High Phosphate Serum Levels Correlate With the Severity of Experimental Severe Acute Pancreatitis

Insight Into the Purinergic System

Guilherme S. Mazzini, MD, PhD,† Daniel T. Jost, MS,† Denise B. Ramos, MS,† Jean P. Oses, PhD,‡ Mateus A. Zeni,* Rafael Machoseki,† Luiza W. Kist, PhD,§‖ Mauricio R. Bogo, PhD,§‖ Carla D. Bonan, PhD,‖¶ Maria I. Edelweiss, MD, PhD,* Marta M. Duarte, PhD,# Luis V. Portela, PhD,† Diogo O. Souza, MD, PhD,† and Alessandro B. Osvaldt, MD, PhD***

Objectives: Extracellular purines are a component of the systemic inflammatory response, and their levels are modulated by ectonucleotidases. In addition, nucleotide hydrolysis releases phosphate. We studied serum phosphate levels as a predictor of severity in acute pancreatitis (AP) and their correlation with extracellular purinergic metabolism.

Methods: Acute pancreatitis was induced by the retrograde injection of sodium taurocholate. The AP group was compared with animals submitted to a model of sepsis by cecal ligation and puncture. The sham group was submitted to laparotomy and closure. We measured the phosphate and purine levels in serum and the expression of 5′-nucleotidase (CD73) and the adenosine A2a receptor in pancreatic tissue by quantitative real-time polymerase chain reaction.

Results: Serum phosphate levels were higher in severe AP and correlated with severity. Severe AP led to increased serum levels of adenosine diphosphate, adenosine monophosphate, and adenosine. In addition, adenosine monophosphate conversion to adenosine in serum was accelerated in the AP groups. We found a positive correlation between serum adenosine and phosphate in the AP groups. The expression levels of CD73 and the adenosine A2a receptor in the pancreas were not altered.

Conclusions: Our study suggests that serum phosphate correlates with severity in AP and implicates extracellular purines in the systemic response to severe AP.

Key Words: pancreatitis, purine nucleotides, phosphate

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Predicting the severity of acute pancreatitis (AP) early in the course of the disease remains a challenge to clinicians and researchers.[1] Because systemic inflammatory response syndrome is one of the main causes of morbidity in severe AP, several studies have investigated inflammatory markers as potential predictors of severity.[2,3] However, although the purinergic system is a well-described component of the systemic inflammatory response and signaling pathway in disorders such as ischemia reperfusion injury and inflammatory bowel disease (IBD),[4,5] extracellular purines have not been examined as potential peripheral markers of severity in AP.

The purine nucleotides adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are released from the endothelium, neutrophils, platelets, and necrotic and apoptotic cells under acute pathological conditions such as ischemia, hypoxia, and inflammation, acting as extracellular signaling molecules.[6] In the extracellular space, ATP and ADP are degraded to adenosine monophosphate (AMP), which in turn is degraded to adenosine (ADO), sequentially releasing 1 phosphate after each conversion. Enzymes called ectonucleotidases regulate this process.[6] Adenosine triphosphate and ADP are both converted to AMP by the ectonucleoside triphosphate diphosphohydrolase 1 (CD39). Subsequently, the 5′-nucleotidase (CD73) rapidly converts extracellular AMP to ADO, which exerts its functions by binding to extracellular receptors (A1, A2a, A2b, A3).[7]

As a result of this well-coordinated enzymatic process, ectonucleotidases contribute to the modulation of the inflammatory response and control the extracellular levels of purines.[8] An increase in extracellular ADO production has been described as a physiologic anti-inflammatory mechanism secondary to systemic inflammation.[9] This has been demonstrated in experimental sepsis and colitis, for example, with ADO promoting tissue-protective responses.[5,10] Specifically regarding AP, 2 studies using rats demonstrated the modulation of AP severity via ADO receptors. Using an experimental model of AP, the authors described the attenuation of the histologic inflammatory findings in the pancreas after administration of an agonist of the A2a ADO receptor.[11] A similar effect was described using an agonist of the A3 ADO receptor.[12]

Considering the possible relationship between extracellular purines and AP, our group hypothesized that extracellular purinergic metabolism is accelerated in AP and is correlated with severity. In addition, serum phosphate may be increased due to nucleotide hydrolysis by ectonucleotidases. In support of this hypothesis, a case report from Birkenfeld et al[13] described a patient with an inexplicable hyperphosphatemia, and the computed tomography revealed severe necrotizing AP. The authors could not explain what they called a puzzling business.

In the present study, we examined the role of serum phosphate levels as a predictor of AP severity using a sodium taurocholate–induced rat experimental model of AP. In addition, we correlated phosphate alterations with extracellular purinergic metabolism. To exclude the influence of a nonspecific systemic inflammatory response and to isolate the effect of AP, we compared results from

From the *Programa de Pós-Graduação em Ciências Cirúrgicas, Faculdade de Medicina, and †Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre; ‡Laboratório de Neurociências Clínicas, ICBS, Centro de Ciências Da Vida E Da Saúde, Universidade Católica de Pelotas, Pelotas; §Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul; ‖Instituto Nacional de Ciência e Tecnologia Translacional em Medicina; ¶Laboratório de Neuroquímica e Psicofarmacologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre; #Universidade Luterana do Brasil, Canoas; and **Serviço de Cirurgia Digestiva, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.

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Reprints: Guilherme S. Mazzini, MD, PhD, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600 Anexo, 90035-000, Porto Alegre, Rio Grande do Sul, Brazil (e-mail: guimazzini@gmail.com).

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rats submitted to AP with those of rats submitted to an experimental model of abdominal sepsis.

MATERIALS AND METHODS

Animals and Surgery

Male Wistar rats (aged 100-120 days, weighing 300-350 g) were obtained from our breeding stock and maintained on a 12-hour light/12-hour dark cycle in a constant room temperature (22°C) colony room. Water and a 20% (weight per weight) protein commercial chow diet were provided ad libitum. All animal research protocols and care conditions were approved by the local institutional animal care and use committee and were in accordance with the national guidelines on animal care. Surgical procedures were performed after the animals were anesthetized with an intraperitoneal injection of ketamine and xylazine. Acute pancreatitis was induced by a retrograde injection of sodium taurocholate solution into the biliary-pancreatic duct.[14] The AP group was divided into 2 subgroups—the Pan 3% and Pan 1.5% groups—injected with different concentrations of sodium taurocholate (3% and 1.5%, respectively), leading to different grades of severity (the 3% group should be of greater severity than the 1.5% group). The sepsis group was submitted to an experimental model of abdominal high-grade sepsis by cecal ligation and puncture,[15] involving ligation of 75% of the cecum and 2 punctures with an 18-gauge needle. The sham-operated group was submitted to anesthesia, laparotomy, and closure.

Blood was collected from the tail before surgery (time, 0 hour) and at 3 hours. At 12 hours, the animals were anesthetized, blood was collected by cardiac puncture, the pancreas was excised, and the rats were euthanized. A portion of the pancreas was placed in Trizol reagent (Invitrogen, Carlsbad, Calif) for gene expression analysis, and the rest of the tissue was fixed in 10% buffered formalin solution for histologic analysis. Blood samples were centrifuged, and serum was maintained at −70°C for later analysis.

A diagnosis of AP was made when the serum amylase concentration was 3 times higher than the basal level. Animals submitted to taurocholate injection with no increase in serum amylase were excluded.

Severity of Pancreatitis

Hematoxylin and eosin–stained pancreatic slices from all the groups were randomly numbered and examined by a blinded pathologist. Pancreatitis severity was determined using a scale adapted from Schmidt et al,[16] and 7 features were scored from 0 to 4 (total range, 0-28); these are as follows: edema, acinar necrosis, fat necrosis, parenchymal hemorrhage, fat hemorrhage, intralobular inflammation, and perivascular inflammation.

Biochemical Assays

Serum phosphate was determined using a colorimetric assay with malachite green, as described by Chan et al.[17] Serum tumor necrosis factor α (TNF-α) was determined with the commercial enzyme-linked immuno assay Ready-SET-Go! kit (eBioscience). Serum calcium was determined by a colorimetric assay using the Cálcio Arsenazo III (Katal Biotecnológica) kit. Amylase serum levels were determined by a colorimetric assay with the Amilase kit (Labtest).

Serum Purines

Purine concentrations in deproteinized serum were determined by high-performance liquid chromatography, according to Böhmer et al.[18] The following purines were evaluated: ATP, ADP, AMP, ADO, and uric acid (UA). Analyses were performed with a Shimadzu Class-VP chromatography system, consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, a Shimadzu SIL-10AF auto injector valve with a 50-μL loop, and a UV detector. Separations were achieved on a Supelcosil LC-18, 5 μm to 250 × 4.6 mm column (Supelco, St Louis, Mo). The mobile phase flowed at a rate of 1.2 mL/min, and the column temperature was 24°C. The buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following composition of buffer B in the mobile phase: 0% at 0.00 minute, 2% at 0.05 minute, 7% at 2.45 minutes, 50% at 10.00 minutes, 100% at 11.00 minutes, and 0% at 12.40 minutes. Fifty-microliter samples were injected into the injection valve loop. The absorbance was read at 254 nm.

5′-Nucleotidase Activity in Serum

Ten microliters of serum from all the groups at the 12-hour time point was incubated with 130 μL of 205 M Tris-HCl buffer solution at 37°C for 10 minutes. The reaction was initiated by the addition of 60 μL of 0.1 mM AMP solution (final volume for each sample was 200 μL). Immediately after adding AMP, 100 μL of the solution was collected, and the reaction was stopped with 14% trifluoroacetic acid (time, 0). The remaining solution was incubated for 10 minutes at 37°C, and the reaction was stopped with 14% trifluoroacetic acid (time, 10). Samples were centrifuged, and the supernatant was kept at −70°C until analysis by high-performance liquid chromatography, as described previously.

Gene Expression Analysis

Gene expression was determined by quantitative real-time polymerase chain reaction (RT-qPCR). Total RNA was isolated with Trizol in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry, and the complementary DNA (cDNA) was synthesized with the ImProm-II Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR Green I (Invitrogen) to detect double-stranded cDNA synthesis. Reactions were performed in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for *Gapd*, *Rpl13α*, *Hprt1*, *cd73*, and *A2a*), containing a final concentration of 5 M betaine (Sigma-Aldrich), 0.2 × SYBR Green I (Invitrogen), 100 μM dNTP, 1 × PCR buffer, 3 mM $MgCl_2$, 0.25 U Platinum Taq DNA Polymerase (Invitrogen), and 200 nM of each reverse and forward primer (Table 1). The PCR cycling conditions were as follows: an initial polymerase activation step for 5 minutes at 95°C, 40 cycles of 15 seconds at 95°C for denaturation, 35 seconds at 60°C for annealing, and 15 seconds at 72°C for elongation. At the end of the cycling protocol, a melting curve analysis was included, and the fluorescence was measured from 60°C to 99°C. The relative expression levels were determined with the 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl), and the stability of the references genes, *Gapd*, *Rpl13α*, and *Hprt1* (*M* value), and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

TABLE 1. Primer Sequences for the RT-qPCR Experiments Included in the Study

Sequence	Sense	Antisense
*Gapd**	5′-TCACCACCATGGAGAAGGC-3′	5′-GCTAAGCAGTTGGTGGTGCA-3′
*Rpl13α**	5′-ACAAGAAAAAGCGGATGGTG-3′	5′-TTCCGGTAATGGATCTTTGC-3′
*Hprt1**	5′-GCAGACTTTGCTTTCCTTGG-3′	5′-CGAGAGGTCCTTTTCACCAG-3′
cd73†	5′-GGCCAGTCCACAGGGGAGTTCC-3′	5′-TTGACCAGATAGCTTGGGAGGACC-3′
A2a†	5′-GGGCATCATTGCAATTTGCTGGGTG-3′	5′-TGTAATTCATGGGCACCACGTCCTCG-3′

*According to Bonefeld et al.[19]
†Designed by the authors.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows 18 (IBM Corporation). The results are expressed as the mean (SD). After the Shapiro-Wilk test, the results were analyzed by the generalized estimating equation, followed by Bonferroni post hoc test. 5′-Nucleotidase activity in serum was analyzed with the Kruskal-Wallis test, followed by Dunn post hoc test. Correlations were made using the Spearman coefficient. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Basic pancreatic histologic findings are summarized in Figure 1. Serum amylase reached the diagnostic levels of AP in all but 1 animal submitted to taurocholate infusion, which was then excluded. A statistically significant increase at 3 hours and 12 hours occurred only in the Pan 3% and Pan 1.5% groups and was not different between the AP groups (Fig. 2A). The severity scale was altered only in the AP groups but was not significantly different between the AP groups. However, there were predominantly less severe animals in the Pan 1.5% group and predominantly more severe animals in the Pan 3% group, expanding the range of severity in our experiment (Fig. 2B). To demonstrate that both experimental models resulted in systemic inflammation, we measured the TNF-α serum levels. There was a significant increase in all groups at 12 hours compared with that in time 0 hour, reflecting a systemic inflammatory status because of AP, sepsis, or surgery. In addition, serum TNF-α was significantly different among all the groups at 12 hours, with the highest levels in the Pan 3% group, followed by the Pan 1.5% group, the sepsis group, and the lowest levels in the sham group (Fig. 2C). Calcium serum levels did not show statistically significant differences among the groups (data no shown).

Serum Phosphate and Severity of AP

Serum phosphate levels were not different among the groups at times 0 hour and 3 hours. However, at 12 hours, there was a statistically significant increase in serum phosphate only in the Pan 3% group (Fig. 3A). Furthermore, the analysis of both AP groups

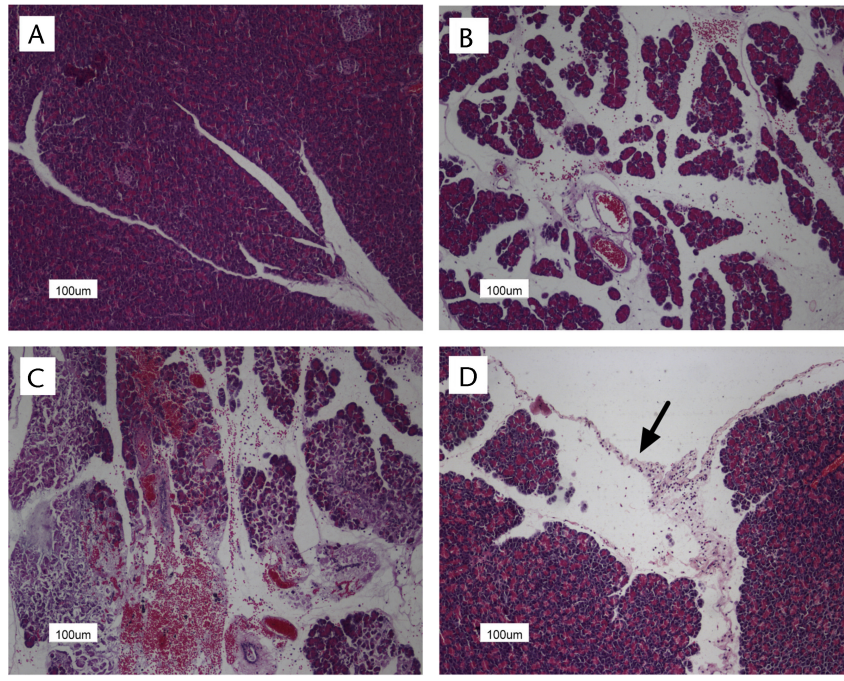
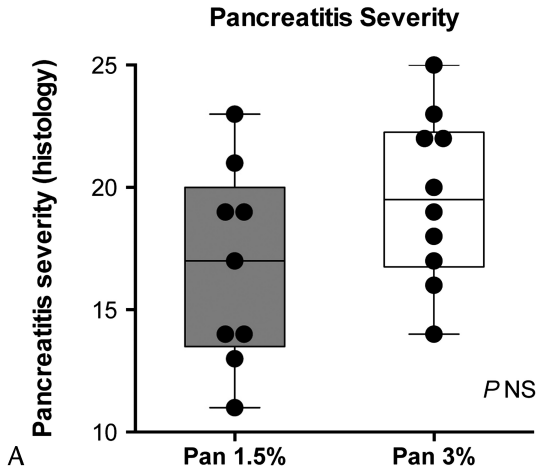
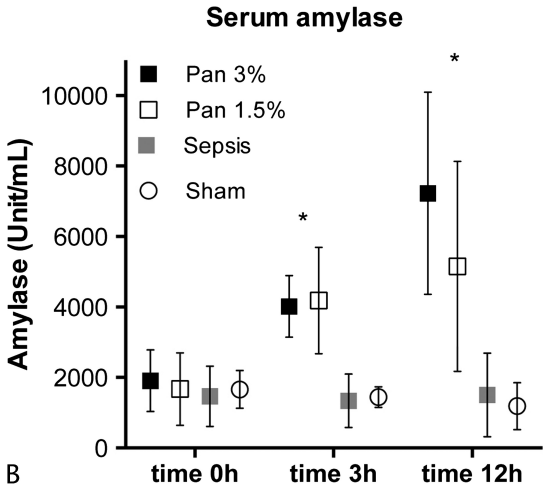


FIGURE 1. The panel shows the basic histologic alterations found in the pancreas (hematoxylin and eosin–stained slices). A, Normal-appearing pancreas from the sham group, with no edema or inflammatory infiltration. B, Mild AP, with diffuse edema and minimum inflammatory infiltration. C, Severe AP, with extensive necrosis, hemorrhage, and inflammatory infiltration. D, Pancreas from the sepsis group, with normal glandular appearance and peritonitis. The arrow indicates peritoneal inflammatory infiltration. **Editor's note:** A color image accompanies the online version of this article.





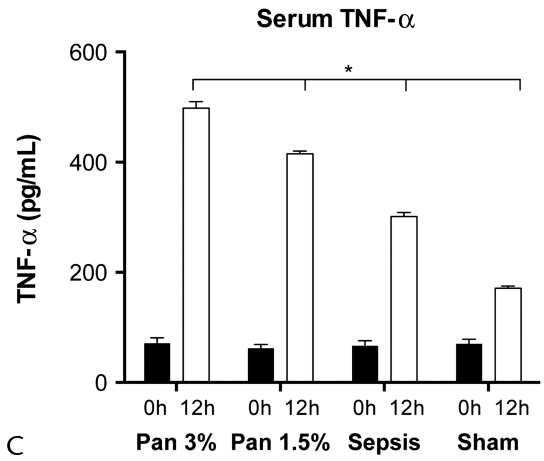
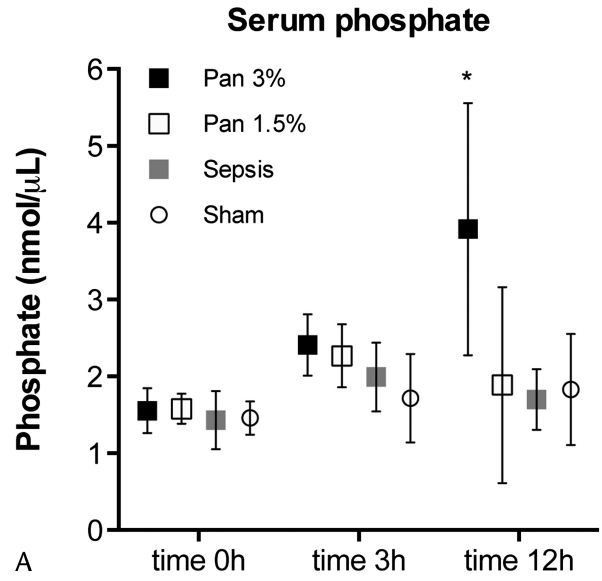


FIGURE 2. A, AP severity accessed by histology is presented as the median ± minimum to maximum range (dots represent individual animals). There was no statistically significant difference between the AP groups. B, Serum amylase was similarly higher in the Pan 3% and Pan 1.5% groups at 3 hours and 12 hours, but there was no difference between the AP groups; *$P < 0.05$. C, Serum TNF-α increased in all the groups at 12 hours compared with 0 hour and was significantly different among all the groups at 12 hours; *$P < 0.05$.

combined showed a statistically significant correlation between serum phosphate at 12 hours and AP severity ($r = 0.61$) (Fig. 3B). There was no statistical correlation in the sepsis or sham group (data not shown).

Purinergic System

The ATP concentration in serum was under the detection limit of our method in all groups. Adenosine diphosphate, AMP, ADO, and UA levels were not different among the groups at times



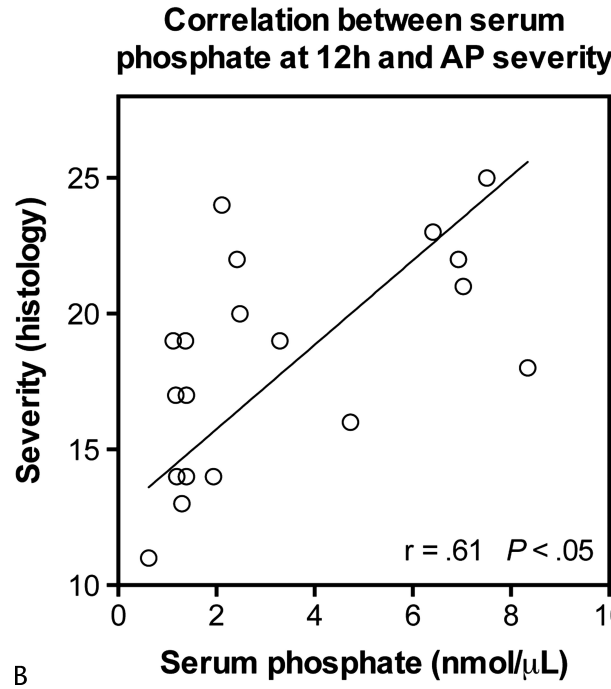


FIGURE 3. A, Statistically significant increase in serum phosphate at 12 hours in the Pan 3% group; *$P < 0.05$. B, Correlation between serum phosphate at 12 hours and AP severity when analyzing the Pan 1.5% and Pan 3% groups together. Spearman coefficient, $r = 0.61$, $P < 0.05$.

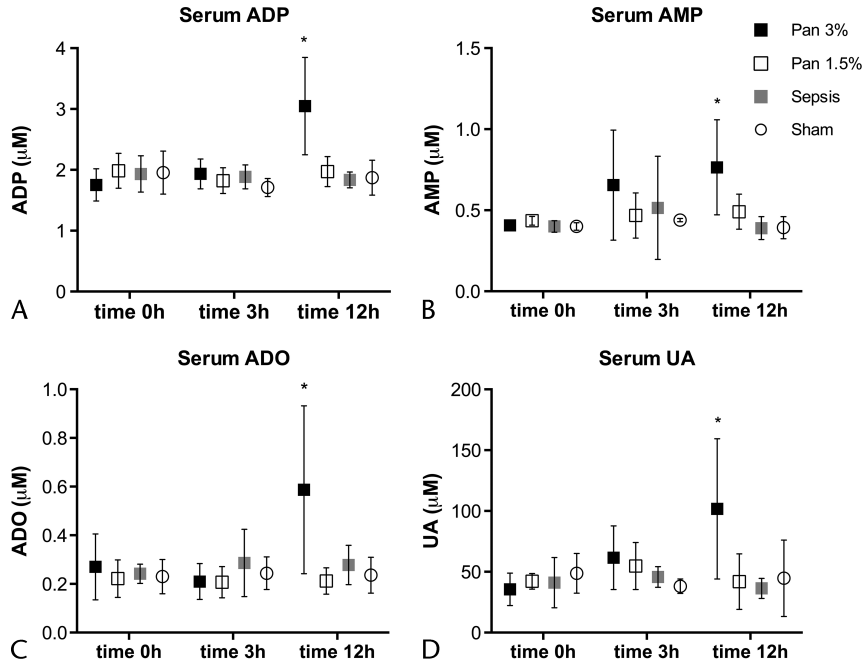


FIGURE 4. A–D, Statistically significant increase in the serum levels of ADP, AMP, ADO, and UA at 12 hours only in the Pan 3% group; *$P < 0.05$.

0 hour and 3 hours. At 12 hours, there was a statistically significant increase in purine serum levels only in the Pan 3% group (Fig. 4). To determine whether serum phosphate is increased due to release from nucleotide hydrolysis, we analyzed the Pan 3% and Pan 1.5% groups together at 12 hours and found a statistically significant correlation between serum phosphate and ADO, with $r = 0.84$ (Fig. 5). In the Pan 3% group, this statistically significant correlation was even higher, with $r = 0.91$ (data not shown).

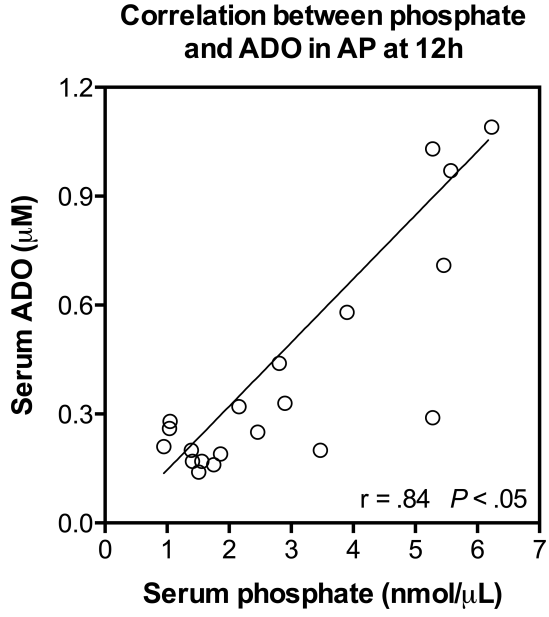


FIGURE 5. Correlation between serum ADO and phosphate at 12 hours, when analyzing the Pan 1.5% and Pan 3% groups together. Spearman coefficient, $r = 0.84$, $P < 0.05$.

Systemic Versus Local Pancreatic Alterations

Considering that increased purines and phosphate were detected specifically in severe AP, we determined if these results originated from systemic or local pancreatic reactions. Using RT-qPCR in pancreatic tissue, we determined the expression of 5′-nucleotidase (CD73), which is directly responsible for ADO production. In addition, we determined the expression of the ADO receptor A2a in the pancreas. However, the expression of CD73 or A2a in the pancreas was not different among the groups (Fig. 6), indicating a possible systemic extracellular mechanism.

Therefore, we incubated serum samples from time 12 hours with 0.1 mM AMP solution and measured ADO formation after 10 minutes, as an indirect measure of 5′-nucleotidase activity in serum. Because we added substrate to the incubation medium, all the groups were expected to increase serum ADO after 10 minutes. However, the variation (delta ADO) was significantly higher in the Pan 3% and Pan 1.5% groups compared with that in the other groups, suggesting increased extracellular enzymatic activity in AP. Delta ADO was not significantly different between the sepsis and sham groups and between the AP groups (Fig. 7).

DISCUSSION

Using an animal model of AP, we demonstrated for the first time that the serum phosphate levels rise in severe AP and correlate with severity. In determining the source of the phosphate, we demonstrated that severe AP led to increased serum levels of ADP, AMP, and ADO. In addition, we demonstrated experimentally that AMP hydrolysis in serum was accelerated in the AP groups. Because nucleotide hydrolysis releases phosphate, it is reasonable to propose that extracellular nucleotide degradation is responsible for our findings. In support of this hypothesis, we found a strong positive correlation between serum ADO and phosphate in the AP groups.

Severe AP is associated with systemic inflammatory response syndrome due to sterile inflammation,[20] with local damage and the systemic release of proinflammatory cytokines,

resulting in increased vascular permeability, thrombosis, hemorrhage, selective pancreatic ischemia, and ischemia reperfusion injury.[21] These alterations are nonspecific and common to other inflammatory conditions. We controlled this variable by comparing our results from experimental AP with an experimental model of high-grade abdominal sepsis. Both groups showed increased serum TNF-α levels, confirming a systemic inflammatory status, but the alterations in serum phosphate and purines occurred only in the AP groups. In addition, AMP hydrolysis in serum was higher in the AP groups, most likely representing accelerated extracellular purine degradation. Despite the fact that the alterations observed were specific for AP, the expressions of 2 molecular targets in pancreatic tissue were not affected (CD73 and ADO A2a receptor), suggesting that our findings are part of a systemic extracellular response favoring the production of ADO.

In vivo and in vitro studies have described a critical role for extracellular ADO in protecting tissue from inflammatory and ischemic damage.[22] Similar to AP, IBD involves microvascular changes, thrombosis, and severe hypoxia of the inflamed mucosa.[23] Experimental studies indicate that ADO production is increased in IBD, and ADO receptor activation promotes anti-inflammatory actions,[5] with pharmacologic therapeutic implications.[7] In line with our findings, a study of rats submitted to AP by retrograde infusion of 5% sodium taurocholate by Celiński et al[11] showed that the expression of A2a receptors evaluated by immunohistochemistry was not altered. Nevertheless, the administration of CGS 21680, an A2a receptor agonist, resulted in a favorable decrease of inflammatory cell infiltration, hemorrhagic changes, necrosis, and vacuolization of acinar cells in the pancreas. Accordingly, in our study, we demonstrated that severe AP increased serum ADO levels, most likely as an endogenous protective response. This mechanism should be considered in further studies as a possible therapeutic target.

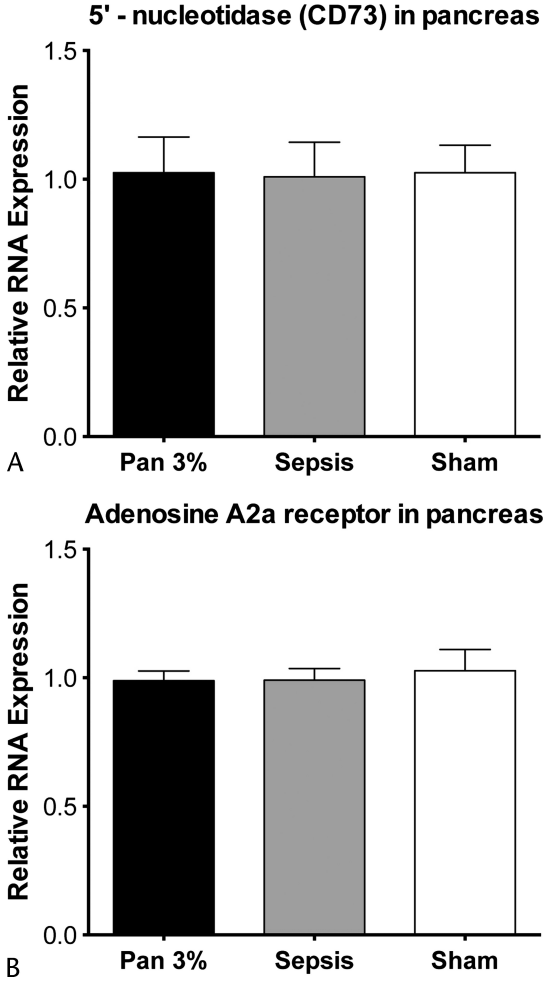


FIGURE 6. RT-qPCR analysis of the pancreas at 12 hours. A, The relative RNA expression of CD73 was not significantly different among the groups. B, The relative RNA expression of the ADO A2a receptor was not significantly different among the groups.

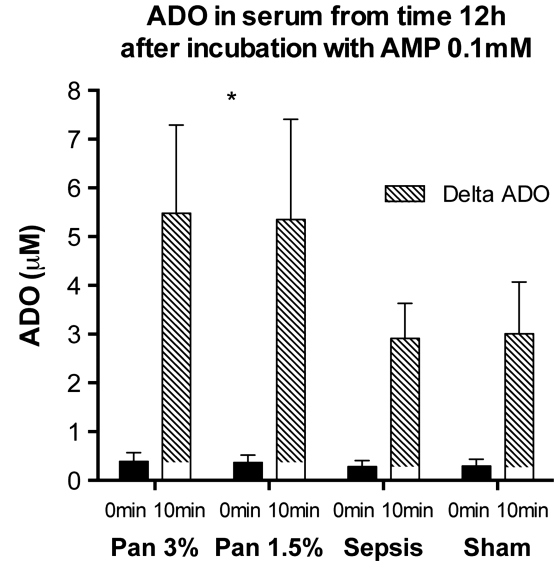


FIGURE 7. ADO levels measured at 0 minute and 10 minutes after the incubation of serum from the 12-hour time point with 0.1 mM AMP. Conversion of AMP to ADO (delta ADO) was significantly higher in the Pan 3% and Pan 1.5% groups but not different between the Pan 3% and Pan 1.5% groups

A recent systematic review of 184 studies in humans concerning new prognostic markers for AP reported that, despite the profusion of studies in this area, hardly any new markers have been adopted into routine clinical use.[1] Although our experimental findings may not directly translate to the clinical setting, retrograde infusion of sodium taurocholate is an appropriate model to study severity markers because it causes local and systemic inflammation, and AP severity may be modulated by changing the concentration of the solution.[24] In addition, we determined the AP severity by histology, allowing a comparison of serum levels of phosphate with a numeric scale of severity. This is experimentally useful but not feasible in clinical practice, where severity is assessed by a combination of laboratory, image, and clinical evaluation.[25] Despite these limitations, we present here the novel finding of a potential marker of severity that is not expensive and can be used in clinical practice for different purposes. This work should facilitate other studies and encourage clinical applications.

In conclusion, our study identifies serum phosphate as a promising marker of severity in AP. In addition, our work implicates the extracellular purinergic system in the systemic response to severe AP and suggests that ADO and ADO receptors are putative targets for further studies of pharmacological therapeutics for severe AP.

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