC can also block leukocyte trafficking by decreasing the expression of adhesion molecules (ICAM-1, E-selectin, and VCAM-1) on the endothelium [32–35].

Several clinical trials have been established to evaluate the APC treatment in sepsis patients. In the PROWESS trial they found that recombinant human activated protein C (drotrecogin alfa) reduced mortality in patients with severe sepsis [36]. In this randomized multicentre trial they found decreases in D-dimer levels and IL-6 levels in patients' plasma that has been taken as evidence of anti-inflammatory action of APC [23]. HLA-DR expression, as marker of immunosuppression, has been shown to correlate with PC and APC levels in SAP [22]. Preliminary, still unpublished results from the PROWESS-SHOCK trial indicated that APC did not have a beneficial effect on 28-day survival in patients with septic shock.

No previous randomized trial has scrutinized the effect of APC in SAP patients, nor is there such evidence of APC's effect on inflammation in SAP. The present study of phlogistic markers supports the view that APC treatment does not affect either the course of systemic inflammation in agreement with no detected effect on organ dysfunction [24].

*Limitations of the study.* The sample size for the study (16 + 16) was determined according to the primary end point and not for comparing systemic inflammatory response between the groups. In addition the number of follow-up samples/patients was decreased at day five (12 + 14) and at day 14 (11 + 10); therefore it is not possible to exclude a type II error.

## 5. Conclusion

The recombinant APC (drotrecogin alfa activated) treatment of patients with SAP did not alter the course of systemic inflammation, as determined using soluble and cellular markers of systemic inflammation.

## Abbreviation

| | |
|---|---|
| APCAP: | Activated protein C in acute pancreatitis |
| AP: | Acute pancreatitis |
| SAP: | Severe acute pancreatitis |
| PC: | Protein C |
| APC: | Activated protein C |
| PCT: | Procalcitonin |
| OD: | Organ dysfunction |
| SOFA: | Sequential organ failure assessment |
| EIA: | Enzyme immuno assays |
| RFU: | Relative fluorescence units |
| ICU: | Intensive care unit |
| PROWESS: | Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis. |

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*Clinical Study*

# Patterns of Pathomorphological Changes in Acute Necrotizing Pancreatitis

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Acinar necrosis is the basic microscopic sign of acute necrotizing pancreatitis (ANP). Microcirculation disorder is one of the major factors in the pathogenesis and morphogenesis of ANP besides free radicals and damage of enzymatic origin. This study is dedicated to the description of microscopic changes in the pancreatic stroma in ANP, which leads to destruction of the exocrine pancreas with a putative mechanism of endocrine function preservation. This study has been carried out on histological samples of pancreas from 224 patients with ANP. Histological staining was performed with hematoxylin-eosin (H&E), Masson, Gomori methods, and PAS. Microscopy was performed with magnifications of 40×, 100×, and 400×. Vascular endothelial desquamation, stasis, and sludge are typical changes in microcirculation observed in early stages of ANP. Initially, parietal circular intravascular microthrombosis accompanied by endothelial desquamation as early as stromal swelling occurs with no detectable necrosis. Residual stroma appears between areas of necrosis and intact pancreatic tissue. Mucoid swelling is first seen in the perivascular spaces extending to the parenchyma and changing into fibrinoid imbibition causing further necrosis. Reticulin argyrophilic backbone surrounding the pancreatic acini and small ducts decompose. Pancreatic structures, which may be preserved in necrotic tissue, include nerves, major ducts, and Langerhans islets.

## 1. Introduction

Necrosis and tissue degradation are the basic microscopic signs of acute necrotizing pancreatitis (ANP). These changes can be focal or diffuse. We believe that the microcirculation disorder is one of the strongest factors in the pathogenesis and morphogenesis of ANP besides free radicals and of enzymatic origin.

## 2. Aim

This study is dedicated to investigation and description of microscopic changes in the pancreas in ANP which leads to the destruction of the exocrine pancreas, changes in the pancreatic stroma, and a presumable mechanism of preservation of endocrine function.

## 3. Materials and Methods

This study has been carried out on histological samples of pancreas from 224 patients with ANP. All these patients were operated at the Kyiv Center for Hepatopancreatobiliary Surgery (named after V. S. Zemskov) from 1997 through 2004. Postmortem examinations of autopsy tissue were performed in 21 of the 224 patients. The samples were investigated at varying times compared to the onset of the disease. 82.5% of the patients presented with alimentary and 17.5% with biliary pancreatitis. The study of the histologic

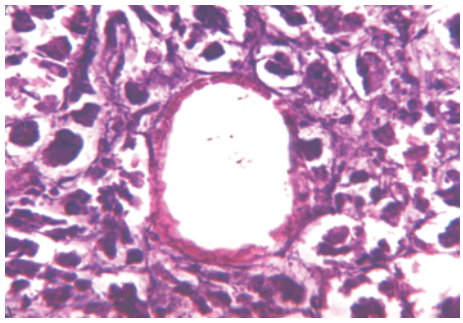

Figure 1: Interstitial swelling of pancreatic parenchyma. Microcirculation peripheral thrombosis. H&E (×400).

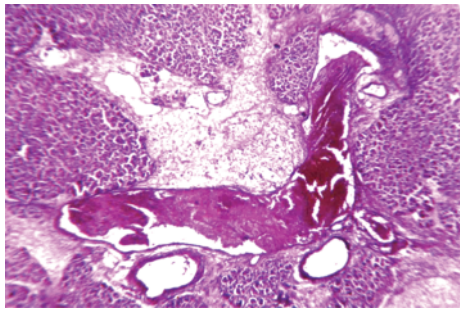

Figure 2: Focal necrosis of pancreatic parenchyma associated with massive thrombosis in venules. H&E (×40).

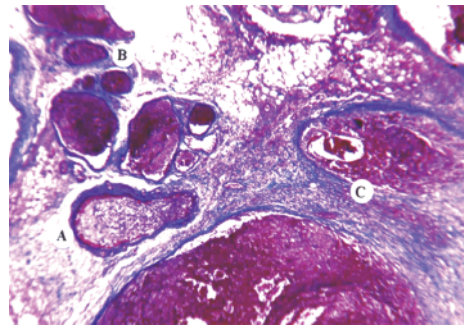


Figure 3: Sequence of thrombus formation: parietal thrombus progressing centripetally (A); total thrombosis (B); vessel remodeling (C). Masson staining (×40).

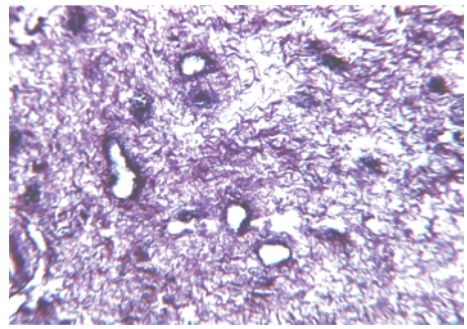

Figure 4: Argyrophilic fibers are preserved only around major structures on the background of necrotic changes in the pancreas. Gomori staining (×100).

tissues was routinely performed by utilizing hemotoxylin-eosin, Masson, Gomori, and PAS stains. Microscopy was performed at 40×, 100×, and 400× magnification.

## 4. Results and Discussion

The pictures of a pancreatic disorder in ANP are very diverse.

Vascular endothelial desquamation, stasis, and sludge are typical changes that are seen in the microcirculation that occurs in early stages of ANP and which subsequently evolves into thrombosis. We believe that thrombosis of the pancreatic microcirculation is one of the leading factors in the course of ANP. This hypothesis correlates with recent data of other investigators [1–4]. Initially, intravascular microthrombosis are seen as parietal circular changes (Figure 1) accompanied by endothelial desquamation. These changes are detected in the early stages when stromal swelling appears and there is no necrosis as yet. Apparently parietal circular thrombosis does not cause complete arrest of the circulation. That is why the first minimal signs of necrosis appear after thrombosis progresses into total obstruction of the vessels (Figure 2).

Masson's trichrome staining is the histochemical method of choice for investigation of thrombotic morphogenesis.

Thrombi start appearing at the periphery and extend towards the center and appear as an internal "death mask" of the vessel. Thereafter, fibrin masses grow at the periphery of the thrombus as a gracile net, then shrinkage and cicatrization follow. Thrombolysis or recanalization can frequently be seen together with the above-mentioned changes in a single sample or even in a single microscopic field (Figure 3).

In normal pancreatic tissue a thin black net is clearly seen surrounding each acinar cell or cell clusters. This net reflecting the shape of an acinus is the reticulin argyrophilic backbone. The brown coarse fibrotic folds that divide the reticulin net are collagen. The changes in the focus of necrosis affect, first of all, the reticulin argyrophilic backbone. In ANP the gracile structure of the argyrophilic reticulin carcass disappears and foci of decomposition are seen as brown structureless fibrotic components surrounding intact vessels or acinar ducts (Figure 4).

Mucoid changes of the stromal carcass which at first are reversible, progress and become visible with routine hematoxylin-eosin staining. Most often mucoid swelling is located in perivascular spaces, but it may also extend and change into fibrinoid imbibition. Fibrin and fibrinoids are easily detected by Masson staining wherein fibrinoids are seen as a bright red and fibrin as a dark red or claret color that shows a fasciculated structure. Intact connective tissue appears as blue or cyan fibers. Foci with fibrinoid changes progress into necrosis and assume PAS-positive features. When stained by Shiff reagent they turn into deep crimson color (Figure 5). Later these foci may accumulate calcium

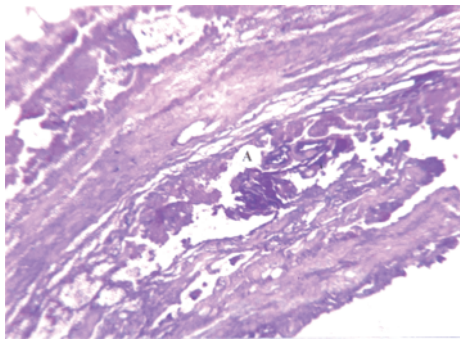


Figure 5: PAS-positive substance accumulation (A) in zones of fibrinoid necrosis. PAS reaction for neutral mucopolysaccharides (×100).

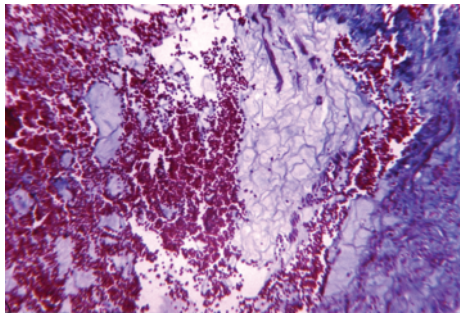

Figure 6: Fresh massive diapedesis (hemorrhages) in pancreas with ANP. Masson staining (×100).

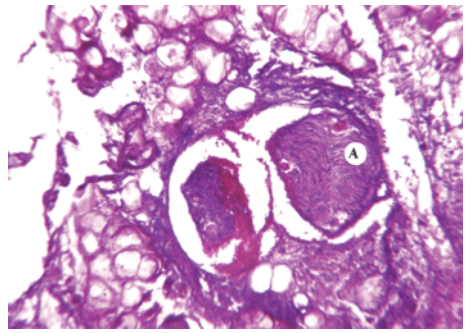


Figure 7: Neural fibers resistant to necrotic changes (A). H&E (×100).

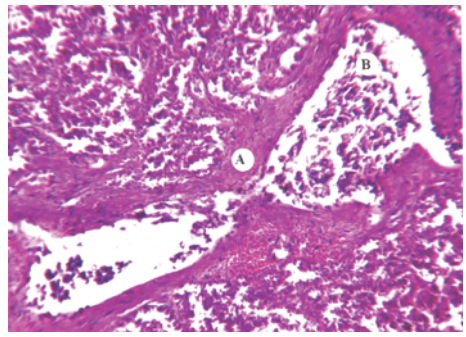


Figure 8: Pancreatic ducts resistant to necrotic changes (A). Desquamation of ductal epithelium (B). H&E (×100).

and that is why calcinosis found in pancreatic parenchyma may indicate earlier ANP.

As microcirculatory paresis persists and vascular permeability increases, the mucoid swelling is complicated by hemorrhage. In ANP, hemorrhages in the pancreatic parenchyma are an obligatory event and their manifestation varies widely from minimal to massive. The mechanism of these hemorrhages is considered diapedesis (Figure 6). Extravasated red blood cells undergo lysis and the extracellular hemoglobin consequently degrades resulting in catabolic hemosiderin formation in the pancreatic parenchyma.

Stromal components arise between the necrotic hemorrhages and the intact pancreatic tissue. Early parenchymal changes in ANP are usually discrete and are probably associated with certain stromal features even though the stroma itself is not clearly distinguishable in any given sample.

In ANP, nerves are resistant to destruction (Figure 7). Nerve structure is well preserved and nerve function is presumably intact in fields of total necrosis. This fact may explain the persistent pain syndrome in ANP patients.

Amidst acinar necrosis, lobular ducts appear to be relatively stable structures (Figure 8). Their resistance to necrosis is apparently proportional to their diameter. However, in all our findings, there was evidence of desquamation of ductal epithelium in samples from ANP patients.

Microscopic changes in the islets of Langerhans, in ANP patients, are also of great interest.

It is well known that in follow-up studies of ANP patients, only about 10–26% develop pancreatogenic diabetes after recovery [5, 6]. Diabetes is not necessarily present in patients with total exocrine pancreatic insufficiency. Microscopically, the structure of the islets of Langerhans is intact among masses of detritus (Figure 9). When stained with Masson, the islets of Langerhans are frequently surrounded by a gracile net of fibrin (Figure 10) which shows a deep claret color. Hence we may assume that this formation of a fibrin net around the islet of Langerhans contributes to its resistance and preservation of its structure in the aggressive microenvironment in patients with ANP.

Table 1 represents quantitative distribution of the microscopic changes in pancreas among patients with APN.

In conclusion, our study correlates with recently published data [1–4] and underlines the importance and supremacy of early pancreatic microcirculation and local coagulation disorders (44.2 to 100%) in the pathogenesis of ANP. Our study shows that despite aggressive surrounding necroses the islets of Langerhans are preserved in 74.1% of cases, most probably due to fibrin capsule formation. Therefore, surgical removal of necrotic tissue should be performed with maximal prudence and tissue preservation in order

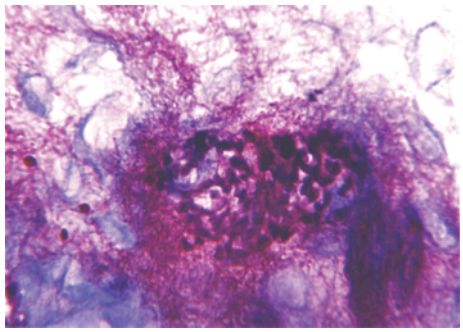

Figure 9: Islets of Langerhans resistant to necrotic changes (A). H&E (×400).

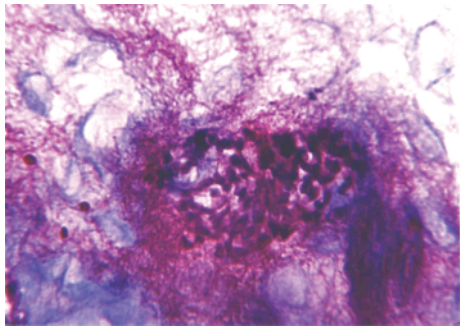

Figure 10: Fibrin masses noted around Langerhans islet in the zone of necrosis. Masson staining (×100).

Table 1: Early, late, and protective changes in pancreatic tissue in patients with APN.

| Microscopic changes | Number of patients | Percentage |
|---|---|---|
| *Early changes* | | |
| Vascular endothelial desquamation | 224 | 100% |
| Intravascular stasis and sludge | 215 | 95.9% |
| Circular thrombosis | 99 | 44.2% |
| Mucoid swelling in stromal carcass | 40 | 17.9% |
| Parenchymal hemorrhages | 208 | 92.9% |
| *Late changes* | | |
| Parenchymal necrosis | 224 | 100% |
| Total thromboses | 206 | 92% |
| Thrombolysis and recanalization | 49 | 21.9% |
| Decomposition of argyrophilic reticular carcass | 211 | 94.2% |
| Fibrinoid swelling | 164 | 73.2% |
| Calcium accumulation | 34 | 15.2% |
| *Protective changes* | | |
| Nerves resistant to necrosis | 179 | 79.9% |
| Lobular ducts resistant to necrosis | 175 | 78.1% |
| Islets of Langerhans resistant to necrosis | 166 | 74.1% |

to decrease the risk of pancreatic endocrine insufficiency in patients with ANP. On admission it may be advisable to use anticoagulation therapy in the early stages of acute pancreatitis, irrespective of the severity of the disease, in order to minimize the extent of pancreatic tissue ischemia and necrosis.

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*Review Article*

# Protective Effect of Melatonin on Acute Pancreatitis

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Melatonin, a product of the pineal gland, is released from the gut mucosa in response to food ingestion. Specific receptors for melatonin have been detected in many gastrointestinal tissues including the pancreas. Melatonin as well as its precursor, L-tryptophan, attenuates the severity of acute pancreatitis and protects the pancreatic tissue from the damage caused by acute inflammation. The beneficial effect of melatonin on acute pancreatitis, which has been reported in many experimental studies and supported by clinical observations, is related to: (1) enhancement of antioxidant defense of the pancreatic tissue, through direct scavenging of toxic radical oxygen (ROS) and nitrogen (RNS) species, (2) preservation of the activity of antioxidant enzymes; such as superoxide dismutase (SOD), catalase (CAT), or glutathione peroxidase (GPx), (3) the decline of pro-inflammatory cytokine tumor necrosis $\alpha$ (TNF$\alpha$) production, accompanied by stimulation of an anti-inflammatory IL-10, (4) improvement of pancreatic blood flow and decrease of neutrophil infiltration, (5) reduction of apoptosis and necrosis in the inflamed pancreatic tissue, (6) increased production of chaperon protein (HSP60), and (7) promotion of regenerative process in the pancreas. *Conclusion*. Endogenous melatonin produced from L-tryptophan could be one of the native mechanisms protecting the pancreas from acute damage and accelerating regeneration of this gland. The beneficial effects of melatonin shown in experimental studies suggest that melatonin ought to be employed in the clinical trials as a supportive therapy in acute pancreatitis and could be used in people at high risk for acute pancreatitis to prevent the development of pancreatic inflammation.

## 1. Melatonin in Pineal Gland and in the Gastrointestinal Tract

More than 50 years ago, Aaron Lerner, a dermatologist from Yale University, discovered melatonin (5-methoxy-N-acetyltryptamine) in the pineal gland. The name of this indoleamine comes from its effect on melanocytes [1]. Melatonin is produced from amino acid precursor; L-tryptophan and its production and release from the pineal gland undergo rhythmic diurnal/nocturnal fluctuations, with the peak at night and lowest level on the light phase [2–4].

Although melatonin has been recognized as the pineal hormone, subsequent studies have shown that melatonin could be synthesized in many tissues and that the gastrointestinal tract appears to be the main source of this substance [5–7]. Two main enzymes involved in the control of melatonin production, *arylalkylamino-N-acetyl-serotonin-transferase* (AA-NAT) and *hydroxyindolo-O-methyl-transferase* (HIOMT), have been detected in the gastrointestinal system [8, 9]. In addition, the estimated level of melatonin in the gastrointestinal tract was 400 times higher than that in the pineal gland [10]. In the gut, this indoleamine is produced mainly in the enteroendocrine (EE) cells; however, high concentration of melatonin has also been found in the bile [11–14]. Melatonin is released from the gastrointestinal mucosa in response to ingested food and this process is independent from the light/dark cycle and from the pineal gland [7, 15].

Previous studies have suggested that melatonin could be produced in the pancreas; the mRNA signal for AA-NAT, an enzyme involved in the synthesis of melatonin from L-tryptophan, has been detected in the isolated rat

pancreatic acinar cells [16], and gene expression for HIOMT, another enzyme controlling the above reaction has been, discovered in the human pancreas [17]. Recent studies revealed that melatonin and its receptors are present in the pancreatic gland [18]. Even though melatonin production in the pancreas is independent from the pineal gland, the content of melatonin in the pancreatic tissue undergoes rhythmic diurnal/nocturnal fluctuations [19].

It is interesting to compare diurnal/nocturnal fluctuations of melatonin in the pancreas, pineal gland, and in the plasma. Nocturnal concentration of melatonin in the pineal gland was about 1600 pg/100 mg, whereas, in the daytime, this concentration was reduced to about 150 pg/100 mg. This corresponds to the plasma levels of melatonin, which reaches 150 pg/mL at night, whereas, throughout the day, it was much lower (60 pg/mL). In the pancreatic tissue, the above-mentioned differences between nocturnal concentration of melatonin and its daily content were less pronounced and achieved 10 and 5 pg/100 mg of tissue, respectively [18].

Melatonin membrane receptors have been identified in the human endocrine pancreas, and this indoleamine has been proposed as one of the modulators of insulin secretion [20]. The abnormal secretion of melatonin was observed in type 2 diabetes, which may contribute to the pathogenesis of this disease [21–24]. In spite of the presence of melatonin in the pancreas and melatonin receptors in the pancreatic tissue, the role of this substance in the physiological regulation of pancreatic functions is still not complete.

Melatonin has also been recognized as a potent pancreatic secretagogue. Administration of melatonin or its amino acid precursor, L-tryptophan, to the animals resulted in the spectacular enhancement of pancreatic enzyme secretion accompanied by a significant increase of CCK plasma level [25]. Stimulatory effects of melatonin or L-tryptophan were much stronger following intraduodenal than intraperitoneal administration of investigated substances. The results of experimental studies suggest that melatonin activates cholinergic enteropancreatic reflex to increase pancreatic enzyme secretion [26]. It is very likely that melatonin produced in the gut lumen in response to food ingestion is implicated in the physiological regulation of pancreatic exocrine function.

## 2. Anti-Inflammatory Effects of Melatonin

The physiological significance of melatonin was the subject of several studies. Because of its rhythmic diurnal/nocturnal fluctuations, melatonin was believed to regulate the circadian rhythms such as hormones release, sleep/wake, and changes of body temperature [2–4, 27]. However, the most interesting property of melatonin appears to be its anti-inflammatory effect. Melatonin has been recognized as a beneficial substance, effectively protecting the tissues from inflammatory damage [16, 17, 28–33]. The favorable effect of melatonin, which has been reported in several studies, depends on two main mechanisms: (1) antioxidant effects of this indole and (2) modulation of immune defense induced by melatonin [34–41].

Melatonin is best known as the scavenger of radical oxygen (ROS) and nitrogen (RNS) species and activator of antioxidant enzymes [29, 42–47]. ROS and RNS are products of mitochondrial metabolism, and, under normal conditions, they are immediately neutralized by natural nonenzymatic scavengers and antioxidant enzymes. Melatonin together with reduced glutathione, vitamins C and E, uric acid, selenium, and creatinine belong to nonenzymatic scavengers [44, 48, 49]. Antioxidant enzymes such as; superoxide dismutase (SOD), catalase (CAT), glutathione peroxydase (GPx), or glutathione reductase (GR) are another line of defense against the noxious effect of ROS and RNS [17, 29, 43–45]. Oxidative stress in acute pancreatitis resulted in excessive production of ROS and RNS leading to the impaired ability of tissue to detoxify above intermediates. ROS and RNS are accumulated in the tissue leading to its damage [50–52]. The harmful effects of ROS and RNS in acute pancreatitis have been confirmed in previous studies [53, 54]. Melatonin, which is a highly lipophilic substance, penetrates inside the cells to maintain antioxidant enzymes activities, to keep the mitochondria from oxidative injury, and to prevent lipid membranes from peroxidation [55].

Recently melatonin has been shown to trigger signal transduction pathways leading to the activation of antioxidant enzymes and to the reduction of inflammatory mediators in the pancreas [56]. In acute pancreatitis, melatonin was demonstrated to inhibit nuclear binding of NF-$\kappa$B, the transcription factor, which controls the expression of genes involved in immunity and inflammation, production of prostaglandins, cytokines, cell adhesion molecules, nitric oxide (NO), and inhibitors of apoptosis [57, 58]. Melatonin has been demonstrated to reduce gene expression and synthesis of proinflammatory cytokine; tumor necrosis factor $\alpha$ (TNF$\alpha$), proinflammatory interleukins; IL-1$\beta$, IL-6, IL-8, and prostaglandins [56, 59, 60]. In addition, melatonin was also reported to modulate the processes of apoptosis and necrosis, to stimulate the production of vascular endothelial growth factor (VEGF), and to activate the process of angiogenesis [32, 61, 62]. All of the above effects could be related to the inhibition of NF-$\kappa$B by melatonin [56].

Beside the reduction of the above proinflammatory molecular pathway, melatonin enhanced expression of nuclear factor erythroid 2-related factor (Nrf2), which activates the signal transduction lane of antioxidant genes [56]. Since Nrf2 is critically involved in the induction of antioxidant enzymes such as SOD, CAT, GPx, GST, GR [63] and melatonin is known to activate above enzymes, it is quite possible that the antioxidative effect of melatonin is mediated by Nrf2.

Melatonin binds to the specific G-protein-coupled receptors: $MT_1R$, $MT_2R$, $MT_3R$, which have been identified in many tissues [64]. Perhaps melatonin exerts its effects also through intracellular orphan receptors (ROR/RZR), which have been detected in the nucleus, cytoplasm, and mitochondria, but physiological significance of these receptors remains unclear [64, 65]. It is important to emphasize that melatonin could very easily cross the cell membranes because

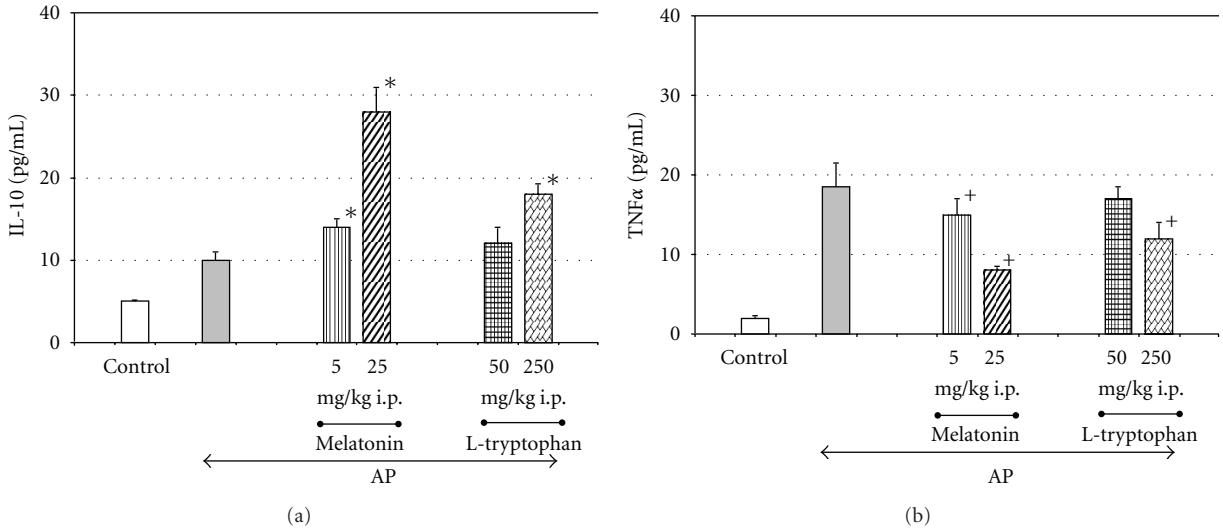


Figure 1: Effect of melatonin on plasma levels of interleukin 10 (IL-10) and tumor necrosis factor $\alpha$ (TNF $\alpha$) in the rats with acute pancreatitis (AP). Melatonin or its precursor, L-tryptophan, was given to the rats 30 min prior the induction of acute pancreatitis produced by caerulein overstimulation (5 $\mu$g/kg-h $\times$ 5 h). Control-intact rats. Means $\pm$ SEM from the separate experiments, each performed on 8–10 rats. Asterisk indicates significant increase above the value detected in AP rats alone. Cross indicates significant decreases below the value detected in AP rats alone.

it is highly lipophilic, and on this way melatonin could exert its biological receptor-independent effects [66].

Production of melatonin is decreased in older individuals, and it is believed that the reduction of melatonin level contributes to the process of aging [67]. Application of this substance reverses some of degenerative processes related to old age, and, for this reason, melatonin was suggested to be the "hormone of youth" [3, 31, 67].

## 3. Beneficial Effects of Melatonin on Acute Pancreatitis

Experimental studies have shown that application of melatonin significantly attenuated the development of acute pancreatitis and protected pancreatic tissue against the damage caused by acute inflammation [16, 17, 59–61, 68–75]. In the rats pretreated with melatonin prior to the induction of acute pancreatitis, the morphological signs of inflammation such as edema, leukocyte infiltration, and cell vacuolization were dramatically reduced [16, 60, 70–73]. Also other parameters of acute pancreatitis severity such as blood levels of amylase or lipase were significantly diminished in the animals pretreated with melatonin, as compared to the rats with acute pancreatitis alone [16, 17, 59, 68, 70–75]. The beneficial effect of this indoleamine on acute pancreatitis was also manifested by the dose-dependent reduction of proinflammatory cytokine TNF$\alpha$ blood level, accompanied by a marked rise of anti-inflammatory interleukin 10 (IL-10) in the animals subjected to acute pancreatitis and pretreated with melatonin [16, 69, 70] (Figure 1).

Melatonin is able to diminish the generation of ROS in the pancreatic tissue, as was demonstrated by the reduced amount of lipid peroxidation products: MDA + 4HNE in the pancreas of animals with acute pancreatitis pretreated with this indoleamine [16, 17, 68, 70–73, 75]. In addition, the application of the mentioned protective substance resulted in the significant and dose-dependent increase of antioxidant enzyme (SOD) activity in the pancreatic tissue taken from the rats with acute pancreatitis [17, 61, 72–74] (Figure 2).

The protective action of melatonin on acute pancreatitis was confirmed in several studies, using different models of experimental pancreatitis. Melatonin attenuated acute pancreatitis severity and diminished harmful effects of acute inflammation induced by L-arginine [72], ischemia/reperfusion, or caerulein overstimulation [16, 59, 61, 68, 70, 74, 76]. Melatonin protected the pancreas against acute pancreatitis caused by taurocholic acid [60] or by obstruction of pancreatic duct [75]. However, in the model of necrotizing pancreatitis induced by glycodeoxycholic acid melatonin appears less effective, because increased serum amylase level and high mortality rate of experimental animals was unaffected by this indoleamine [77].

It is worth remembering that this substance was also found to promote the regeneration of pancreatic tissue following the damage caused by acute pancreatitis. Treatment with melatonin improves the rate of DNA synthesis, as well as pancreatic enzyme content in the rats with arginine-induced pancreatitis [76].

Studies on melatonin revealed that not only melatonin but also its amino acid precursor, L-tryptophan, is able to attenuate pancreatic tissue damage caused by acute inflammation and to reduce lipid peroxidation in two models of acute pancreatitis: caerulein-induced and ischemia/reperfusion pancreatitis [16]. It is likely that the protective effect of L-tryptophan on acute pancreatitis was

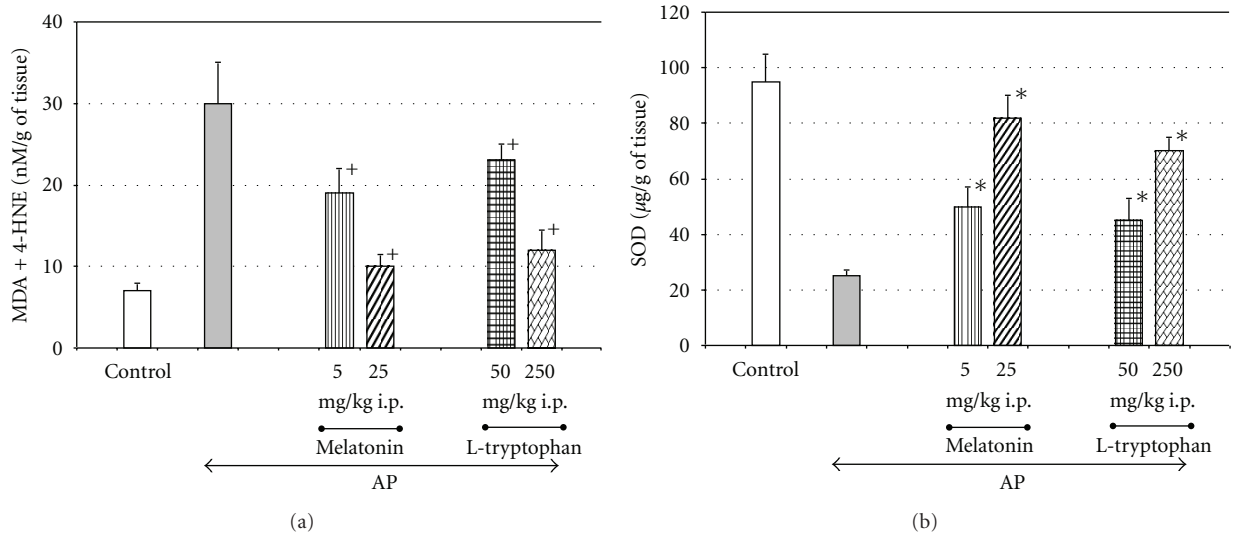


Figure 2: Effect of intraperitoneal (i.p.) application of melatonin or its precursor, L-tryptophan, on concentrations of antioxidative enzyme superoxide dismutase (SOD) and lipid peroxidation products (MDA + 4-HNE) in the pancreatic tissue taken from rats with acute pancreatitis (AP). Melatonin or its precursor, L-tryptophan, was applied as explained on Figure 1. Control—intact rats. Means ± SEM from the separate experiments, each performed on 8–10 rats. Asterisk indicates significant increase above the value detected in AP rats alone. Cross indicates significant decreases below the value detected in AP rats alone.

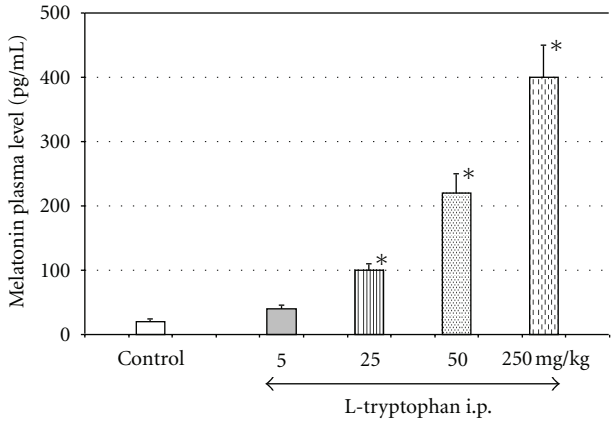


Figure 3: Plasma level of melatonin in response to intraperitoneal (i.p.) administration of increasing doses of L-tryptophan. Melatonin plasma level was measured by RIA. Means ± SEM from the separate experiments, each performed on 8–10 rats. Asterisk indicates significant increase above the control value.

dependent on the conversion of this amino acid into melatonin, because intraduodenal administration of L-tryptophan resulted in the significant and dose-dependent increase of plasma melatonin level (Figure 3). These results lead to the conclusion that endogenous melatonin, which is produced from L-tryptophan effectively protects the pancreas from the damage caused by acute inflammation [16].

Application of L-tryptophan at doses of 25 or 50 mg/kg raised the plasma level of melatonin up to 100 or 220 pg/mL, respectively. It is important to underline that pretreatment of the animals with the above doses of L-tryptophan significantly attenuates the inflammatory process in the pancreas [16]. Since normal blood level of melatonin fluctuates from 50 pg/mL (during the light phase) to about 160 pg/mL (at night) [18], it could be assumed that melatonin at physiological concentrations is able to protect the pancreas against acute inflammatory damage.

This observation indicates that endogenous melatonin could be one of the physiological protectors of the pancreas. This notion is supported by the study showing that blockade of the melatonin receptor aggravated pancreatic damage caused by caerulein overstimulation. In the rats subjected to acute pancreatitis and pretreated with melatonin MT1/2 receptor antagonist, luzindole, the histological and biochemical manifestations of pancreatitis were significantly higher than in the group with acute pancreatitis alone [70].

Recent observation from humans with acute pancreatitis supported and reinforced this hypothesis. It was observed that melatonin serum level, measured in the first 24 hours after the onset of acute pancreatitis, negatively correlated with the severity of this illness. In the patients with mild pancreatitis, serum level of melatonin was markedly higher than in these with severe form of this disease [78]. This study presents additional evidence that melatonin could be one of the natural pancreatic protectors and that high blood level of this indoleamine has a protective value against acute pancreatic inflammation.

A considerable amount of melatonin is produced in the gastrointestinal system in response to food ingestion, and this melatonin is absorbed into the blood stream and represents the daily pool of this indoleamine, whereas nocturnal level of melatonin depends on its synthesis in the pineal gland [5, 7, 12, 33]. The possible involvement of this pineal melatonin in

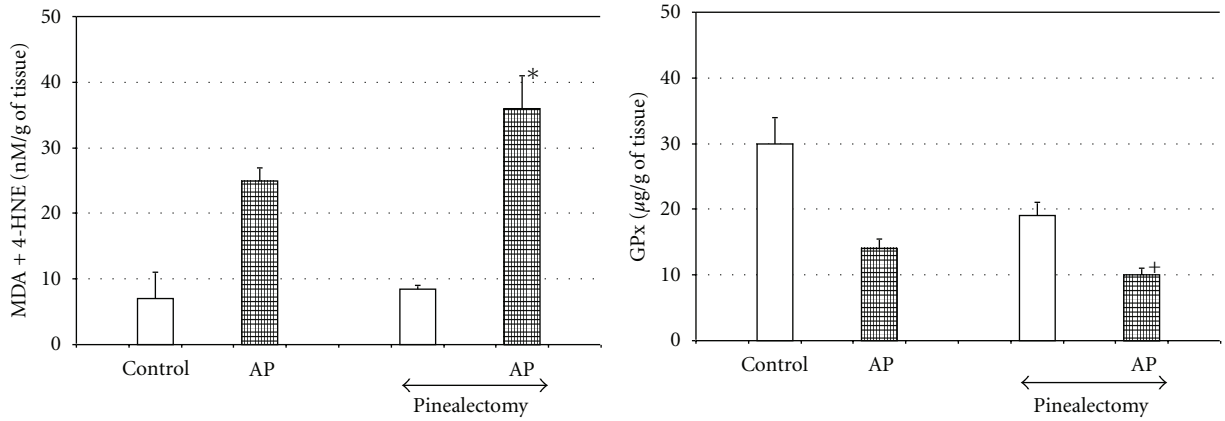


Figure 4: Effect of pinealectomy on pancreatic contents of glutathione peroxidase (GPx) and lipid peroxidation products (MDA + 4-HNE) in the pancreatic tissue of rats with acute pancreatitis. Control—intact rats. Means ± SEM from the separate experiments, each performed on 8–10 rats. Asterisk indicates significant increase above the value detected in AP with intact pineal gland. Cross indicates significant decrease below the value detected in AP with intact pineal gland.

the pancreatic protection should be taken into consideration, and, to solve this problem, subsequent experiments have been performed. It has been observed that acute pancreatitis induced in the dark phase was more severe than during the day [74]. Because nocturnal melatonin plasma level depends on the production and release of this substance from the pineal gland, it is possible that pineal melatonin takes part in the protection of the pancreas against acute inflammation. A recent study on rats with removed pineal glands give further evidence for this hypothesis. As we have observed, acute pancreatitis was much more severe in pinealectomized animals, than in those with intact pineal glands. This was manifested by significant decrease of an antioxidant enzyme GPx in the pancreas of pinealectomized rats subjected to acute pancreatitis, as compared to the animals with intact pineal glands (Figure 4). Application of melatonin to the rats deprived of the pineal gland and subjected to acute pancreatitis significantly reduced pancreatic tissue lesions and attenuated the course of acute inflammation [75].

The results of experimental studies and clinical observations indicate that several mechanisms are involved in the beneficial effect of melatonin on acute pancreatitis.

*3.1. Antioxidative Mechanism.* Melatonin as an effective scavenger of free radicals is able to neutralize above toxic products directly [16, 17, 68, 70–73]. In addition, this indoleamine could activate the antioxidant enzymes such as SOD, CAT, GPx, and GSH and protects them from inactivation by reactive intermediates. Thus, melatonin could improve the oxidative status of the pancreatic tissue indirectly [16, 61, 70, 71, 73–75].

*3.2. Modulation of the Immune System.* Melatonin is able to affect the immune system and to strengthen the immune defense. This substance has been demonstrated to inhibit neutrophile infiltration [16, 70–74], to decrease myeloperoxidase (MPO) activity [56], and to diminish the prostaglandin generation [59] in the inflamed pancreas. Recently, melatonin has been shown to reduce mRNA expression of many proinflammatory cytokines such as IL-1 $\beta$, IL-6, IL-8, and TNF$\alpha$ in the pancreatic tissue subjected to acute inflammation [56]. The inhibitory effect of melatonin on proinflammatory cytokine production has been confirmed by marked reduction of the blood level of TNF$\alpha$ in the rats with acute pancreatitis pretreated with this indoleamine [16, 69, 70]. On the contrary; anti-inflammatory IL-10 was increased in these animals [16]. Melatonin enhanced the expression of nuclear factor erythroid 2-related factor (Nrf2) and diminished the nuclear binding of NF-$\kappa$B, and it is likely that above effects could be involved in the curtailing of acute pancreatitis by melatonin [56].

*3.3. Improvement of Pancreatic Blood Flow.* Melatonin has been demonstrated to increase the blood flow and to remove the toxic substances from pancreatic tissue [16, 69, 70, 76, 79].

*3.4. Effect on Apoptosis.* Melatonin is able to reduce processes of apoptosis and necrosis in the pancreas [61]. However in the tumor cells, this substance promotes apoptosis maintaining the viability of normal pancreatic units [80].

*3.5. Stimulation of Heat Shock Protein (HSP).* HSPs are known to protect the cell compartment against the damage. Production of these proteins is augmented in response to high temperature, oxidative stress, or inflammation [81]. Melatonin has been reported to increase mRNA signal for HSP60 in pancreatic acinar cell line AR42J [82]. It could be expected that melatonin works to save acinar cells from acute damage through the stimulation of HSP production. As was observed, melatonin prevented these cells from mitochondrial and nuclear damage caused by acute pancreatitis, reduced the dilatation of endoplasmatic

reticulum and Golgi apparatus, and diminish formation of autophagosomes [72].

*3.6. Promotion of Pancreatic Regeneration.* It should be emphasized that administration of melatonin following the induction of acute pancreatitis not only reduced the severity of inflammation, but also promotes the spontaneous regeneration process of the pancreatic tissue. This was manifested by an increased rate of DNA and protein synthesis and supported by histological examination [77].

The results of previous experimental studies and clinical test indicate that melatonin should be employed in clinical trials as a supportive agent for the treatment of patients with acute pancreatitis. Melatonin has been used previously as part of composed therapy in patients with tumors and neurological diseases [82–84]. In patients with cancer melatonin significantly decreased thrombocytopenia, asthenia, neuro- and cardiotoxicity induced by chemotherapeutic treatment [82]. It is important to emphasize that the use of this indoleamine is safe, it has been reported that melatonin given at doses of 20 mg/day for several weeks in patients with dyskinesia or with sleep disturbance did not produce any side effects [84, 85]. It was suggested that melatonin at doses as high as 50–100 mg/day could be applied for treatment of insomnia and depression [85]. Regarding the beneficial effects and safety of melatonin use, this substance could also be introduced as a component of early jejunal feeding in patients with acute pancreatitis.

## 4. Conclusion

Endogenous melatonin could be one of the native mechanisms protecting the pancreas from acute damage and accelerating regeneration of this gland. The beneficial effects of melatonin shown in experimental studies suggest that melatonin ought to be employed in the clinical trials as a adjuvant therapy in acute pancreatitis.

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*Review Article*

# Pancreatic Pseudocyst: Therapeutic Dilemma

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Pancreatic pseudocyst develops in both acute and chronic pancreatitis. It is an entity likely to either remain asymptomatic or develop devastating complications. Despite being diagnosed easily, treatment exercise is still at crossroads whether in the form of internal or external drainage or endoscopic, laparoscopic, or open intervention with a good radiological guidance. The therapeutic dilemma whether to treat a patient with a pancreatic pseudocyst, as well as when and with what technique, is a difficult one. This paper is intended to get information about diagnostic and therapeutic exercises most appropriate for acute and chronic pancreatic pseudocyst.

## 1. Background

The first description of pseudopancreatic cyst dates back almost two and half centuries to 1761 A.D. by Cannon et al. [1]. The management of cystic changes of the pancreas is an old problem. Eugene Opie, at the beginning of twentieth century, was the first to distinguish true pancreatic cysts, which are, by definition, lined by epithelium, from pseudocysts, which are surrounded by a wall composed of collagen and granulation tissue. More than two centuries after the first description, some clear consensus and guidelines were evolved in the Atlanta classification of 1993 [2]. The Atlanta classification consists of four distinct disease entities: acute fluid collections that develop early in the course of acute pancreatitis and do not yet have a cyst wall; acute pancreatic pseudocysts, which arise as sequelae of acute pancreatitis or trauma, and whose wall consists of granulation tissue and extracellular matrix; chronic pancreatic pseudocysts, which arise as sequelae of chronic pancreatitis and are likewise surrounded by a wall; pancreatic abscesses, which are intra-abdominal collections of pus immediately adjacent to the pancreas, without any large areas of necrosis. Acute fluid collections, pancreatic pseudocysts, and pancreatic abscesses can be distinguished from one another by the history, imaging studies of the wall of the abnormality and its contents, and, if necessary, a needle aspiration of the content [2].

## 2. Introduction

Pseudocysts are formed after acute as well as chronic pancreatitis but more common after acute exacerbations of chronic pancreatitis than acute pancreatitis. There is lack of data containing randomized case-control studies, but numerous case series and reports indicate that pancreatic injury leads to pseudocyst formation. Pancreatic pseudocysts often arise as a complication of acute or chronic pancreatitis. The prevalence of pancreatic pseudocysts in acute pancreatitis has been reported to range from 6% to 18.5% [3, 4]. The prevalence of pancreatic pseudocysts in chronic pancreatitis range 20% to 40% [5]. Pancreatic pseudocysts most commonly arise in patients with alcoholic chronic pancreatitis (from 70% to 78%) [6]. The second most common cause is idiopathic chronic pancreatitis (from 6% to 16%), followed by biliary pancreatitis (from 6% to 8%). The incidence of pseudocyst is low ranging from 1.6 to 4.5% or 0.5 to 1 per 100000 adults per year [7, 8].

## 3. Classification

D'Egidio and Schein, in 1991, described a classification of pancreatic pseudocyst based on the underlying etiology of pancreatitis (acute or chronic), the pancreatic ductal anatomy, and the presence of communication between the cyst and the pancreatic duct and defined three distinct types

of pseudocysts [9]. Type I, or acute "postnecrotic" pseudocysts that occur after an episode of acute pancreatitis and are associated with normal duct anatomy, rarely communicates with the pancreatic duct. Type II, also postnecrotic pseudocysts, which occurs after an episode of acute-on-chronic pancreatitis (the pancreatic duct is diseased but not strictured, and there is often a duct-pseudocyst communication). Type III, defined as "retention" pseudocysts, occurs with chronic pancreatitis and is uniformly associated with duct stricture and pseudocyst duct communication.

Another classification, based entirely on pancreatic duct anatomy, is proposed by Nealon and Walser [10].

Type I: normal duct/no communication with the cyst.

Type II: normal duct with duct-cyst communication.

Type III: otherwise normal duct with stricture and no duct-cyst communication.

Type IV: otherwise normal duct with stricture and duct-cyst communication.

Type V: otherwise normal duct with complete cutoff.

Type VI: chronic pancreatitis and no duct-cyst communication.

Type VII: chronic pancreatitis with duct-cyst communication [10].

## 4. Diagnosis

Pseudocyst of pancreas must be preceded by attacks of pancreatitis in either acute or chronic form. Most of the times, clinical, biochemical, and radiological evidence of pancreatitis present, but still a large number of patients may present with features of pancreatic pseudocyst without any documentary evidence of pancreatitis. One should always consider the possibility of a pseudocyst in a patient who has persistent abdominal pain, anorexia, or abdominal mass after a case of pancreatitis. Rarely, patients present with jaundice or sepsis from an infected pseudocyst [11]. In patients presenting with pancreatic cyst incidentally discovered on imaging, a crucial point is to define whether the patient has had prior history of pancreatitis [12]. Rarely, patients with large pancreatic pseudocyst may be asymptomatic occasionally. Tender abdomen with palpable mass is the positive finding on physical examination. Fever, icterus, and pleural effusion may be present in complicated pseudocyst. If pseudocyst ruptures then features of secondary peritonitis set in and presentation may be like septicaemic shock [11]. The diagnosis of a pancreatic pseudocyst is usually established by imaging studies, among which transabdominal ultrasonography is important as an initial investigation [13]. Computerized tomography (CT) is often the imaging method of choice, with 82% to 100% sensitivity and 98% specificity [14].

Biochemical parameters have limited role in diagnosis. Among remarkable parameters are serum amylase and serum lipase, which will be elevated in most cases. Liver functions are normally unchanged but may be deranged in cases where obstruction to the biliary tract occurs. Another thing to be considered is strong possibility of biliary peritonitis if liver parameters deranged. Other inflammatory marker C-reactive protein is raised and is of prognostic significance only. Elevated triglycerides and low serum calcium are indirect indictors of panreatic pseudocyst.

Differential diagnosis of pancreatic pseudocyst always may be of two possibilities either intrapancreatic lesions or extrapancreatic lesions.

Intrapancreatic diseases mimicking pancreatitis are:

(1) pancreatitis (acute and chronic),

(2) pancreatic necrosis,

(3) pancreatic abscess,

(4) adenocarcinoma of pancreas,

(5) cystic neoplasm of pancreas,

(6) pancreatic artery pseudoaneurysm.

Extrapancreatic diseases mimicking pancreatitis are

(1) peptic ulcer disease,

(2) acute cholecystitis and cholelithiasis,

(3) gastric cancer,

(4) abdominal aortic aneurysm,

(5) ovarian cysts and carcinoma,

(6) acute myocardial infarction,

(7) pneumonia,

(8) intestinal obstruction,

(9) intestinal ischemia.

Among different imaging modalities, ultrasound (USG) is the foremost diagnostic tool and also useful pointer of diagnosis in most of the cases. It may be used in

(a) transabdominal USG,

(b) colour doppler study,

(c) duplex scanning,

(d) endoscopic USG.

Pancreatic pseudocyst appears as anechoic structure usually round or oval and surrounded by a smooth wall associated with distal acoustic enhancement on US examination. They are well defined and round or oval, and they are contained within a smooth wall. During the early phases of their development, pseudocysts can appear more complex, with varying degrees of internal echoes. If the earliest detection missed sometimes it may be due to excessive bowel gas. When necrotic debris or hemorrhage presents inside cyst or infection sets in then the interpretation on USG may be difficult. Color Doppler or duplex scanning should always be performed in cystic lesions to ensure that the lesion in question is not a giant pseudoaneurysm. Sensitivity rates for US in the detection of pancreatic pseudocysts are from 75% to 90%. Therefore, US is inferior to CT, which has a sensitivity rate of 90%–100%. US has several limitations, as compared with CT, in the initial diagnosis of a pseudocyst:

the presence of overlying bowel gas decreases the sensitivity of US, and unlike CT, US examinations are highly operator dependent [15].

A thick-walled, rounded, and fluid-filled mass adjacent to the pancreas on an abdominal CT scan in a patient with a history of acute or chronic pancreatitis is virtually pathognomonic for pancreatic pseudocyst. In acute manifestations when ileus or excessive gas shadow or bowel obstruction is a problem in USG evaluation, CT scan is definitely better and is purposeful in diagnosing pseudocyst. It is almost diagnostic and no other supplementary investigation that is required to confirm the diagnosis. Major advantage of CT scan is the detection of an objective and detailed anatomy as well as pathology. In addition to pancreas, extrapancreatic pathology as well as status of adjoining organs, for example, gallbladder, liver, common bile duct, stomach, and duodenum can be perfectly assessed. Contrast-enhanced CT is now the primary tool of investigation for initial diagnosis of pancreatic pseudocysts. USG should be done for the followup of asymptomatic pseudocysts or when diagnosis is uncertain. The only major limitation of CT scan is that it is unable to differentiate cystic neoplasm of pancreas from pseudocyst, and the main pathology to be missed is mucinous cystadenomas and intraductal papillary mucinous cystadenoma (IPMN) [16].

MRI and MRCP are accurate and sensitive diagnostic aids for defining the anatomy of duct better than any other diagnostic tool. But these are not used routinely as adequate information is obtained in maximum cases by CT, and very rarely ductal anatomy is needed to be calibrated with too much precision and MRI/MRCP is required. Pancreatic duct and biliary system are best visualized in detail although interpretation of integrity of pancreatic duct may be difficult [17]. MRCP techniques can also depict subtle branch-chain dilatation in chronic pancreatitis. MRI is also highly sensitive to the detection of bleeding with complex fluid collections.

The role of endoscopic retrograde cholangiopancreatography (ERCP) is limited to some extent for therapeutic intervention rather than diagnostic purpose. It may help in planning an intervention after the increased use of endoscopic USG its role is gradually decreasing.

Endoscopic ultrasound (EUS) is a test of choice to differentiate between cystic neoplasms of pancreas from pseudocyst. EUS is usually used as a secondary test to further evaluate pancreatic cyst detected by other imaging modality (US, CT or MRI). For the distinction of acute fluid collections from pancreatic abscesses and acute pancreatic pseudocysts, endosonography (EUS) has the highest sensitivity (93% to 100%) and specificity (92% to 98%). The diagnostic puncture of a pseudocyst under EUS guidance helps distinguish cystic malignancies from pseudocysts. A malignant lesion is more likely present when the carcinoembryogenic antigen (CEA) value exceeds 192 ng/mL and when the cyst contents are highly viscous [18].

Visualization of the pancreas via EUS provides high quality images due to the close proximity analysis, which are helpful to detect malignancy. An elevated CEA level on FNAC within the cyst fluid strongly suggests mucinous lesion [19, 20]. Amylase levels are usually high in pseudocysts and low in serous cystadenoma of the ultrasound transducer to the area of interest. Criteria suggestive of cystic neoplasm include a cyst wall thickness of greater than 3 mm, macroseptation (all cystic components more than 10 mm), the presence of a mass or nodule, and cystic dilation of the main pancreatic duct [19–21].

Aspiration of cyst fluid under EUS guidance and biochemical analysis with molecular analysis helps in differentiating different cystic neoplasms of pancreas. Mutational changes and DNA content point towards malignancy.

## 5. Treatment

Treatment of pseudopancreatic cyst comprises two aspects: supportive care or medical management and definitive care or surgical drainage.

Intravenous fluids, analgesics, and antiemetics are the basic requirements. Low-far diet is given to patients who tolerate and intake. In patients with low or poor oral intake, support can be provided via nasoenteral feeding or total parenteral nutrition (TPN). To date, no studies have compared these two approaches in the seating of pancreatic pseudocyst, and the choice is based on availability and local preferences. If one can extrapolate from studies comparing the two modalities in the seating of acute necrotizing pancreatitis, one can expect that jejunal feeding will be related with fewer complications (infection) but may not be able to provide as much calories as TPN.

The role of octreotide is still dubious as this has not been tested much with strong evidence in the literature. The rationale of using octreotide as a therapy for pancreatic pseudocyst is that it will decrease pancreatic secretions and aid in pseudocyst resolution. Unfortunately, this strategy has not been rigorously tested and only a handful of case series have been published [22, 23].

Most pseudocysts resolve with supportive medical care. Vitas and Sarr followed over a period of 5 years 114 patients with the diagnosis of pancreatic pseudocyst [24]. Forty-six patients underwent primary operative therapy, with 13% undergoing emergency operations for pseudocyst-related complications. Morbidity occurred in 26% of patients (emergency operations, 67%; elective procedures, 10%) without any mortality. The remaining 68 patients were initially treated with a nonoperative expectant approach. Severe and life-threatening complications in this group (followup for a mean of 46 month) occurred in only six patients (9%); 19 patients eventually underwent elective operations directed at either the pseudocyst or other complications related to pancreatitis. Overall, in patients managed by a nonoperative approach, resolution of the pseudocyst occurred in 57% of the 24 patients with satisfactory radiographic followup, with 38% resolving more than 6 months after diagnosis. Although patients eventually undergoing operation tended to have larger pancreatic pseudocysts than the patients managed successfully nonoperatively (6.9 cm versus 4.9 cm), no serious complications occurred in seven patients with pancreatic pseudocysts greater than 10 cm who were treated expectantly [24].

Large-sized and long-standing cysts are not likely to respond on conservative treatment and more likely to have complications during the course of the disease. Morbidity and mortality are more commonly found in this group. These patients need surgical intervention and usually managed surgically. But some studies say that size and duration never matter, and actually these patients too have excellent surgical results and do well. There are two definite conclusions that the presence and the severity of symptoms and complications are determinants of prognosis and course in pancreatitis [25–27].

## 6. Drainage Procedures

Most of the symptomatic and complicated pancreatic pseudocysts need intervention in any form during the course of the disease. Intervention options are either guided endoscopically, radiologically, laparoscopically, or open/direct. To date, no prospective controlled studies have compared directly percutaneous, surgical, and endoscopic drainage approaches. As a result, the management varies based on local expertise, but in general endoscopic drainage is becoming the preferred approach followed by laparoscopic approach.

There is no consensus regarding methods of intervention in pancreatic pseudocyst although there is no controversy with conservative treatment. Minimal intervention with maximal conservative approach remains the most widely acceptable option of therapeutic intervention in pancreatic pseudocyst. Small sized asymptomatic cysts need no intervention at all. Asymptomatic large-sized cyst should be intervened after six weeks only and in the meantime is must be under close monitoring to detect the earliest symptoms or complications. Only in symptomatic cases or if any complication develops, intervention is required before six weeks. Cyst of any size should be intervened once it becomes symptomatic or if complications develop irrespective of duration, size, or site. So two things are important determinants the regarding plan of management: size when it is more than five cm and duration when it is more that six weeks.

*6.1. External Drainage.* External drainage can be achieved radiologically by using CT or US guidance. In this technique, a drainage pigtail catheter is placed percutaneously into the fluid cavity, and the fluid is drained. Three-dimensional ultrasonography has been reported useful for the guidance of catheters into cyst cavities and avoiding vessels. When the drainage output becomes minimal, the catheter is removed. Contrast injection into the cyst cavity will demonstrate the size of the remaining cavity, and this finding can be used to monitor the progress. This technique is successful in resolving pseudocysts, but it has a high risk of infections. This technique is definitely a failure if the catheter tends to block repeatedly. It tends to create significant discomfort to the patient. Furthermore, the catheter tends to clog and may require repositioning and exchange. The reported long-term success rate of pseudocyst resolution for US-guided pseudocyst drainage is around 50%. Unsuccessful drainages are usually caused by large ductal leaks or obstruction of the main pancreatic duct. Percutaneous catheter drainage is contraindicated in patients who are poorly compliant and cannot manage a catheter at home. It is also contraindicated in patients with strictures of the main pancreatic duct and in patients with cysts containing bloody or solid material [28–30].

*6.2. Surgical Drainage.* In cases of failure of external percutaneous drainage radiologically, this approach is applied either by open method or by laparoscopy. It can be a good option for the patients who cannot tolerate endoscopic drainage. Stoma is created between the most dependent part of the cyst and the adjoining stomach, jejunum, or ileum to provide effective drainage.

For surgical drainage, either lap or open method can be opted as both are effective for relief, but laparoscopic approach definitely carries low morbidity and mortality as compared to open techniques. Surgical drainage, which is increasingly done laparoscopically with a cholecystectomy if needed is the preferred mode then open approach.

External drainage of pseudocyst should only be carried out in case of emergency relief of severe symptoms and sepsis. Otherwise, EUS or surgical drainage are the procedures of choice. Blind external drainage when duct status is unsure results in difficult-to-manage pancreatic fistulae [31].

*6.3. Endoscopic Drainage.* Endoscopic drainage of pseudocysts is becoming the preferred therapeutic approach because it is less invasive than surgery. The intervention done is minimal and avoids the need for external drain and has a high long-term success rate. Internal drainage is accomplished with either a transpapillary approach with ERCP or direct drainage across the stomach or duodenal wall. A transpapillary approach is preferable when the pseudocyst communicates with the main pancreatic duct, usually in the gene of the pancreatic duct. This approach is also successful for patients with pancreatic duct disruption. The endoscopic approach is guided by the presence of a bulge into the lumen of the stomach or duodenum in order to determine the entry site for catheterization. This approach has several inherent risks, including missing the pseudocyst, injuring intervening vessels, and suboptimal placement of the drainage catheter [32]. Therapeutic echoendoscopes now make it possible to treat pseudocysts with EUS-guided transmural stenting [33]. Several series have described the deployment of a 7 Fr stent that is introduced with a needle-knife catheter [34]. A new large-channel echoendoscope allows the use of 10 Fr stent across the stomach or duodenum [35].

In a large retrospective analysis of 603 patients who were undergoing EUS-FNA of pancreatic cysts, possible infection developed in only a single patient. The majority of patients in this series (90%) received antibiotic prophylaxis, most commonly a fluoroquinolone given for 3 days after the procedure, and this may possibly explain the low infection rate. The benefit of prophylactic antibiotics before an FNA of cystic lesions has not been evaluated by prospective randomized studies [36].

The ASGE, in 2008, published the guidelines for prophylactic use of antibiotics for GI endoscopy. According to these guidelines, prophylaxis with an antibiotic, such as a fluoroquinolone, is administered before EUS-FNA of cystic lesions along the GI tract including pancreatic cyst. Antibiotics may be continued for 3–5 days after the procedure (supported by observational studies). The administration of antibiotic prophylax, a fluoroquinolone administered before the procedure and continued for 3 days after the procedure, is a reasonable regimen [37].

Cahen et al. concluded that endoscopic drainage is an effective treatment for pancreatic pseudocysts and offers a definitive solution in almost three-quarters of the cases. The majority of the major complications might have been prevented by using pigtail stents instead of straight stents and by taking a more aggressive approach to the prevention and treatment of secondary cyst infection [38].

Final decision on EUS versus surgical drainage is important and interesting as the decision making depends upon the profile of the patient. It is important to know that multiple procedures are sometimes necessary to ensure adequate drainage. Also when there is a large amount of solid debris, EUS drainage does not give good results. There has been significant technical advancement in EUS-guided drainage procedures with improved equipments and skill base. It is certain that EUS drainage will be more and more a preferred option over surgical drainage in the future too.

## 7. Complications

Pancreatic pseudocyst needs close followup to early detect the most dreadful complications, which may be devastating if it remain unrecognized for long.

(A) *Infection*: infection occurs either spontaneously or after therapeutic or diagnostic manipulations. While infected pseudocyst can initially be treated with conservative means, a majority of patients will require intervention. Traditionally, surgery has been the preferred modality but endoscopic treatment is gaining acceptance. An external drainage may be necessary in selected situations such as when there is evidence of gross sepsis and the patient is too unstable to undergo surgical or endoscopic drainage [39].

(B) *Hemorrhage*: hemorrhage can greatly complicate the course of a pseudocyst and can be devastating [40]. The morbidity and mortality is very high because it can appear without warning and is usually due to erosion of a major vessel in the vicinity of the pseudocyst. If not recognized immediately, life of the patient may be jeopardized. Interventional radiology can play an invaluable role both in locating the source of bleeding and in embolisation of the bleeding vessel [41]. Without prior information of the bleeding point, surgical exploration can be hazardous and challenging.

(C) *Splenic infarction and thrombosis*: complications of pseudocyst include massive hemorrhage into the pseudocyst, sepsis with splenic infarction, and splenic vein thrombosis. The diagnosis of intrasplenic pseudocyst, based on clinical findings alone, is difficult to arrive at but should be suggested by the presence of a mass in the left upper quadrant. Sonography and computerized axial tomography may be particularly helpful in confirming splenic involvement. Selective celiac arteriography should be performed whenever splenic involvement is suggested in order to confirm the diagnosis and to search for pseudoaneurysm formation. Urgent surgical intervention is usually warranted in view of the high incidence of serious complications and the propensity toward rapid clinical deterioration. Resection of the pseudocyst by splenectomy and distal pancreatectomy is the treatment of choice [42].

(D) *Rupture*: rupture of a pseudocyst can have either a favorable or an unfavorable outcome, and this depends on whether it ruptures into the gastrointestinal tract, into the general peritoneal cavity, or into the vascular system. Rupture into the gastrointestinal tract either results in no symptoms or leads to melaena or hematemesis that usually requires urgent measures. Rupture into the general peritoneal cavity results in features of peritonitis and occasionally hemorrhagic shock. Emergent surgical exploration is usually required. While an internal drainage should always be aimed for, usually a thorough abdominal lavage and external drainage are all that can be achieved safely [43, 44].

(E) *Biliary complications*: biliary complications occur due to a large cyst in the pancreatic head region obstructing the common bile duct and resulting in obstructive jaundice. Therapeutic endoscopy with short-term biliary stenting is valuable in this situation. It can be retained until either the pseudocyst resolves or is treated by intervention [45, 46].

(F) *Portal hypertension*: portal hypertension can result from compression or obstruction of the splenic vein/portal vein either by the cyst alone or by the cyst in conjunction with underlying chronic pancreatitis. In this situation, surgery appears to be the only treatment modality available, and an appropriate surgical procedure can effectively treat this form of portal hypertension [47].

(G) *Gastric outlet obstruction*: pseudocysts around the head of the pancreas are likely to cause gastric outlet obstruction. Once the features of gastric outlet obstruction develop, it needs certainly intervention and decompression or drainage of the cyst.

## 8. Conclusion

Pancreatic pseudocysts are the most common cystic lesions of the pancreas, accounting for 75%–80% of such lesions. The most common symptoms are abdominal pain, nausea, and vomiting, although they can be asymptomatic.

Abdominal CT is an excellent choice for initial imaging. EUS plays an important role in differentiating pseudocyst from other cystic lesions of the pancreas and can greatly assist in transmural endoscopic drainage. Initial management consists of supportive care. Persistent symptoms and the development of complications warrant invasive intervention. The endoscopic and minimally invasive therapeutic procedures for the drainage of pancreatic pseudocysts are superior to open surgical techniques with respect to their success, morbidity, and mortality rates, but they cannot always be performed. In making treatment decisions, it is important to recall that 50% of pancreatic pseudocysts do not require any intervention and can be successfully managed by a wait-and-watch approach. Laparoscopic and endoscopic drainages have comparable success rates, while that of transcutaneous drainage are somewhat worse. Thus, the choice of technique depends very heavily on the experience of the treatment center. The surgical, percutaneous, and endoscopic pseudocyst drainage procedures have not been directly compared in high-quality prospective randomized studies and the preferred approach varies based on patient preferences and local expertise.

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*Research Article*

# Cytosolic Double-Stranded DNA as a Damage-Associated Molecular Pattern Induces the Inflammatory Response in Rat Pancreatic Stellate Cells: A Plausible Mechanism for Tissue Injury-Associated Pancreatitis

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Pancreatitis is an inflammatory disease of unknown causes. There are many triggers causing pancreatitis, such as alcohol, common bile duct stone, virus and congenital or acquired stenosis of main pancreatic duct, which often involve tissue injuries. Pancreatitis often occurs in sterile condition, where the dead/dying pancreatic parenchymal cells and the necrotic tissues derived from self-digested-pancreas were observed. However, the causal relationship between tissue injury and pancreatitis and how tissue injury could induce the inflammation of the pancreas were not elucidated fully until now. This study demonstrates that cytosolic double-stranded DNA increases the expression of several inflammatory genes (cytokines, chemokines, type I interferon, and major histocompatibility complex) in rat pancreatic stellate cells. Furthermore, these increase accompanied the multiple signal molecules genes, such as interferon regulatory factors, nuclear factor-kappa B, low-molecular-weight protein 2, and transporter associated with antigen processing 1. We suggest that this phenomenon is a plausible mechanism that might explain how cell damage of the pancreas or tissue injury triggers acute, chronic, and autoimmune pancreatitis; it is potentially relevant to host immune responses induced during alcohol consumption or other causes.

## 1. Introduction

In 1998, star-shaped cells in the pancreas called pancreatic stellate cells (PSCs) were identified and characterized [1, 2]. In a normal pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, they are transformed from their quiescent phenotype into myofibroblast-like cells, which actively proliferate, express $\alpha$-smooth muscle actin ($\alpha$-SMA), and produce extracellular matrix components such as type I collagen [3–5]. Although the transition from quiescent to activated PSCs is triggered by various types of molecules, recent evidence suggests that components of dead/dying host cells may also trigger this transition [6].

This study aimed to determine whether host double-stranded DNA (dsDNA) contributes to the functions of PSCs, particularly in inflammation. Although DNA was historically believed to be immunologically inert, it is now appreciated that DNA can be recognized by the immune system [7, 8]. For example, unmethylated CpG motifs, which are expressed at high frequency in bacterial DNA, cause

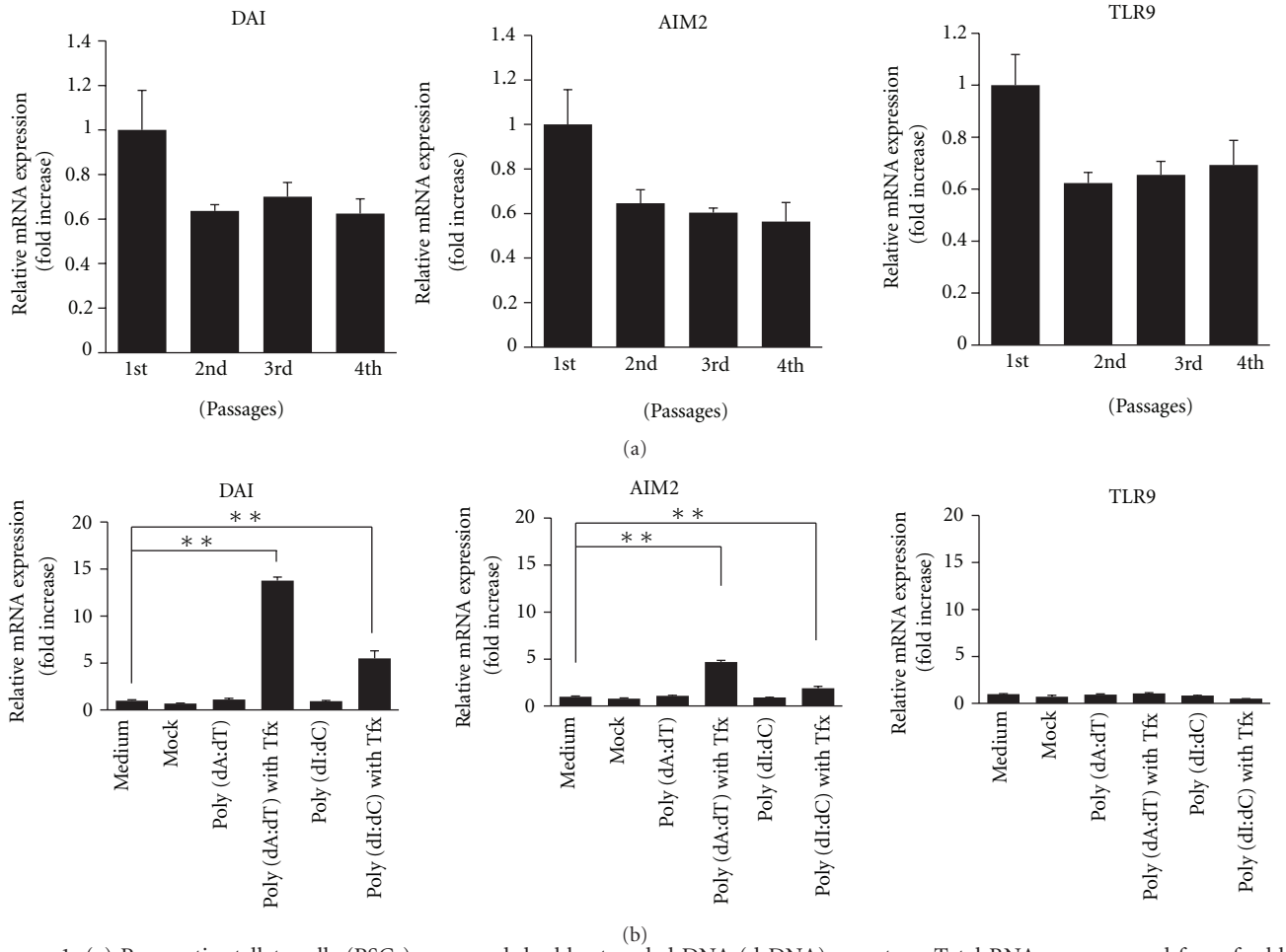



Figure 1: (a) Pancreatic stellate cells (PSCs) expressed double-stranded DNA (dsDNA) receptors. Total RNA was prepared from freshly isolated (3 days after isolation) culture-activated PSCs (passages 2 and 4). Expression of the dsDNA receptors was assessed by real-time PCR. All PSCs constitutively expressed DNA-dependent activator of IFN-regulatory factors (DAI), absent in melanoma 2 (AIM2), and toll-like receptor 9 (TLR9). (b) Extracellular DNA stimulation had no effect on DNA receptors, such as DAI and AIM2. In contrast, intracellular dsDNA increased the expression of all dsDNA receptors except TLR9. PSCs: pancreatic stellate cells, TFx: + transfection reagent lipofectamine. $^{*}P < 0.05$, $^{**}P < 0.01$.

lymphocytes, dendritic cells, and pancreatic stellate cells to proliferate and secrete immunoglobulin and/or cytokines [9–11], and dsDNA upregulates surface expression of major histocompatibility complex (MHC) molecules in thyroid cells [12]. Although DNA is normally sequestered in the nucleus, it can be released into the systemic circulation when cells undergo necrosis/apoptosis. Exposure to DNA has been implicated in the development of autoimmune and inflammatory diseases and has been observed in DNase-deficient mice [13]. These findings led us to hypothesize that dsDNA released by injured host cells may act as a "danger signal," which affects PSCs.

Here, we report that cytosolic dsDNA induces the expression of various inflammatory genes, which play a role in the tissue damage that mediates the inflammatory activity of host dsDNA.

## 2. Materials and Methods

*2.1. Materials.* Poly (dA : dT), Poly (dI : dC), mouse antirat alpha-smooth muscle actin antibody, and lipofectamine 2000 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antimouse IgG Alexa 555-conjugated antibody was obtained from Invitrogen (Carlsbad, CA, USA).

*2.2. Isolation of PSCs and Cell Culture.* PSCs were isolated from male Wistar rats by density-gradient centrifugation method. Cells were maintained in complete DMEM/F-12: this is a mixture of DMEM (Dulbecco's modified Eagle medium) and Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 50 units/mL of penicillin, and 50 mg/mL of streptomycin (all from Invitrogen, Carlsbad, CA, USA). All experiments were performed with cells between passages three and four. Unless specifically described, we incubated PSCs in serum-free medium for 24 h before the addition of experimental reagents. All animal procedures were performed in accordance with the guidelines of the Committee on Animal Care of the Kyushu University.

*2.3. Transfection.* Unless otherwise noted, 10 $\mu$g of DNA was mixed with 5 $\mu$L of Lipofectamine2000 and 985 $\mu$L of

serum-free medium and then incubated for 15 min at room temperature. A duplicate mixture without DNA and/or lipofectamine2000 also was incubated for 15 min at room temperature. Cells were washed with serum-free medium, and the combined mixtures were added for DNA transfection (Tfx).

*2.4. Expression of Cytosolic DNA Receptors and the Effects of dsDNA on the Functions of PSCs: Real-Time Reverse-Transcription Polymerase Chain Reaction.* Total RNA was extracted from PSCs using an RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 20–100 ng of total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using a PrimeScript RT reagent kit (Takara Bio, Inc, Otsu, Shiga, Japan) according to the manufacturer's instructions. RT-PCR was performed using a LightCycler Real-Time PCR system (Roche, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 $\mu$L) contained SYBR Premix Ex Taq II (TLi RNAseH Plus; Takara Bio, Inc, Otsu, Shiga, Japan), 4 mM $MgCl_2$, 0.5 mM of the upstream and downstream PCR primers (Table 1), and 2 $\mu$L of first-strand cDNA template. To control for variations in the reactions, all PCR data were normalized against GAPDH expression.

*2.5. Quantification of Soluble Monocyte Chemoattractant Protein-1 (MCP-1): MCP-1 ELISA.* After 24 h of incubation, the levels of MCP-1 in the culture supernatants were measured by ELISA (Rat MCP-1 ELISA from Thermo Scientific, Rockford, IL, USA) according to the manufacturers' instructions.

*2.6. Cell Viability Assay: MTS Assay.* Cell viability was assessed by the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Madison, WI, USA). After treatment with dsDNA for 24 h, MTS solution was added to the cells and the incubation continued at 37°C for 1 h. After the incubation period, cell viability was quantified by the differences in absorbance at wavelengths of 570 and 690 nm.

*2.7. Cell Cytotoxicity Assay: LDH Assay.* Cell cytotoxicity was assessed by the LDH assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Madison, WI, USA). After treatment with dsDNA for 24 h, the cell supernatants were transferred to another microplate, and then LDH substrate was added to the supernatants and the incubation continued at 37°C for 30 min. After the incubation period, stop solution was added and cell cytotoxicity was quantified by the differences in absorbance at wavelengths of 570 and 690 nm.

*2.8. Expression of Alpha-Smooth Muscle Actin and M30 Cytokeratin: Immunofluorescent Confocal Microscopy.* Cell activation and cell apoptosis was assessed by immunofluorescent cytochemistry. Mouse antirat alpha-smooth muscle actin antibody were used to evaluate cell activation, and FITC-labelled M30 antibody was used to evaluate apoptosis. After incubation, cells were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, and analyzed for fluorescence under a confocal laser scanning microscope (Nikon A1/C1, Tokyo, Japan).

Table 1: Sequences of primers used in this study.

| Gene | Sequence |
|---|---|
| Rat DAI: sense | 5′- TGTCCCGCAGTAAAAGATGG -3′ |
| Antisense | 5′- TTCCAGCCAATGACAACCTC -3′ |
| Rat AIM2: sense | 5′- CATCACGGAGGAGGAACTGA -3′ |
| Antisense | 5′- CGTCCTGTCTGCAATGTTCA -3′ |
| Rat TLR9: sense | 5′- CCGAAGACCTAGCCAACCT -3′ |
| Antisense | 5′- TGATCACAGCGACGGCAATT -3′ |
| Rat TNF-$\alpha$: sense | 5′- CTGGTGGTACCAGCAGATGG -3′ |
| Antisense | 5′- GGAGGCTGACTTTCTCCTGG -3′ |
| Rat IL-6: sense | 5′- CCACCAGGAACGAAAGTCAA -3′ |
| Antisense | 5′- CAGTCCCAAGAAGGCAACTG -3′ |
| Rat MCP-1: sense | 5′- ACGTGCTGTCTCAGCCAGAT -3′ |
| Antisense | 5′- GTTCTCCAGCCGACTCATTG -3′ |
| Rat CINC-1: sense | 5′- CCACACTCAAGAATGGTCGCG -3′ |
| Antisense | 5′- AGACGCCATCGGTGCAATC -3′ |
| Rat NF-$\kappa$B p65: sense | 5′- TTCTGGGCCATATGTGGAGA -3′ |
| Antisense | 5′- CCTCGCACTTGTAACGGAAA -3′ |
| Rat RelB: sense | 5′- GCCACGTAGCCTCTGAGTTG -3′ |
| Antisense | 5′- ATGGAGTGCTGGACCACAAG -3′ |
| Rat IFN-$\beta$: sense | 5′- TCCAGTTCCGACAAAGCACT -3′ |
| Antisense | 5′- CTTCCATTCAGCTGCCTCAG -3′ |
| Rat IFN-$\alpha$: sense | 5′- TCTTCACACTCCTGGCACAAATG -3′ |
| Antisense | 5′- CTCTCAGTCTTCCCATCAAGTTGG -3′ |
| Rat IRF1: sense | 5′- GAGGGGACATCGAGATAGGC -3′ |
| Antisense | 5′- CTGGTAGAGTTGCCCAGCAG -3′ |
| Rat IRF2: sense | 5′- CCCGACATTGAGGAAGTGAA -3′ |
| Antisense | 5′- TTCTTGGAAGGTCGCTCAGA -3′ |
| Rat IRF3: sense | 5′- CCAGACCTGTCAACCTGGAA -3′ |
| Antisense | 5′- GGTCAAAAGGGTCCTTGCTC -3′ |
| Rat IRF7: sense | 5′- GCGACAAGGATCACCACATT -3′ |
| Antisense | 5′- CTCCAGCTTCACCAGGATCA -3′ |
| Rat MHC I: sense | 5′- GACACAGATCGCCAAGGGA -3′ |
| Antisense | 5′- ATATCCGCGGAGGAGGCT -3′ |
| Rat MHC II: sense | 5′5′- GAGGCGACCGTGTTTTCC -3′ |
| Antisense | 5′- TCTGTGACTGGCTTGCTGTT -3′ |
| Rat TAP1: sense | 5′- CCACCACATCCTCTTCCTCA -3′ |
| Antisense | 5′- ACCCTCCTCTCTCCATGAGC -3′ |
| Rat LMP2: sense | 5′- GGTGTAATGGGCAGAGGTGA -3′ |
| Antisense | 5′- AAGAATGGGAGGTGCTTGCT -3′ |
| Rat $\alpha$SMA: sense | 5′- CCTCAGGGTGCTCGTGGAT -3′ |
| Antisense | 5′- CAGGACTGCCAGGCTCTCC -3′ |
| Rat type I collagen: sense | 5′- AGTTGGTGATGATGCCGTGTT -3′ |
| Antisense | 5′- ATGGGCCAAAAGGACAGCTAT -3′ |
| Rat GAPDH: sense | 5′- GCTCTCTGCTCCTCCCTGTT -3′ |
| Antisense | 5′- CACACCGACCTTCACCATCT -3′ |

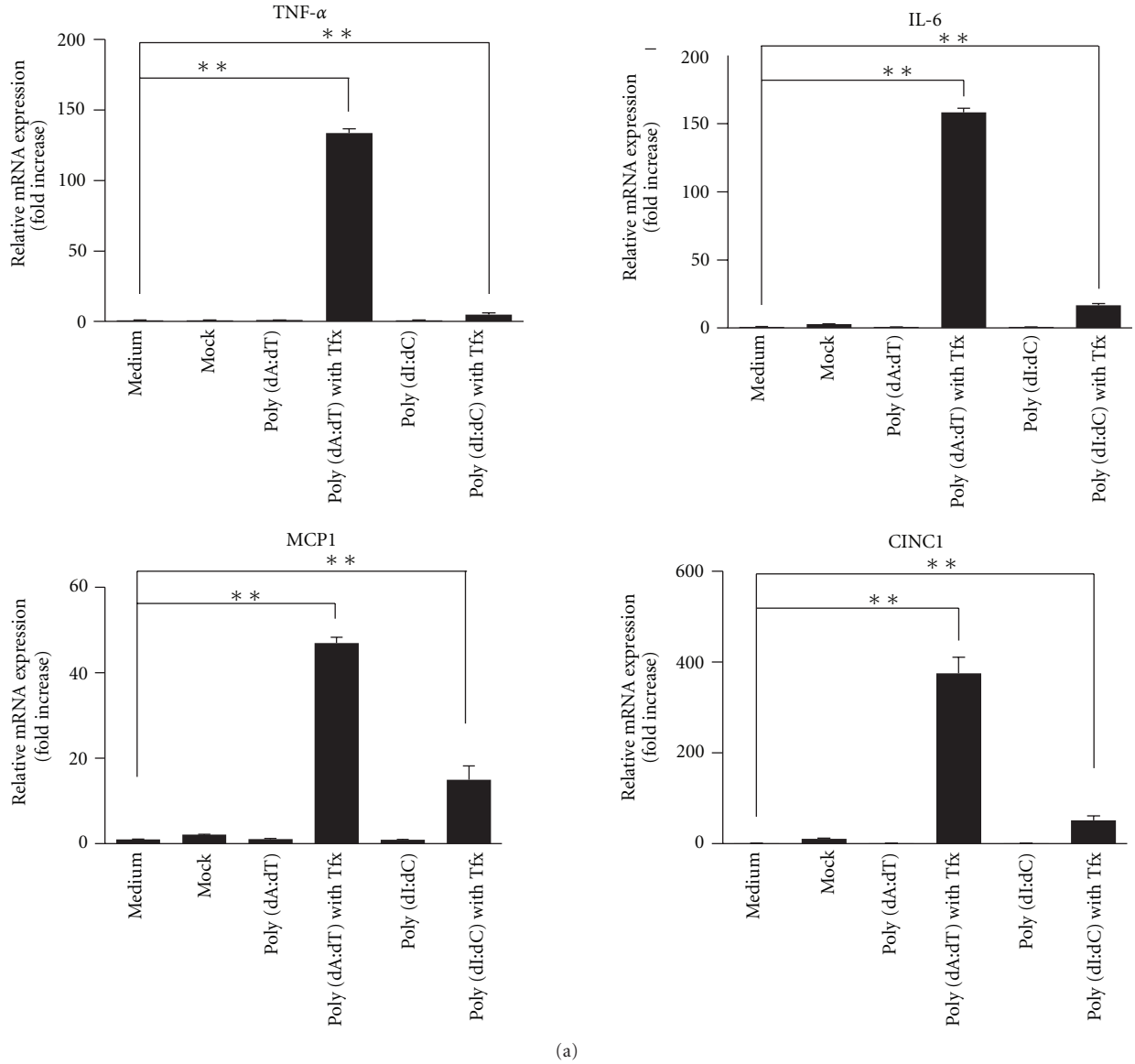
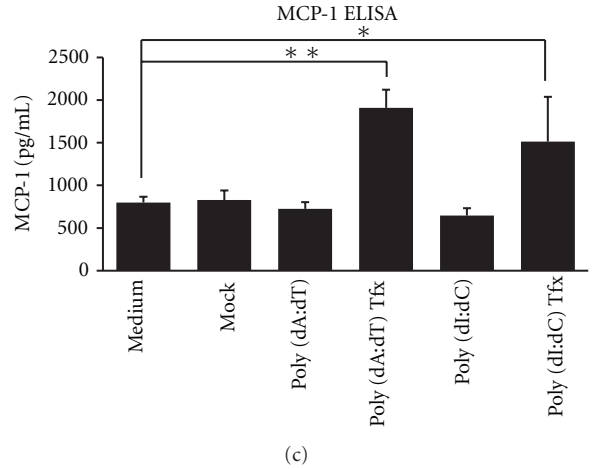



Figure 2: Continued.




(c)

Figure 2: Transcription of cytokine and chemokine mRNA was induced by synthetic double-stranded DNA (dsDNA). (a) Extracellular DNA stimulation had no effect on inflammatory cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-$\alpha$), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and cytokine-induced neutrophil chemoattractant 1 (CINC-1). In contrast, intracellular dsDNA (at 10 $\mu$g/mL) had stimulatory effects on their expression, including the expression of their transcriptional factors nuclear factor-kappa B (NF-$\kappa$B) and reticuloendotheliosis viral oncogene homolog B (RelB) (b). Release of MCP-1 was also confirmed by ELISA (c). PSCs: pancreatic stellate cells, TFx: + transfection reagent lipofectamine. $^{*}P < 0.05$, $^{**}P < 0.01$.

*2.9. Statistical Analysis.* Results are expressed as the means (SEM) of 3-4 separate cell preparations per experimental protocol. Student's *t*-test was used for the statistical analyses. *P* values of <0.05 were considered statistically significant.

## 3. Results

*3.1. Rat PSCs Expressed Cytosolic DNA Receptors.* There have been no previous reports on the expression of foreign DNA receptors in PSCs other than toll-like receptor 9 (TLR9) [2]. Therefore, we first measured the mRNA expression of DNA-dependent activator of IFN-regulatory factors (DAI) and absent in melanoma 2 (AIM2), which recognize cytosolic dsDNA using real-time PCR. PSCs expressed both the DAI and AIM2 receptors regardless of the passage and could recognize cytosolic DNA (Figure 1(a)). Next, synthesized dsDNA was introduced into the cytoplasm by lipofection to determine whether the number of receptors increased in response, that is, whether inflammation was initiated. The synthesized dsDNA used in this study had a structure similar to that of host dsDNA, and has been widely used to imitate host dsDNA that is derived from cell and tissue injury. Poly (dA : dT) has been reported to induce type I interferon (IFN) cytokines, and chemokines, and triggers the inflammatory response. However, it is also known that dsDNA is transformed into double-stranded RNA (dsRNA) by RNA polymerase III and is detected by the RIG-I receptor, which recognizes dsRNA. Therefore, this is not true DNA stimulation [14]. In contrast, poly (dI : dC) lacks the 3′-ppp structure that is sensed by RIG-I and is sensed only by receptors that recognize dsDNA. Although poly (dA : dT) and poly (dI : dC) are not influenced by extracellular DNA stimulation, introduction of intracellular dsDNA by lipofection has been shown to significantly increase the number of the receptor expression and induce inflammation (Figure 1(b)).

*3.2. dsDNA Increased Cytokine and Chemokine Expression.* Next, we determined whether the expression of inflammatory cytokine and chemokine genes was induced using RT-PCR. The results indicated that although extracellular DNA stimulation did not induce expression of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-$\alpha$) and interleukin-6 (IL-6) and chemokines such as MCP-1 and cytokine-induced neutrophil chemoattractant 1 (CINC-1), intracellular dsDNA did stimulate their expression at 6 h (Figure 2(a)). Gene expression is regulated by transcription factors such as nuclear factor-kappa B (NF-$\kappa$B). This study showed that the expression of such genes was increased by intracellular dsDNA stimulation (Figure 2(b)), which suggested that release of excess host dsDNA due to viral infection and tissue injury might trigger inflammation.

*3.3. dsDNA Induced MHC Expression.* We also determined the presence or absence of expression of gene-controlled antigen presentation, which activates T-cell-mediated cellular immunity. The results revealed that intracellular dsDNA stimulation increased MHC class I gene expression and was involved in not only the inflammation but also the activation of lymphocytes and others (Figure 3(a)). MHC class II expression was also examined because PSCs reportedly have phagocytic activity [15]; however, the expression was not increased. Transporter associated with antigen processing 1 (TAP1) and low-molecular-weight protein 2 (LMP2) play an important role in the induced expression of MHC class I [12]. Our study showed that TAP1 and LMP2 expression also increased (Figure 3(b)), which suggested that the presence of excess host dsDNA due to tissue injury might induce the

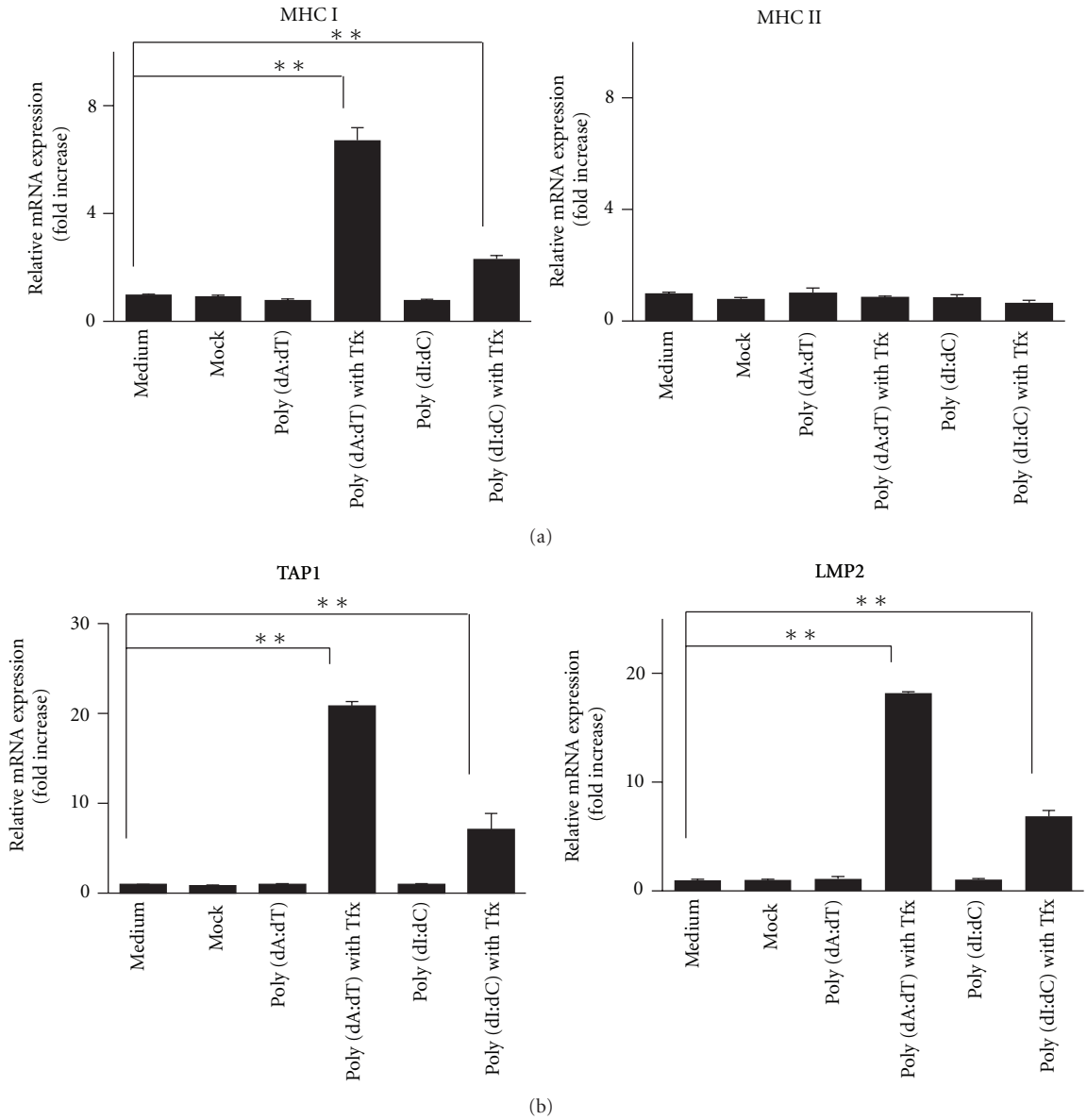



Figure 3: Major histocompatibility complex (MHC) mRNA transcription is induced by synthetic double-stranded DNA (dsDNA). (a) Extracellular DNA (at 10 $\mu$g/mL) stimulation has no effect on MHC class I and class II. In contrast, intracellular dsDNA (at 10 $\mu$g/mL) increased expression of their transcriptional factors transporter associated with antigen processing 1 (TAP1) and low-molecular-weight protein 2 (LMP2) (b). TFx: + transfection reagent lipofectamine. $^{*}P < 0.05$, $^{**}P < 0.01$.

abnormal MHC expression observed in patients with both chronic and autoimmune pancreatitis.

*3.4. dsDNA Induced Type I IFN Induction.* Like MHC, type I IFN is involved in the activation of cell-mediated immunity; either IFN-$\alpha$ or IFN-$\beta$ is predominantly induced depending on the cell type. In case of PSCs, IFN-$\beta$ induction, which has also been reported in fibroblasts, has been observed (Figure 4(a)). Various interferon regulatory factors (IRFs) are involved in the expression of the above-mentioned genes [12]. In this study, the expression of IRF 1, 2, and 7 increased, while IRF3 expression was not induced (Figure 4(b)).

*3.5. dsDNA Impaired Cell-Specific Functions.* It has been reported that engulfment of necrotic acinar cells attenuated the activation and collagen synthesis of PSC [6]. We examined whether this phenomenon was reproducible when PSCs were stimulated with synthetic dsDNA. Intracellular dsDNA attenuated activation, type I collagen gene induction and proliferation (Figures 5(a) and 5(c)). Furthermore, extracellular poly (dI : dC) attenuated type I collagen gene induction, indicating the possible function of extracellular dsDNA. We confirmed the decrease of $\alpha$-SMA at protein level by immunofluorescent cytochemistry (Figure 5(b)). LDH assay and M30 staining revealed the concomitance of cell death, including necrosis and apoptosis (Figures 5(d) and 5(e)).

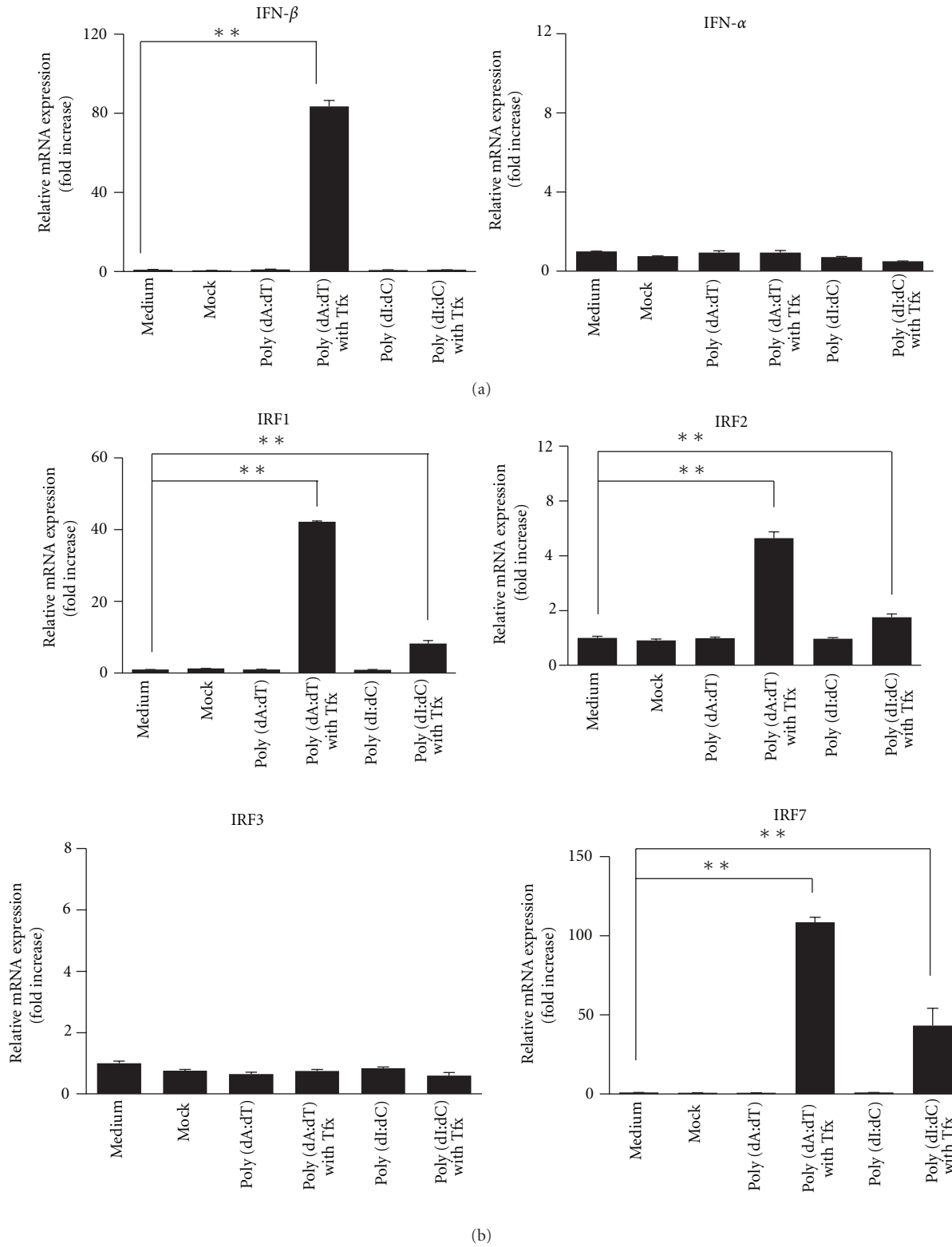
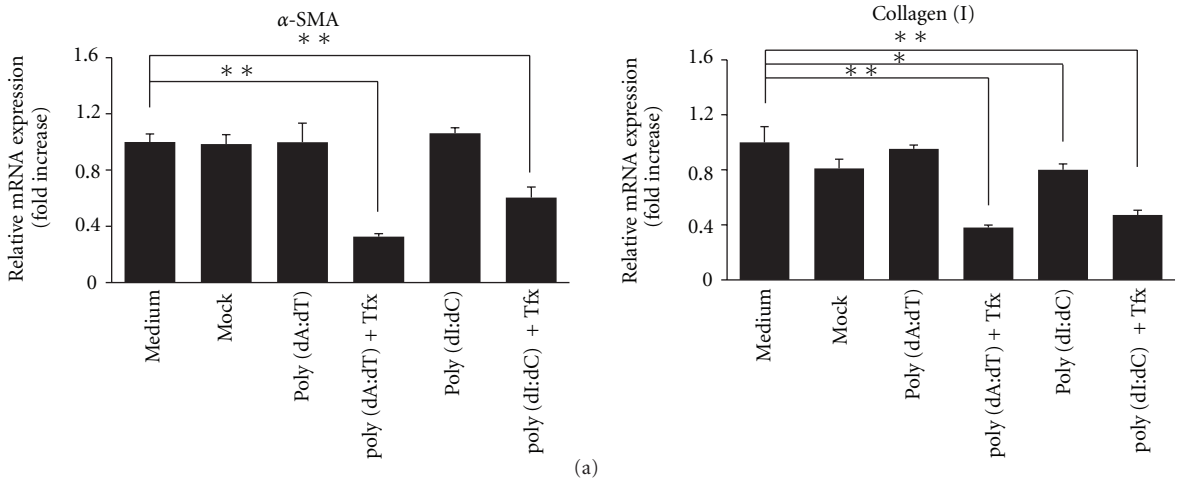
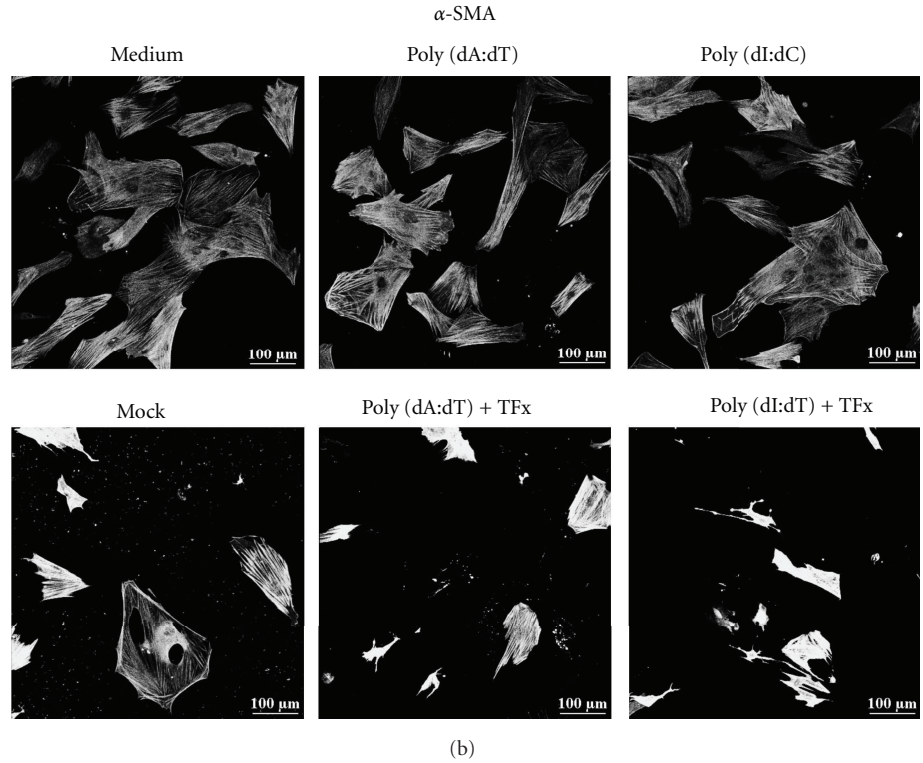


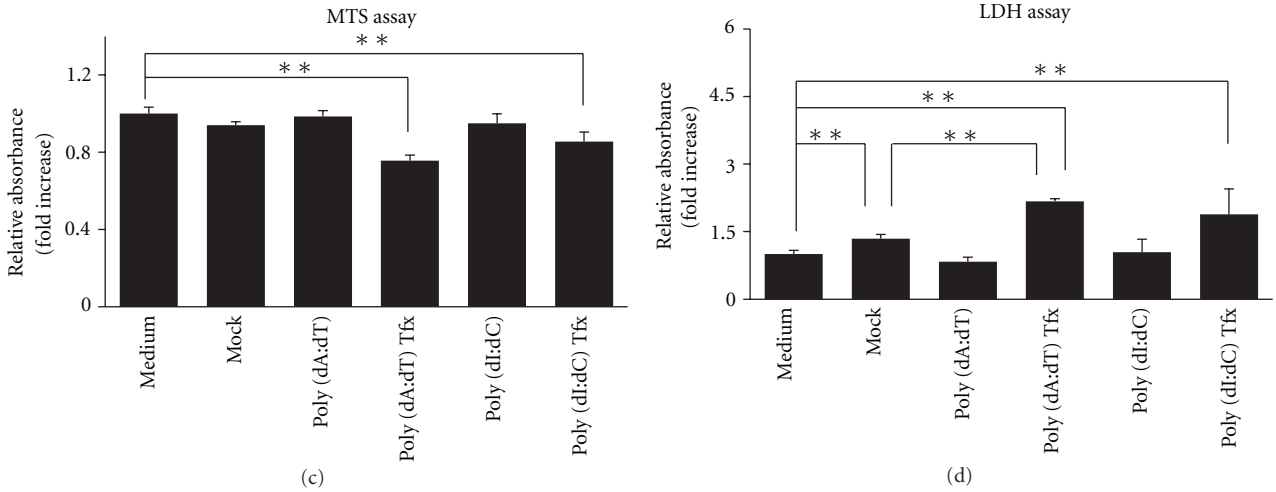

Figure 4: Transcription of type I interferon mRNA is induced by synthetic double-stranded DNA (dsDNA). (a) Extracellular DNA stimulation had no effect on the induction of type I interferons (IFNs), such as IFN-$\alpha$ and IFN-$\beta$. In contrast, intracellular dsDNA (at 10 $\mu$g/mL) has stimulatory effects on their expression, including the expression of their transcriptional factors interferon regulatory factor 1 (IRF1), IRF2, and IRF7 (b). TFx: + transfection reagent lipofectamine. $^{*}P < 0.05$, $^{**}P < 0.01$.




(a)




(b)




(c) (d)

Figure 5: Continued.

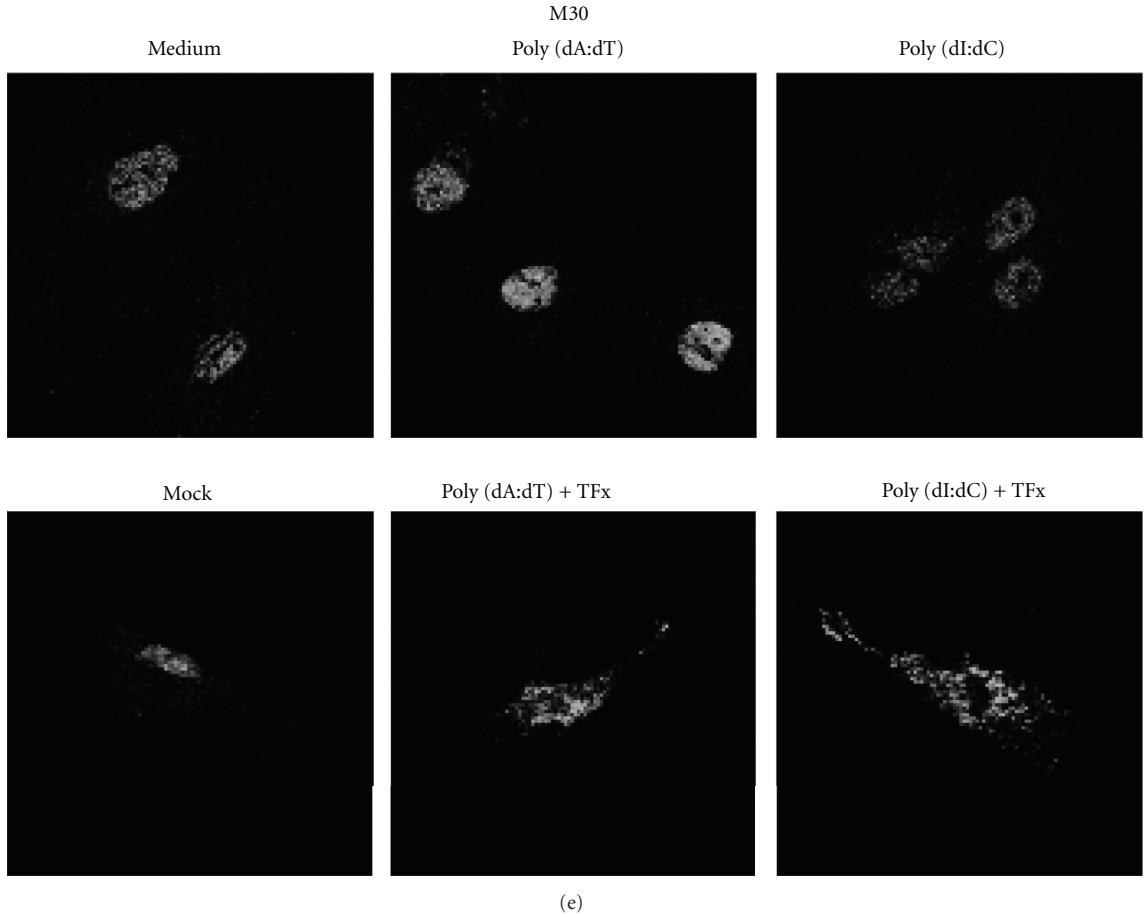



Figure 5: Double-stranded DNA (dsDNA) impaired cell-specific function. Cell-specific functions were impaired by intracellular dsDNA (at 1–10 $\mu$g/mL) and extracellular poly (dI : dC) (at 10 $\mu$g/mL) (a), (b). Intracellular dsDNA (at 10 $\mu$g/mL) attenuated cell proliferation of PSCs (c)–(e). TFx: + transfection reagent lipofectamine. $^{*}P < 0.05$, $^{**}P < 0.01$.

## 4. Discussion

At the time of their discovery, PSCs were identified as fibroblasts that maintained the homeostasis of the extracellular matrix [1, 2]. However, PSCs have recently been recognized as a multifunctional cell type [5, 16–18] that differ slightly from the well-differentiated cells of the pancreas, such as acinar cells, duct cells, and endocrine cells. The innate immune response of PSCs is particularly important in the induction of pancreatic inflammation. Furthermore, phagocytosis of various extracellular bacteria and dead cells by PSCs leads to activation of cellular immunity through antigen presentation, and there have been some reports on phagocytosis and endocytosis by PSCs [15, 17]. Stimulation of the innate immunity is generally divided into infectious and noninfectious stimulation [19], and the pathways that sense the stimulation include the phagolysosomal pathway, the endosomal-lysosomal pathway, and other autophagy pathways that function through phagocytosis. Some types of stimulation are recognized by diverse nucleic acid receptors that induce inflammatory responses through activation of common downstream transcriptional factors such as NF-$\kappa$B and IRF [20, 21]. In contrast, there are pathological conditions that induce inflammatory responses in an uninfected environment, which is similar to the infectious environment. The mechanism of inflammation was previously unknown. However, it is currently widely understood that inflammatory diseases develop because the innate immunity is activated by endogenous molecules released as a result of tissue injury, which are referred to as damage-associated molecular patterns (DAMPs) [22, 23].

The release of DAMPs is induced by various tissue injuries, for example, ischemia and reperfusion injury [24], trauma [25], and other harmful stimulations (alcoholic pancreatitis, drug-induced pancreatitis, and others) that induce critical apoptosis and necrosis. As a result, tissue injury induces the release of intracellular molecules (nucleic acids, HSP, UA, HMGB1, and others) and the degradation of extracellular matrix (hyaluronic acid), which results in induction of inflammation and repair of the injured sites [26]. However, uncontrolled tissue injury, for example, autodigestion caused by massive necrosis of pancreatic acinar cells, produces many necrotic cells. DAMPs, such as genomic DNA fragments, activate innate immunity and acquired immunity and induce autoimmune inflammation while inhibiting cell-specific functions [12, 27, 28]. Dead cells are usually removed by resident phagocytes; however, the pancreas does not have interstitial macrophages ($M\phi$) similar to hepatic Kupffer

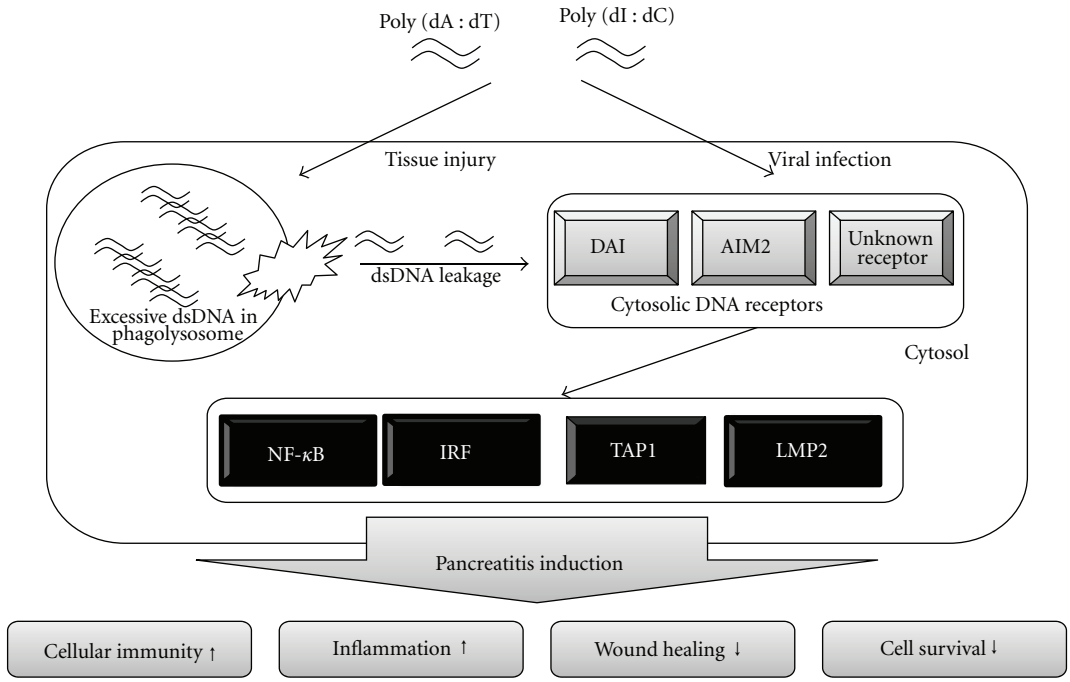



Figure 6: Model for mechanisms triggered by intracellular double-stranded DNA (dsDNA) in PSCs. The scheme depicts the induction of the innate immune response by dsDNA reflecting the onset and exacerbation of pancreatitis under sterile an nonsterile conditions.

cells, and so the PSCs phagocytose the dead cells. Therefore, PSCs are considered to be the primary cells that induce inflammation. The intrahepatic $M\phi$ of DNase II knockout mice lack the ability to degrade the nucleic acids of apoptotic phagocytosis and produce many type I IFNs, which leads to chronic inflammation [13]. DNase I knockout mice develop antinuclear antibody-positive SLE-like symptoms through this same mechanism [29], and the intracellular DNA receptors in DNase III knock-out mice are activated by the accumulation of extranuclear DNA, which results in the onset of lethal inflammatory myocarditis associated with massive IFN induction [30]. The above-mentioned findings demonstrate that excessive nucleic acid accumulation from dead cells induces breakdown of the intracellular DNA processing system. This leads to the production of cytosolic dsDNA, which normally does not exist and not only triggers inflammation but also leads to the development of autoimmune disease. Furthermore, these findings suggest that the processing mechanism of DNase as well as its stimulation by dsDNA should be studied.

Most previous studies on the innate immune response in PSCs have focused only on infectious stimulation, and there have been no reports on DAMPs. Although it is known that an increase in the number of receptors due to stimulation by PAMPs or DAMPs primes inflammation, the change in the number of receptors expressed, such as the TLRs of PSCs, has not been studied. There are at least 2 receptors that recognize intracellular dsDNA and trigger inflammation. In particular, the expression of DAI and AIM2, which are most responsible for the inflammation, increased (Figure 1(b)), which suggests that a minor tissue injury could spread to entire organ [31, 32]. However, the number of TLR9 receptors did not increase. Instead, receptors that are specifically sensitive to certain stimulation increased, which could differentiate between self and nonself. In addition to the 2 above-mentioned receptors, there are other nucleic acid receptors, and extracellular H2B is thought to play an important role in the onset of autoimmune thyroid disease [27, 33, 34]. Which receptor or receptors are responsible for the onset of pancreatitis should be clarified in the future. The induction of cytokines and chemokines has a significant effect on the onset and clinical cure of pancreatitis. There have been reports that stimulation by a component of gram-negative bacilli, such as LPS or flagellin, a component of gram-positive cocci, such as LTA, or stimulation similar to that in viral infection, such as Poly (I : C), has effects similar to stimulation by cytosolic dsDNA. Therefore, common transcriptional factors, such as NF-$\kappa$B, are thought to induce expression of inflammatory cytokine and chemokine genes [17, 35, 36]. We previously reported that dsDNA from bacteria such as *Escherichia coli* has no inflammatory effect [11]. This may be because the intracellular DNA receptors are not stimulated due to various factors, including the length and amount of bacterial DNA and the cellular uptake pathway for the bacterial genomic DNA. In this study, we demonstrated for the first time that expression of cellular immunity activation factors associated with antigen presentation, such as IFN-$\beta$, and MHC class I, were induced by cytosolic dsDNA stimulation. We consider that these data will be useful for the evaluation of aberrant MHC expression and lymphocyte activation in the pancreatic tissue of patients with chronic and autoimmune pancreatitis [37–40]. Furthermore, these data suggest the possibility that host dsDNA from tissue injury may be involved in the onset of

the above-mentioned diseases via innate immunity activation. Aberrant MHC expression is not observed in pancreatic acinar cells but does exist in inter- and intralobular ductules [38]. PSCs are likely to be partially responsible for this expression. However, since it is likely that MHC class II may also be involved in the onset of autoimmune pancreatitis [40], stimulation other than host dsDNA may be related to the onset.

Since intracellular and extracellular dsDNA impaired the cell-specific function of PSC, functional loss of tissue repair was anticipated in environment where abundant necrotic dsDNA fragments were released from injured tissue and cells. Cell survival also decreased with intracellular dsDNA, which might recruit bone-marrow-derived PSC increasing the total number of PSC in the pancreas [41, 42]. It has been reported that engulfment of necrotic acinar cells attenuated the cell-specific function of PSC [6], which might reflect that the excessive amount of dsDNA in phagolysosome induced the leakage of dsDNA fragment and was recognized by cytosolic dsDNA sensor. We could not define the type of cell death because of the minimal effect of lipofectamine toward cell necrosis, but we thought that dsDNA induced both cell necrosis and apoptosis, which were often observed in pancreatitis.

In this study, we found that induction of the innate immune response by dsDNA reflects the onset and exacerbation of pancreatitis under sterile conditions (Figure 6). The results of this study will be very useful in elucidating the pathology of new pancreatitis and deciding on treatment targets for these diseases, including autoimmune pancreatitis. In the case of acute pancreatitis, DAMPs other than host dsDNA such as HSP [43] and uric acid [44] may be involved in the pathology; therefore, future studies should be performed from the viewpoint of the innate immunity.

## Conflict of Interests

The authors have no potential conflict of interests.

## Acknowledgments

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*Review Article*

# Pancreatic Perfusion CT in Early Stage of Severe Acute Pancreatitis

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Early intensive care for severe acute pancreatitis is essential for improving SAP mortality rates. However, intensive therapies for SAP are often delayed because there is no ideal way to accurately evaluate severity in the early stages. Currently, perfusion CT has been shown useful to predict prognosis of SAP in the early stage. In this presented paper, we would like to review the clinical usefulness and limitations of perfusion CT for evaluation of local and systemic complications in early stage of SAP.

## 1. Introduction

Severe acute pancreatitis (SAP) is a fatal disease [1]. The Atlanta Symposium criteria for the severity of acute pancreatitis define SAP as acute pancreatitis with local complications (pancreatic necrosis, abscess, and pseudocysts) and/or with systemic complications (organ failure, disseminated intravascular coagulation, and severe metabolic disturbances) [2] (Figure 1). Both acute necrotizing pancreatitis (ANP) and multiple-organ failure (MOF) have been shown to be significant prognostic factors [3–6]. Mortality rates for SAP patients developing ANP and MOF are 32% and 30%, respectively [7]. Early intensive care for SAP is essential for improving SAP mortality rates [8–10]. However, intensive therapies for SAP are often delayed because there is no ideal way to accurately evaluate severity in the early stages [11–13].

Perfusion CT has been used for evaluation of various pancreatic diseases [14–22]. Perfusion CT has been shown useful to predict prognosis of SAP in the early stage [17, 18]. In this presented paper, we would like to review the clinical usefulness and limitations of perfusion CT for evaluation of local and systemic complications in early stage of SAP.

## 2. Perfusion CT Technique

Previously published perfusion CT protocols are summarized in Table 1. Multidetector CT (MDCT) is essential for performing perfusion CT of pancreas. With a 4–64 slice MDCT scanner, perfusion CT scans are obtained with the patient in a stationary position. The craniocaudal CT scan coverage is limited to 20 to 32 mm (4 slices of 5 to 8 mm thickness). Therefore, scan location must be carefully chosen to cover as much of the pancreas as possible as it is often difficult to cover the entire pancreas. Since most pancreatic necrosis occurs in the neck region, it is probably uncommon to exclude the area of necrosis due to the scanning coverage limitation. With the use of a recently developed 256–320-slice MDCT scanners [19], craniocaudal coverage has increased to 80–160 mm. Alternative way to increase the craniocaudal coverage is by using the so-called shuttle or toggle mode. In this mode, similar to conventional CT scans, patient table moves back and forth as the multiple scans are performed.

First, noncontrast transaxial images of the upper abdomen are obtained using low-dose technique. This scan is performed to localize the pancreas, and it determines the scan range of the perfusion CT.

Perfusion CT is performed after a bolus injection of intravenous contrast material. Unlike conventional CT, the perfusion CT requires smaller dose (40–50 mL) of contrast material injected at a high rate (4–10 mL/sec). Higher concentration of contrast material (350–370 mgI/kg) is preferred [23–25].

Perfusion CT images are obtained multiple times through the pancreas. In most of previous reports, scan interval ranges from 0.5 to 1.5 second, and the scan duration ranges from 30 to 150 seconds, respectively (Table 1). Total scan duration necessary for calculation of perfusion parameters may depend on the algorithm used. For example, maximum slope method needs shorter duration scan time than the deconvolution method [14, 19, 26]. Because the scan duration is long, the scans are usually performed under free breathing.

Perfusion CT scan is obtained at a low tube current (mAs) to reduce radiation dose. At 120 kV, mAs of 100 is commonly used. There is increased interest in the use of low tube voltage setting, as it reduces radiation dose and improves iodine contrast material conspicuity. In a smaller patient, the use of 100 kV or 80 kV is recommended. In a larger patient, the use of low-kV scan may result in noisy images due to photon deficiency.

*2.1. Radiation Dose and Scan Parameters.* Radiation dose is dependent on the tube current (mAs), tube voltage (kV), number of scans, and scan coverage [29]. Tube current (mAs) and tube voltage (kV) are largely dictated by the patient size to maintain adequate image quality. Radiation dose should be kept as low as reasonably achievable (ALARA) by reducing the scanning parameter settings but achieving image dataset adequate for calculating CT perfusion parameters [27, 30]. Further study is necessary to optimize the scanning protocol.

From a European study, the effective dose of pancreatic perfusion CT was 3.54 mSv with 90 KV, 100 mAs, and 40 scans [17]. A study from Japan reported that mean radiation dose of pancreatic perfusion CT was approximately 204.8 mGy·cm (dose-length product (DLPw)), 3.07 mSv (effective dose), and 64 mGy (CT dose index volume (CTDIvol)) with 80 kV, 60 mAs, and 106 scans [27]. In the national survey, the radiation exposure of a single-phase abdominal CT was 13–25 mGy (CTDIvol) [31]. Therefore, the radiation dose of perfusion CT is slightly higher than that of biphasic (two phase), which is commonly used for pancreatic or liver imaging. Average abdominal transverse diameter of the Japanese patients in our experience was 32 cm, while transverse diameters of patients in the Unites States are usually larger [32]. Therefore, the radiation dose will likely be higher in the western countries

*2.2. Perfusion CT Data Analysis.* Pancreatic perfusion CT image data are analyzed by using perfusion CT analysis software. There are various perfusion algorithms to calculate perfusion parameters. Maximum slope method, deconvolution method, single-compartment method, and the Patlak method are commonly used perfusion algorithms. Which perfusion best suits in the evaluation of SAP is yet to be determined. As different perfusion algorithms are suited for different disease processes and require different scanning protocol, determination of scanning protocol and perfusion algorithms should be considered together. For example, maximum slope method may require shorter scanning duration, but higher rate of contrast injection is required, while deconvolution method may require longer scanning duration but slower rate of contrast injection rate [14, 26].

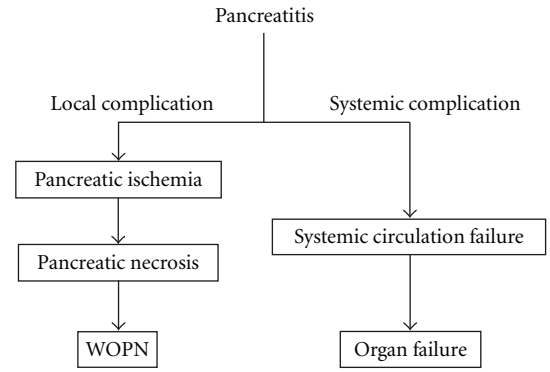


Figure 1: Schema of complications of severe acute pancreatitis. WOPN: walled-off pancreatic necrosis.

The software requires placement of small regions of interest (ROI) on an artery to generate arterial input function. Venous outflow function is required in deconvolution method. This process is required because the computer algorithm compares the shape and height of the time-density curve of each pixel of the pancreatic CT time series with shape and height of the arterial and/or venous time-density curves to calculate pancreatic perfusion parameters. Calculated pancreatic perfusion parameters are displayed using color maps [14].

## 3. Perfusion CT for Predicting Development of Pancreatic Necrosis in the Early Stage of Severe Acute Pancreatitis

Development of pancreatic necrosis is the critical event of acute pancreatitis that determines the prognosis of the patients. The overall mortality rate of acute pancreatitis is reported to be between 2.1% and 9.2% worldwide [1]. Pancreatic necrosis occurs in 10–15% of patients with SAP, with a mortality rate of 23% [1]. This rate is nearly twice that for patients with SAP who do not develop pancreatic necrosis (i.e., 11%) [1].

There is a report that dynamic contrast-enhanced CT is more accurate than either the Ranson criteria for pancreatitis mortality or the APACHE II scoring system in predicting the development of pancreatic necrosis [33]. However, the accuracy of contrast-enhanced CT in predicting necrosis at an early stage of SAP is not satisfactory [34]. The United Kingdom guidelines for the management of acute pancreatitis, the most popular clinical guideline of acute pancreatitis, recommends that contrast-enhanced CT should be performed at day 3 or later after onset of SAP because of its low sensitivity of CT [11].

In our experience, perfusion CT performed within 3 days of onset of symptoms had a sensitivity and specificity of 100% and 95.3% for predicting development of pancreatic necrosis [18]. The area of necrosis was depicted as area

Table 1: Scanning protocols of pancreatic perfusion CT.

| Authors | Disease | CT | The number of detector | kv | mA | Images | Contrast matter | | Duration time (sec) | Algorithm |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Injection rate (mL/sec) | Amount | | |
| Miles [25] | — | — | — | — | 50–100 | 60 | 4–7 | 40 mL | 60 | Deconvolution |
| | | | | | 100–250 | 15 | 7–10 | 50 mL | 45 | Compartment |
| | | | | | 100–250 | 6 | 4 | 100 mL | 120 | Patlak plot |
| Tsushima and kusano [15] | Normal | S | 1 | — | — | 19 | 5 | 40 mL | 85 | Maximum slope |
| Abe et al. [16] | PC | G | 1 | 120 | 60 | — | 5 | 0.5 mL/kg | 40 | Deconvolution |
| Bize et al. [17] | AP | P | 16 | 90 | 100 | 40 | 5 | 40 mL | 40 | Maximum slope |
| Tsuji et al. [18] | AP | T | 16/64 | 120 | 30–50 | 30–48 | 4 | 40 mL | 33–48 | Deconvolution |
| Tsuji et al. [27] | AP/NET | T | 64 | 80 | 40 | 106 | 4 | 40 mL | 54 | Deconvolution |
| Sheiman and Stick [28] | Normal | G | 64 | 100 | 80 | 30 | 4 | 40 mL | 90 | Compartment |
| d'Assignies et al. [20] | NET | G | 64 | 100 | 100 | 70 | 4 | 40 mL | 150 | Compartment |
| Park et al. [21] | PC | S | 64 | 100 | 100 | 30 | 5 | 50 mL | 30 | Patlak plot |
| Kandel et al. [19] | PC | T | 320 | 100 | 45 | 19 | 8 | 60 mL | 80 | Maximum slope |

PC: pancreatic cancer; AP: acute pancreatitis; NET: neuroendocrine tumor; S: Siemens; G: GE Health care; P: Philipse; T: Toshiba.

of pancreatic blood flow decreased by more than 19.3% of surrounding pancreatic parenchyma. The area of perfusion defect was commonly diagnosed by using pancreatic blood flow. The perfusion defects detected by perfusion CT reflected ischemia which was produced by vasospasms of the intrapancreatic arteries [35, 36].

## 4. Perfusion CT for Evaluating Systemic Blood Flow

Perfusion CT could be a useful tool to evaluate abnormal systemic circulation in early stage of SAP. Recent study by Whitcomb et al. showed that elevated serum angiopoietin-2 (Ang-2) on admission is predictive of persistent organ failure in patients with sap [37]. Ang-2 is produced by damaged vessels and increases vascular permeability [38]. In our study, elevated serum Ang-2 is related with hyperdynamic state of systemic circulation [22]. In this study, perfusion CT parameter ($\tau$) was calculated using single-compartment model [28, 39]. $\tau$ is a measure of the mean transit time of contrast material from upper abdominal aorta to pancreas; thus, this could be considered a surrogate of systemic circulation with a lower value indicating hyperdynamic state of systemic circulation [28]. In the result, significant correlation was found between $\tau$ and serum Ang-2 levels ($P < 0.05$); higher serum Ang-2 levels were associated with lower $\tau$ values (hyperdynamic state of systemic circulation).

Hepatic circulation abnormality has been reported in patients with SAP using Perfusion CT [40]. They reported that hepatic arterial perfusion is increased in the early stage of SAP as measured on dual-input maximum slope method.

## 5. Clinical Utility of Pancreatic Perfusion CT

Early diagnosis of pancreatic necrosis is very important in the treatment of patients with SAP. Current methods to predict early pancreatic necrosis or SAP is not satisfactory [11–13]. Perfusion CT is a promising technique that allows accurate diagnosis of pancreatic necrosis. Early diagnosis allows prompt clinical decision such as transferring patients to ICU or institution of aggressive treatment such as anticoagulation therapy [41], continuous regional arterial infusion of antiprothrombin agent [8, 9], early fluid resuscitation [10], and molecular targeted therapy [42, 43].

## 6. Conclusion

Perfusion CT is a promising technique for diagnosis of local and systemic complications of SAP at an early stage.

## Conflict of Interests

The authors declare no conflict of interests.

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*Review Article*

# Effect of Ageing on Systemic Inflammatory Response in Acute Pancreatitis

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Elderly patients show increased incidence of multiple organ dysfunction in acute pancreatitis possibly due to bacterial translocation. This is associated with increased susceptibility to infections in older people. Several reports have related this increased susceptibility to a proinflammatory status called *inflammaging*, which decreases the capacity of the immunological system to respond to antigens. Cellular senescence also contributes to this low-grade chronic inflammation in older subjects. We discuss here the effect of ageing on systemic inflammation, focusing on that induced by acute pancreatitis and some of the mechanisms involved. It is important to understand the immunological changes in the elderly to adjust treatment strategies in order to reduce the morbidity and mortality associated with acute pancreatitis and other conditions that lead to systemic inflammation.

## 1. Introduction

Several reports investigating ageing physiopathology have stated that old age is followed by a low-grade inflammatory process [1], which may be upregulated during sepsis, surgical procedures, and ischaemic/reperfusion injury. This low-grade inflammatory state is called *inflammaging,* a concept supported by several reports [2–4]. This is probably related to antigenic stress throughout life that may have caused exhaustion of immunological cells, thus decreasing the capacity of the immunological system to respond to antigens [5]. A concept that has been established is one of senescent cells being able to secrete many factors including growth factors, proteases, and cytokines that can induce inflammation [6]. Cellular senescence is a situation where the cell has an irreversible proliferative arrest due to potential oncogenic stress. This cellular state, limited to proliferative cells, has been recognized as a tumour suppressive mechanism. Senescent cells remain metabolically active, producing an array of tumour-suppressing and proinflammatory substances. Senescent cells probably contribute to the chronic inflammatory state related to ageing. This subject was extensively discussed in two important reviews, which also analyzed the molecular mechanisms and cellular pathways associated with cellular senescence [6, 7].

There is also clear evidence that elderly patients are more susceptible to infections after surgical procedures than young patients, and this could be related to the proinflammatory status of the elderly patients. This increased susceptibility to infections could be the reason for the increased postoperative morbidity and mortality observed in elderly patients [8]. However, there are reports showing that selection and appropriate care make the outcome of even major surgical procedures in elderly patients similar to that in young patients [9–15]. Thus, it seems that multiple factors influence the postoperative outcome of ageing people, including comorbidities and immunological status. It is therefore important to understand the immunological characteristic changes in the elderly to adjust treatment strategies in order to reduce the morbidity and mortality associated

with surgery, infection, and acute pancreatitis (AP) among other conditions that induce systemic inflammation.

## 2. Molecular Mechanisms of Age-Related Inflammation

The molecular mechanisms involved in the chronic low-grade inflammation of elderly people are poorly understood. Up- or downregulation of genes related to the development of inflammation may be involved in the process. A recent report pointed to the potential role of the poly(ADP-ribose) polymerase-1 gene in inflammation and the ageing process [16]. It has also been found that aged animals with sepsis present higher splenic tissue concentration of alpha-2A adrenergic receptors and phosphodiesterases (related to the autonomic nervous system) and increased CD14 and toll-like receptor-4 expression (related to innate immunity) when compared to young animals. The authors of this report concluded that hyperinflammation in older animals is related not only to the innate immune response but also to the upregulation of the adrenergic autonomic nervous system that may contribute to increased proinflammatory cytokine production [17].

The heat shock protein 70 has been shown to modulate the systemic response of an aged host to sepsis, preventing gut cell apoptosis and systemic inflammation [18]. In a study of partial liver ischaemia/reperfusion injury, the recruited neutrophils from aged rats were observed to have a higher-activation state when compared to those from young animals and that the hepatic heat shock protein 70 was less expressed in old animals [19]. It is possible to speculate that the diminished expression of heat shock protein 70 in the elderly population may be responsible for the higher-bacterial translocation and organ dysfunction observed in elderly patients with AP [20].

## 3. Systemic Inflammation and Ageing

Systemic inflammation such as that induced by sepsis is accompanied by a burst in the production of proinflammatory cytokines, whose serum levels are higher in elderly patients compared to the young [21].

Elderly patients usually develop an exaggerated inflammatory response after surgery and this has been attributed to the proinflammatory status of older people [22]. However, when pro-inflammatory cytokine levels are analyzed, contradictory reports are found in the literature: (a) increased and delayed levels of interleukin (IL)-6 have been observed in elderly patients after surgery [23]; (b) monocyte activation and hypercytokinemia have been observed in elderly patients after surgical intervention [24]; (c) significantly higher levels of tumour necrosis factor (TNF)-alpha, IL-6, and IL-1-beta are produced by mitogen-stimulated peripheral mononuclear cells from the elderly when compared to young subjects [19]. Contrarily, blood monocytes from elderly patients with pneumonia produce less proinflammatory cytokines (IL-1beta, TNF-alpha, and IL-8) upon stimulation than those from young patients [25]. The controversy is further increased by reports showing that young and old patients with community-acquired pneumonia [26] have similar serum levels of IL-6 and IL-10, and that there is no difference in serum pro-inflammatory cytokine levels between elderly and young patients after coronary surgery.

Ischaemia/reperfusion is another condition that induces systemic inflammation. Liver ischaemic/reperfusion injury is increased in older animals due to an exaggerated production of TNF-alpha. Inhibition of TNF-alpha production reverses the effect of ageing in ischaemic/reperfusion injury [27]. Ischaemic preconditioning protects young livers from ischaemic/reperfusion injury; however, this strategy increases liver damage in old livers [27]. This information is extremely important for liver surgeons dealing with hepatic ischaemia.

It is important to consider that during systemic inflammation, organ-specific alterations may take place and contribute to systemic inflammation. The lungs are particularly affected, which release a second wave of mediators that may potentiate systemic inflammation. In an experimental model of systemic inflammation, it was found that lung injury was much more intense in aged compared to young mice, as demonstrated by increased inducible nitric oxide synthase expression and decreased extracellular superoxide dismutase levels [28].

## 4. Acute Pancreatitis and Ageing

Severe AP is associated with high morbidity and mortality due to local pancreatic complications and multiple organ dysfunction [29, 30]. Although the mortality rate of severe AP has decreased in recent years, it is still around 20 to 25% [31, 32].

Advanced age has been considered an independent prognostic factor for mortality in AP [33]. The presence of complications such as infected pancreatic necrosis in the aged population is associated with a mortality rate of up to 50% [34]. In elderly patients with AP, in spite of similar occurrence of local complications, a substantial increase in multiple organ failure has been found [20]. However, it has not been clearly demonstrated that elderly patients with AP have different outcomes when compared to younger patients [35, 36]. Despite the severity of local inflammation being higher in younger compared to older patients, the score that measures the clinical severity of the disease (APACHE II, acute physiology, and chronic health evaluation) is higher in elderly patients [35]. To our understanding, this indicates that the severity of systemic response in elderly patients is more intense.

Although ageing is related to increased mortality due to organ failure in AP, no differences in local complications between young and elderly patients have been observed [37, 38]. Indeed, advanced age and associated diseases do not influence the occurrence of pancreatic necrosis [39]. The factors related to the increased incidence of organ failure in elderly patients, however, are not well understood and may be related to changes in the innate immune system or to an abnormal elevation of proinflammatory cytokines

Table 1: Effect of ageing on acute pancreatic inflammation.

| | Young | Elderly | references |
|---|---|---|---|
| Mortality | ↑ | ↑↑ | [33] |
| Organ failure | ↑ | ↑↑ | [20] |
| Local complications | ↑ | ↑ | [37, 38] |
| Bacterial translocation | ↑ | ↑↑ | * |
| Systemic inflammation | ↑ | ↑↑ | [35] |

*Coelho AMM, Machado MCC, Sampietre SN et al. Aging is related to increased intestinal damage and bacterial translocation in acute pancreatitis in rats (in preparation).

in the bowel, which facilitates bacterial translocation. In a recent study, we found that 2-year-old rats with taurocholic acid-induced AP presented much higher-bacterial translocation than the young ones (in preparation). The available information on the effect of ageing on AP inflammation is summarized in Table 1.

## 5. Future Studies and Conclusions

Studies addressing intestinal alterations during AP in older animals should help to elucidate the mechanisms involved in bacterial translocation that are related to multiple organ failure in AP.

New strategies that reduce the exaggerated inflammatory response in elderly patients are needed to decrease morbidity and mortality in elderly patients with AP. Our group has shown some strategies that reduce the inflammatory response in experimental AP, such as lavage of the peritoneal cavity [40], treatment with hypertonic saline solution [41, 42], treatment with platelet-activating factor antagonists [43], and pentoxifylline [44]. It would be desirable to investigate if these strategies are also effective in reducing the exaggerated inflammatory response in older animals with AP.

Specialized diets have been proposed to increase innate immunity and protect elderly patients from infections and should also be tested in elderly AP patients [45].

In conclusion, advanced age is considered an independent prognostic factor for mortality in acute pancreatitis, but the mechanisms involved in this process are not completely understood. Organ failure seems to be the most important factor responsible for the higher mortality rate observed in elderly patients since local complications are similar in young and older patients.

It is conceivable that different therapeutic strategies should be employed in young and old people to control AP, and clinical trials on anti-inflammatory and antimicrobial drugs in AP should consider patient age as an important issue.

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