Volume replacement with saline solutions during pancreatitis in rats and the hepatic profiles of apoptotic proteins and heat-shock proteins

Reposição volêmica com soluções salinas em pancreatite e perfil hepático de proteínas apoptóticas e de choque térmico

ABSTRACT

Objective: Liver failure can occur as a consequence of the systemic inflammation after acute pancreatitis. We assessed the effect of volume repositioning with hypertonic saline solution or normal saline on hepatic cytokine production and the expression of heat-shock proteins and apoptotic proteins after acute pancreatitis.

Methods: Wistar rats were divided in four groups: C - control animals that were not subjected to insult or treatment; NT - animals that were subjected to acute pancreatitis and received no treatment; normal saline - animals that were subjected to acute pancreatitis and received normal saline (NaCl 0.9%); and HS - animals that were subjected to acute pancreatitis and received hypertonic saline solution (NaCl 7.5%). Acute pancreatitis was induced by retrograde transduodenal infusion of 2.5% sodium taurocholate into the pancreatic duct. At 4, 12 and 24 h following acute pancreatitis induction, TNF-alpha, IL-1-beta, IL-6 and IL-10, caspase-2 and -7, Apaf-1, AIF and HSP60 and 90 were analyzed in the liver.

Results: Casp2 decreased in the normal saline and hypertonic saline groups (p<0.05 versus C) at 12 h. Apaf-1, AIF and HSP90 remained unchanged. At 4 h, Casp7 increased in the NT group (p<0.01 versus C), although it remained at the baseline levels in the reperfused groups. HSP60 increased in all of the groups at 4 h (p<0.001 vs. C). However, the hypertonic saline group showed lower expression of HSP60 than the normal saline group (p<0.05). Hypertonic saline solution maintained the production of cytokines at normal levels. Volume reperfusion with normal or hypertonic saline significantly modulated the expression of Casp7.

Conclusion: Volume replacement with hypertonic or normal saline was effective in reducing caspase 7. However, only hypertonic solution was capable of regulating cytokine production and HSP60 expression at all time points.

Keywords: Heat-shock proteins; Apoptosis; Pancreatitis; Liver

INTRODUCTION

Liver failure can occur as a consequence of the systemic inflammatory response syndrome that occurs in acute pancreatitis. In our previous studies, we have demonstrated increased lipid peroxidation levels and extracellular matrix degradation in the liver after pancreatitis. In addition, we observed increased blood levels of hepatic enzymes, indicating liver cell damage. Acute pancreatitis–associated liver injury is mediated by inflammatory cytokines that are produced within tissue-resident macrophages, which are activated by the inflammatory mediators that are systemically released by the pancreas. The liver, in turn, participates in systemic inflammation, releasing several
inflammatory mediators, leading to injury of other organs. Substances that are systemically released during pancreatitis, such as nitric oxide (NO) and free radicals, can interfere with the respiration of hepatic mitochondria and can induce apoptosis. Apoptotic cell death might play a considerable role in affecting mortality and morbidity in severe acute pancreatitis. The apoptosis pathway, by death receptors or via the mitochondrial pathway, activates the final caspase cascade for cell death. Death receptor signaling has been associated with apoptosis in several hepatic diseases, such as ethanol-induced liver injury and cholestatic liver disease. Apoptosis related to severe acute pancreatitis injury is known to be triggered via the mitochondrial pathway.

Cell death has been observed in both apoptotic and necrotic forms in both clinical and experimental acute pancreatitis. Current evidence suggests that the amounts of and balance between apoptosis and necrosis influence the severity of acute pancreatitis. Recently, heat-shock proteins and their cofactors have been revealed to be associated with apoptotic and necrotic pathways. Heat-shock proteins are molecular chaperones that stabilize and refold damaged intercellular proteins, preventing intracellular protein aggregation and rendering cells resistant to stress-induced cell damage.

Volume replacement, mainly with hypertonic saline alone, has shown benefits in various aspects of the pathophysiology of several diseases due to improvement of tissue hypoperfusion and decreases in oxygen consumption, endothelial dysfunction and cardiac depression, as well as reductions in a broad array of pro-inflammatory cytokines and various oxidant species. We previously reported that hypertonic saline treatment reduces oxidative stress and tissue degeneration in the liver after pancreatitis. Additionally, our group showed the effects of hypertonic saline in the expression and activity of several proteins, including heat-shock proteins (HSPs), in the lung and the liver. However, there are no data in the literature regarding the effect of hypertonic solution on hepatic apoptosis during pancreatitis. In the present study, we assessed the effects of normal (NaCl 0.9%) and hypertonic saline (NaCl 7.5%) on the expression of apoptotic proteins and HSPs, as well as the correlation of these factors with inflammation during pancreatitis.

METHODS

Pancreatitidis induction

All of the experiments were conducted in accordance with the guidelines established by the Research Ethics Committee of the Faculdade de Medicina de the Universidade de Sao Paulo. Male Wistar rats, weighing 270-320 g, were anesthetized subcutaneously with ketamine (10 mg/kg) and xylazine (8 mg/kg). Acute pancreatitis was induced by a well-established method of retrograde infusion of 2.5% sodium taurocholate (1.0 mL/kg; Sigma, St. Louis, MO, USA) transduodenally into the pancreatic duct via a 24-gauge angiocatheter at a constant infusion rate of 1 mL/min. The bile duct was clamped with a microsurgical “bulldog” clamp at the hepatic hilum to prevent leakage of taurocholate solution into the liver. The hepatic hilar clamp was released after the injection. It has been reported in the literature that this model of pancreatitis causes hepatic injury and reproduces the mortality and pathophysiological changes of human pancreatitis. In the present study, analysis was performed on four groups: the control group, consisting of animals that suffered neither insult nor treatment (C); the no treatment (NT) group, consisting of animals in which pancreatitis was induced, but no treatment was given; the normal saline (NS) group, consisting of animals in which pancreatitis was induced, and an intravenous bolus of normal saline (0.9% NaCl, 34 mL/kg) was administered; and the hypertonic saline (HS) group, consisting of animals in which pancreatitis was induced, and hypertonic saline (7.5% NaCl, 4 mL/kg) was administered via the internal jugular vein over a period of 5 min at 1 h after pancreatitis induction. The volume of normal saline infused was equivalent in sodium content to 4 mL/kg of hypertonic saline. The animals were sacrificed and their livers collected at 4, 12, or 24 h after pancreatitis induction.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to determine the mRNA levels in liver tissue. Total RNA was extracted from frozen rat livers with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instruction. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and was quantified spectrophotometrically at 260 nm. First-strand c-DNA was generated by adding RNA (1 μg) to a mixture containing 1 μL of ImProm-II™ reverse transcriptase (Promega, Madison, WI, USA), 1 μL (0.5 μg/μL) of oligo (dT), 20 U/μL of Recombinant RNAsin® RNase inhibitor, 3 mM MgCl₂, 6 μL of ImProm-II™ 5X reaction buffer (Promega, Madison, WI, USA) and 1 μL (0.5 mM) of dNTP mix (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μL. Reverse transcription was performed at 42°C for 50 minutes, followed by heat inactivation of reverse transcriptase at 70°C for 10 min. PCR amplification was performed using a Programmable
Thermal Controller (MJ Research PTC-200, Watertown, MA, USA). The PCR solution contained 1 µL of first-strand cDNA, 2.5 µL of 10X PCR buffer, 2 mM MgCl₂, 0.5 mM dNTP mix, 1 pmol/µL of each specific primer and 2.5 U/µL of TaqDNA polymerase (Invitrogen, Carlsbad, CA, USA) in a final volume of 25 µL. To evaluate the relative abundance of a transcript between samples, the relative RT-PCR was performed with 18S ribosomal RNA primer as an internal control. The PCR products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide (EtBr) and visualized under ultraviolet light with a video imaging system (Pharmacia). Densitometric analyses of EtBr-stained gel bands were performed using Gene Tools software (Syngene, Cambridge, MA, USA). The data were plotted as a function of the log OD of the gene target product against the log OD of 18S rRNA. The sequences of the specific primers (Invitrogen, Carlsbad, CA, USA) were as follows: rRNA (320bp) sense: GAAGATGCGTGAACACTATGCC; and antisense: TTACCCAAAGTGGCCCTA; HSP60 (213pb) sense: GAAAGATGGTGAACTATGCC; and antisense: CTAGCCAACACCCTGAGAGC.

Western blot

Frozen tissue samples (100 mg) were pulverized in liquid nitrogen. The samples were then homogenized in a buffer containing 1% TX-100, 20 mM Tris (pH 8.0), 10% glycerol, 135 mM NaCl and proteolytic enzyme inhibitors (40 µg/mL of phenylmethylsulfonylfluoride and 10 µg/mL of pepstatin; Sigma, St. Louis, MO). After separation of debris by centrifugation for 45 minutes at 14,000 g, the supernatants were collected, and the protein concentration was determined by the Bradford method input into a computer. The samples were stored at -80ºC until assayed. Protein expression was assessed using SDS-polyacrylamide gel electrophoresis under reducing conditions. Liver tissue extracts (25-100 µg/mL) were boiled in equal volumes of loading buffer (150 mM Tris-HCl–pH 6.8, 4% SDS, 20% glycerol, 15% β-mercaptoethanol and 0.01% bromophenol blue) and were subjected to electrophoresis on 10% polyacrylamide gels. Following electrophoretic separation, the proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and 0.5% Tween 20 (TBST) for 1 hour. Primary antibodies against the following were employed: caspase-2 (rabbit, 1:1000, Santa Cruz Biotechnology 623), caspase-7 (rabbit, 1:1000, sc-337773), apoptotic protease activating factor 1 (APAF-1, goat, 1:1000, sc-26685), apoptosis-inducing factor (AIF, rabbit, 1:1000, ab32516, Abcam), HSP60 (goat, 1:1000, sc1052), HSP90 α/β (goat, 1:1000, sc1055) and β-actin (1:10000, Sigma, A5441); they were incubated at 4ºC overnight. After washing twice with TBST, secondary horseradish peroxidase conjugate Ab (goat anti-rabbit polyclonal sc2004 or rabbit anti-goat sc2768, Santa Cruz Biotechnology) was applied at a dilution of 1:5000 for 2 hours. The blots were washed in TBST twice over 30 min, incubated in enhanced Super Signal Detection Kit chemiluminescence reagents (Pierce, Rockford, IL, USA) and exposed to Kodak O-OMAT-AR photographic film (Kodak, Rochester, NY, USA). The band intensity of the original blots was quantified using Image J software (Research Services Branch, National Institutes of Health, Bethesda, MD, USA) and was normalized to control levels (control = 1). (17)

Cytokine measurement

Plasma samples were collected from the animals just before sacrifice. The cytokines TNF-α, IL-6, IL-1β and IL-10 were measured by ELISA, according to the manufacturer’s instructions (R&D Technologies, USA).

Histology analysis

After fixation in 10% formalin, the liver tissue was embedded in paraffin and cut into 4- to 6-µm sections. The sections were stained with hematoxylin and eosin and were analyzed qualitatively by light microscopy. The occurrence of hepatic damage, such as areas of necrosis, hemorrhage, inflammatory infiltrates and vacuolization of the cytoplasm, was assessed. The images were generated by a microscope (Leica) connected to a camera (Sony Trinitron CCD, Sony, Japan) and were input into a computer.

Statistical analysis

The data are expressed as the means ± standard errors of the means (SEMs). The analyses were performed using Sigma Stat statistical software, version 3.1 (Sigma Stat Software Inc., Chicago, IL, USA). Comparisons among experimental groups were performed by analysis of variance (one-way ANOVA), and Tukey’s post hoc test was used to compare individual groups (NT, NS and HS were compared among themselves and with a unique control group at all time points) based on time (4, 12 and 24 hours). A p value of <0.05 was considered to be significant.
RESULTS

Cytokine production

Four hours after the induction of pancreatitis, increased plasma levels of IL-1β (Figure 1A) were observed in the NT and HS groups (p<0.05 versus C). After 12 hours, the plasma IL-1β levels remained increased in the NT group (p<0.001 versus C and HS; p<0.01 versus NS). Treatment with normal saline or hypertonic solution maintained the normal levels.

Plasma IL-10 levels (Figure 1B) increased in the NT group 4 (p<0.05 versus C; p<0.01 versus NS and HS) and 24 hours (p<0.05 versus C) after the induction of pancreatitis. The levels of this cytokine increased in the NS group at 12 h (p<0.05 versus C).

The hepatic levels of IL-1β (Figure 2A) increased significantly in the NS group (p<0.05 versus C) at 4 h. After 12 and 24 h, we could not observe any statistically significant difference in hepatic IL-1β release among the groups. The hepatic production of IL-10 (Figure 2B) did not change in the liver for the first 4 hours after the induction of pancreatitis. However, the IL-10 levels increased in the NT group at 12 h (p<0.05 versus C, NS, HS) and in the NS group at 24 h (p<0.05 versus C).

The plasmatic and hepatic levels of TNF-α and IL-6 did not change in any group over 24 hours (data not shown).

Apoptotic protein expression

To study the cell death process, we investigated the expression of the following apoptotic proteins: Apaf-1, AIF and caspases 2 and 7.

The expression of Apaf-1 (Figure 3) and AIF (Figure 4) remained at baseline levels throughout the 24 hour experimental period.

Precursors of both caspase-2 (51 kDa) and caspase-2L were expressed in the liver. The expression of caspase-2L (Figure 5) was unchanged for the first 4 hours. Twelve
hours after the induction of pancreatitis, the groups treated with hypertonic solution or normal saline showed decreases in caspase-2 expression compared to the control group (p<0.05). After 24 hours, caspase-2 expression decreased in all of the groups subjected to pancreatitis (p<0.01 versus C).

There was an increase in caspase-7 (Figure 6) expression in the NT group at 4 hours (p<0.01 versus C). However, normal saline and hypertonic treatments maintained the baseline expression of this protein. After 12 and 24 hours, caspase-7 expression normalized in all of the groups.

**Gene and protein expression of HSP60 and HSP90**

We did not observe any alterations in the gene expression of HSP60 (Figure 7A) throughout the 24 hour experimental period. However, 4 hours after the induction of pancreatitis, the protein expression of HSP60 (Figure 7B) increased in all of the groups (p<0.001 versus C). At
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this time, HSP60 expression was lower in the HS group than in the NS group (p<0.05). HSP90 expression (Figure 8) was not altered in any of the groups studied.

**Histology analysis**

The qualitative histological analysis (Figure 9) showed areas of necrosis, as well as the occurrence of hemorrhage, inflammatory infiltration and vacuolization of the cytoplasm in the livers of animals sacrificed 12 hours after the induction of pancreatitis that did not receive treatment.

We did not observe any differences among the rats treated with isotonic or hypertonic solutions.

**DISCUSSION**

We have demonstrated the occurrence of hepatic injury during pancreatitis and the benefits of saline solution administration.\(^{(1,16)}\) The present study showed the cytokine profiles and the expression of apoptotic and heat-shock proteins in the liver after the induction of acute pancreatitis. The current data support the proposal of hypertonic saline solution as an immune modulator by demonstrating the effect of sodium tonicity alone, as in our previous research.

Although it is known that the primary alterations in cytokine production occur early, we observed changes in the systemic and local production of two cytokines after the establishment of pancreatitis during the period studied. The group subjected to pancreatitis without volume treatment showed increases in plasma IL-1ß and IL-10 levels at 4
hours. After 12 hours, this group showed increased IL-1β and IL-10 levels in the plasma and tissue, respectively. Another study demonstrated increased levels of plasma cytokines after pancreatitis; however, the hepatic contents of cytokines were not studied.\(^{(18)}\)

Our analysis of hepatic cytokine profiles demonstrated that hypertonic solution maintains normal levels for 24 hours. A slight increase in plasma IL-1β levels occurred during the first 4 hours after pancreatitis induction. IL-1β is known to be one of the main cytokine mediators of the acute inflammatory response. The controlled release of this cytokine might induce NO production, which is important to hepatic perfusion and to the prevention of apoptosis in the liver.\(^{(19)}\) It is interesting to note that animals treated with normal saline showed an elevated level of at least one of the cytokines studied at some time point during the analyzed period.

Increased proinflammatory cytokines have been reported to be related to cell death.\(^{(20)}\) In this context, we measured several elements that participate in apoptotic events. Pancreatitis induces liver damage and causes an increase in the expression of caspase-7. However, in our experiments, the same effect did not occur with caspase-2, AIF or Apaf-1, which are proteins that are related to the intrinsic pathway of apoptosis. We must consider that the signaling pathway that culminates in apoptosis can be regulated and reversed at several points.\(^{(8)}\) Moreover, the regulation of apoptosis is more closely correlated with the activation of caspases than with intracellular protein content,\(^{(21)}\) such as the activity of caspases and their relationship with NO. Caspase nitrosylation modulates the activity of these proteins and, in contrast, could interfere in the final events of the apoptosis pathway.\(^{(22)}\) Both treatments, normal saline and hypertonic solution, were efficient in maintaining the expression of caspase-7 at baseline levels. This modulation of caspases might interfere with the apoptotic potential.\(^{(23,24)}\)

Additionally, we must consider necrosis as an important event in liver injury. Indeed, we observed necrosis in the histological analysis. In addition, in our previous studies, the induction of pancreatitis caused hepatic cell death, with the release of alanine aminotransferase (ALT) in the plasma.\(^{(25)}\) Increases in hepatic enzymes in the bloodstream are correlated with hepatic injury. In the liver, necrosis is usually the consequence of acute metabolic perturbation due to ATP depletion, as occurs in ischemia/reperfusion and acute drug-induced hepatotoxicity.\(^{(26)}\) The improvement in hepatic perfusion with volume administration restores oxygen delivery and consequent ATP production,\(^{(27)}\) thus avoiding cell necrosis.

Pancreatitis induced by cerulein increased the gene expression of heat-shock proteins and, concomitantly, decreased the expression of these proteins.\(^{(28)}\) In our model of experimental pancreatitis, the gene profiles of HSP60 and HSP90 remained unchanged. Conversely, HSP60 protein expression increased 4 hours after pancreatitis induction. HSP60 is involved in the regulation of the immune system, and it is capable of activating the Toll-like receptors, causing NO release. Previously, we reported that animals subjected to pancreatitis and treated with normal saline presented an increase in NO products concomitantly with increases in several inflammatory mediators.\(^{(25)}\) In addition to the increase in HSP60 in all of the groups, the group treated with hypertonic solution presented lower protein expression than the normal saline group. It is known that HSPs are produced in response to stress and are regulated by a heat-shock factor, which is inactive under conditions of no stress.\(^{(29)}\) Indeed, we showed the effect of hypertonic solution in reducing liver injury and inflammation during pancreatitis and its correlation with the reduction of HSP70.

The beneficial effects of hypertonic fluid administration were recently demonstrated in patients with septic shock.\(^{(30)}\) Our present study corroborates and elucidates the role of saline solutions in the regulation of the immune system beyond hemodynamic effects.

**CONCLUSION**

Volume replacement with hypertonic or normal saline was effective in reducing caspase 7. However, only hypertonic solution was capable of regulating cytokine production and HSP60 expression at all time points.

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**RESUMO**

Objetivo: A falência hepática é uma consequência da inflamação sistêmica após pancreatite aguda. Avaliou-se o efeito da reposição volêmica com soluções salinas fisiológicas ou hipertônicas na produção hepática de citocinas e na expressão de proteínas ativadas por choque térmico e proteínas ligadas à apoptose durante a pancreatite aguda.

**Métodos:** Ratos Wistar foram divididos em quatro grupos: C - animais controles não submetidos à lesão e nem ao tratamento; NT - animais submetidos à indução de pancreatite...
aguda e não tratados; SN - animais submetidos à indução de pancreatite aguda e tratados com solução salina normal (NaCl 0,9%); SH - animais submetidos à pancreatite aguda e tratados com solução salina hipertônica (NaCl 7,5%). A pancreatite aguda foi induzida por infusão retrógrada transduodenal de tau-rocolato de sódio 2,5% no ducto pancreático. Após 4, 12 e 24 horas da indução da pancreatite aguda, analisaram-se, no fígado, TNF-α, IL-1β, IL-6 e IL-10, caspase-2, caspase-7, APAF-1, AIF, HSP60 e HSP90.

Resultados: A caspase-2 diminuiu nos grupos SN e SH (p<0,05 versus C) após 12 horas. APAF-1, AIF e HSP90 permaneceram inalterados. Após 4 horas da indução, a caspase-7 aumentou no grupo NT (p<0,01 versus C), embora se mantendo em níveis baixos nos grupos reperfundidos. A HSP60 aumentou significativamente nas regiões analisadas. A reposição volêmica com solução salina normal ou hipertônica foi efetiva em reduzir a caspase-7. Entretanto, somente a solução salina hipertônica foi capaz de regular a produção de citocinas e a expressão de HSP60 em todos os momentos analisados.

Descritores: Proteínas de choque térmico; Apoptose; Pancreatite; Fígado

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