CO$_2$ Abdominal Insufflation Decreases Local and Systemic Inflammatory Response in Experimental Acute Pancreatitis

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Objectives: Acute pancreatitis (AP) is a serious disease that is amplified by an associated systemic inflammatory response. We investigated the effect of CO$_2$ pneumoperitoneum on the local and systemic inflammatory response in AP.

Methods: Acute pancreatitis was induced in Wistar rats by 5% taurocholate intraductal injection. Carbon dioxide pneumoperitoneum was applied for 30 minutes before the induction of AP. Inflammatory parameters were evaluated in the peritoneum (ascites, cell number, and tumor necrosis factor α [TNF-α]), serum (amylase, TNF-α, interleukin-6 [IL-6], and IL-10), pancreas (myeloperoxidase [MPO] activity, cyclooxygenase 2 and inducible nitric oxide synthase expression, and histological diagnosis), liver, and lung (mitochondria dysfunction and MPO activity).

Results: Abdominal insufflation with CO$_2$ before induction of AP caused a significant decrease in ascites volume, cells, and TNF-α in the peritoneal cavity and in serum TNF-α and IL-6 but not IL-10 levels. In the pancreas, this treatment reduced MPO activity, acinar and fat necrosis, and the expression of inducible nitric oxide synthase and cyclooxygenase 2. There were no significant differences on serum amylase levels, liver mitochondrial function, and pulmonary MPO between groups.

Conclusions: Our data demonstrated that CO$_2$ pneumoperitoneum reduced pancreatic inflammation and attenuated systemic inflammatory response in AP. This article suggests that CO$_2$ pneumoperitoneum plays a critical role on the better outcome in patients undergoing laparoscopic pancreatic surgery.

Key Words: acute experimental pancreatitis, carbon dioxide, pneumoperitoneum, SIRS, inflammation

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S evere acute pancreatitis (AP) is one of the most serious diseases with high morbidity and mortality. The pancreatic injury is amplified by an associated systemic inflammatory response syndrome (SIRS) that is ultimately responsible for the morbidity and mortality related to this disease.[1,2]

Systemic inflammatory response syndrome is characterized by elevated serum levels of cytokines such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor α (TNF-α), and nitric oxide (NO) that greatly increase in the serum early after the onset of the syndrome.[3] The initiating event that results in the release of inflammatory cytokines and mediators and in the systemic inflammatory process is still incompletely understood. However, it does involve the activation of several intracellular signaling pathways in leukocytes and the increased expression of specific genes.

Some investigators in the early 1970s proposed that these mediators could be found in the ascitic fluid in AP and therefore advocated peritoneal lavage for the treatment of this disease.[4,5]

Furthermore, a number of experiments demonstrated the toxicity of the pancreatitis-associated ascitic fluid to liver mitochondria and to the kidneys causing acute renal failure.[6–8]

Pancreatic injury leads to activation and leakage of pancreatic enzymes into the organ interstitium and accumulation in pancreatic and peripancreatic tissues and peritoneal cavity.[9]

Resident peritoneal macrophages are an important source of TNF-α.[10] Macrophages obtained from the peritoneal cavity of animals undergoing AP produce higher levels of TNF-α when exposed to lipopolysaccharide (LPS) than their controls.[11]

It has also been shown that injection of trypsin into the peritoneal cavity stimulates the production of TNF-α and IL-1.[12] These studies have suggested that the peritoneum may play an important role in the systemic inflammatory response in AP.[12,13]

Previous studies have demonstrated that peritoneal macrophages stimulated with LPS have a significant decrease in TNF-α and IL-1 production when exposed to CO$_2$ in vitro.[14]

Moreover, it has been reported that CO$_2$ insufflation pretreatment reduces the plasma levels of TNF-α and IL-6 and increases survival after LPS-contaminated laparotomy.[15] The authors of this study suggested that the local tissue acidification caused by peritoneal CO$_2$ insufflation might explain the decrease in local and systemic inflammatory response mediated by peritoneal macrophages.

On the other hand, experience with laparoscopic pancreatic surgery is growing exponentially worldwide, with lower systemic inflammatory response and reduction of postoperative pancreatic fistulas being reported.[16]

In the present study, we investigated the effect of CO$_2$ pneumoperitoneum on local and systemic inflammatory response during AP.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 230 to 270 g were housed in individual cages and kept under standard conditions (12 hours of light-dark cycle and temperature between 22 and 28°C) with free access to a standard rat chow and water ad libitum. The experimental protocol was approved by the ethics commission

of the Hospital das Clínicas: São Paulo University. Surgical anesthesia was induced with ketamine chloride (Ketalar; Parke Davis, São Paulo, Brazil).

Reagents

All chemical reagents were obtained from Sigma Chemical, Co (St Louis, Mo). Tumor necrosis factor α, IL-6, and IL-10 were assayed with kits from Biosource International (Camarillo, Calif).

Induction of AP

Acute pancreatitis was induced by retrograde injection of 0.5 mL of 5% sodium taurocholate in saline into the main pancreatic duct during 1 minute at constant rate by using an infusion pump. A clamp was applied to the proximal part of the hepatic duct during the injection.[17,18]

Experimental Groups

Animals were randomized to the following experimental groups:

Group 1: Ten control rats, nonmanipulated;

Group 2: Ten control rats, with CO_2 pneumoperitoneum applied for 30 minutes at a pressure of 4 mm Hg, not submitted to AP;

Group 3: Forty-six rats without treatment before the induction of AP;

Group 4: Forty-six rats with CO_2 pneumoperitoneum applied for 30 minutes at a pressure of 4 mm Hg before the induction of AP.[15]

Mortality Study

Animals of groups 1 (n = 20) and 2 (n = 20) were observed for 7 days after induction of AP for mortality analysis.

Sample Preparations

At 2 hours after AP induction, animals were killed. Serum samples were assayed for amylase,[19] TNF-α, IL-6, and IL-10 levels by a solid-phase sandwich enzyme-linked immune absorbent assay. Volume, levels of TNF-α, and cell number were determined in ascitic fluid. Cyclo-oxygenase (COX2) and inducible nitric oxide synthase (iNOS) protein expressions and myeloperoxidase (MPO) activities were analyzed in homogenates of pancreas at 2 hours, and pulmonary MPO was analyzed at 2 and 24 hours after AP induction. Hepatic tissue was collected for evaluation of oxidation and phosphorylation of liver mitochondria. A portion of the pancreas was fixed in 10% buffered formalin for histological analysis.

Cell Counting of Peritoneal Cells

Ten milliliters of cooled phosphate-buffered saline solution was injected into the abdominal cavities, and the peritoneum was massaged. The peritoneal lavage fluid was recovered by peritoneal puncture. The lavage fluid was centrifuged at 250g for 10 minutes, and the cell pellet was resuspended in phosphate-buffered saline. Cells were counted in a Neubauer chamber.

Myeloperoxidase Activity

Myeloperoxidase activity was used as an indicator of neutrophils' infiltration into tissues. Samples of pancreatic and pulmonary tissue were homogenized with a Polytron homogenizer using a homogenization buffer that contains 0.5% hexadecyltrimethyl ammonium bromide, 5-mmol/L EDTA and 50-mmol/L phosphate at pH 6.0. Homogenized samples were sonicated and centrifuged (3000g for 30 minutes) at 4°C. Myeloperoxidase activity in the supernatant was assayed by measuring the change in A_{460} resulting from the decomposition of
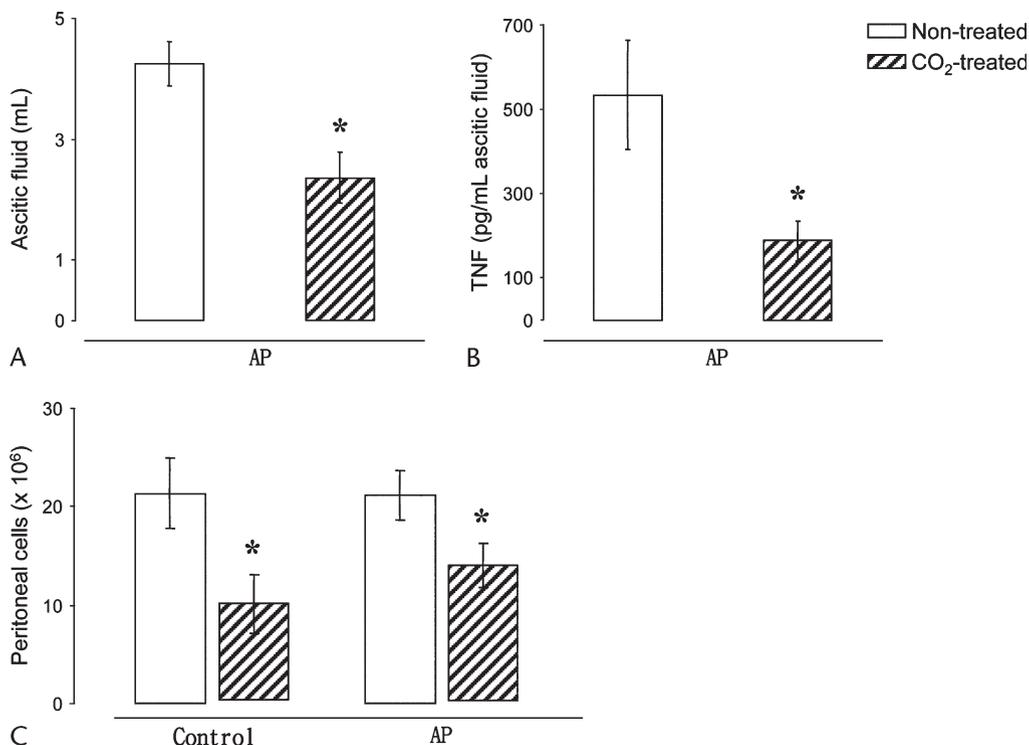
FIGURE 1. Effect of CO_2 insufflation on the peritoneal inflammation induced by AP. Acute pancreatitis was induced in rats by intraductal instillation of 0.5 mL of 5% taurocholic acid. One group received abdominal insufflation with CO_2 30 minutes before induction of AP: volume of ascites (A), number of peritoneal cells (B), and levels of TNF-α in ascitic fluid (C). Data are expressed as mean ± SEM of 10 animals per group. *$P < 0.001$.
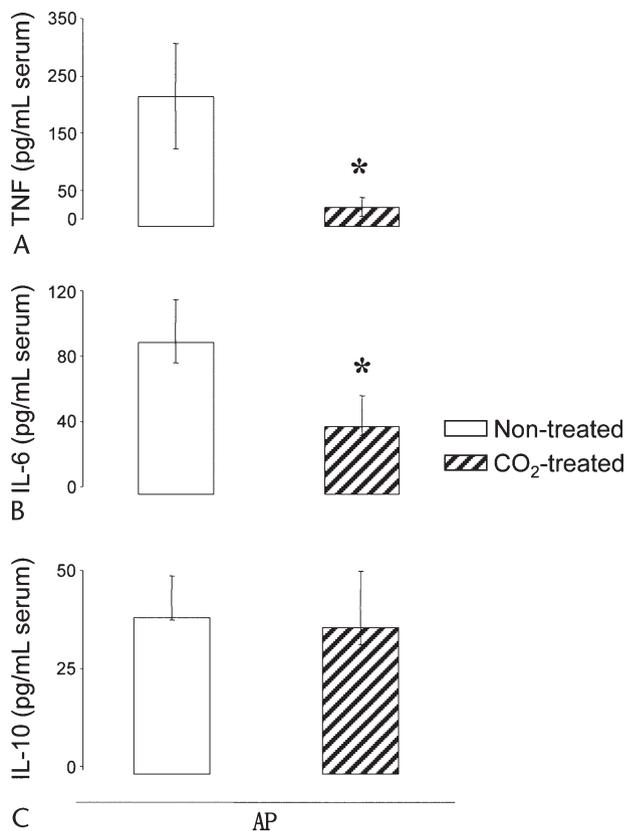
FIGURE 2. Effect of CO_2 on the systemic inflammation induced by AP. Acute pancreatitis was induced in rats by intraductal instillation of 0.5 mL of 5% taurocholic acid. One group received abdominal insufflation with CO_2 30 minutes before induction of AP: serum levels of TNF-α (A), levels of IL-6 (B), and levels of IL-10 (C). Data are expressed as mean ± SEM of 10 animals per group. *$P < 0.05$.

H_2O_2 in the presence of *O*-dianisidine. Results were expressed as optical density at 460 mm.[20,21]

Expression of Inducible NO and Cyclo-Oxygenase

Protein content in the supernatant of pancreas homogenates was determined using the BCA protein assay reagent kit (Pierce), according to the protocol provided by the manufacturer. Samples containing 20 µg of protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and were transferred to nitrocellulose membrane using the Bio-rad Mini-Gel System and Trans-Blot SD-Semidry Transfer Cells. For immunoblotting, the nitrocellulose membranes were incubated in TBS-T buffer (150-mmol/L NaCl, 20-mmol/L Tris, 1% Tween 20, pH 7.4) containing 5% nonfat dried milk for 1 hour. The blot was treated with 1:1000 dilutions of rabbit polyclonal antibodies to COX2 or rabbit antiserum iNOS for 2 hours at room temperature, then washed 3 times with TBS-T, and incubated with 1:2000 dilutions of peroxidase-conjugated monoclonal antirabbit immunoglobulin G for 1 hour at room temperature. Protein bands at 72 kd (COX2) or at 130 kd (iNOS) were identified by comparison with Rainbow Protein Molecular Weight Markers. The immunocomplexed peroxidase-labeled antibodies were visualized by an ECL chemiluminescence kit following the manufacturer's instruction (Amersham) and exposed to photographic film. Finally, blots were stripped with

200-mmol/L glycine, pH 3.0, for 10 minutes, washed with TBS-T 3 times for 30 minutes each, and reprobed with β-actin (1:10,000), followed by antimouse secondary antibody (1:2000). The band densities were determined by densitometric analysis using the AlphaEase FC program. Density values of bands were normalized to the total β-actin present in each lane and were expressed in percentage of control.

## Oxidation and Phosphorylation of Liver Mitochondria

Liver mitochondria were prepared as previously described.[22] Mitochondrial oxygen consumption was measured polarographically[23] using a Gilson 5/6H Oxygraph (Gilson Medical Electronics, Middleton, Wis) in a closed reaction vessel fitted with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio) at 28°C. The respiratory control rate (RCR), considered an index of oxidative and phosphorylative mitochondrial function, was calculated by the rate of oxygen consumption in the presence of adenosine diphosphate (state 3, S3) over the consumption in the absence of adenosine diphosphate (state 4, S4).[24] Respiratory S3 and S4 were measured and reported as nanogram atoms of oxygen per milligram of mitochondrial protein per minute. Mitochondrial protein content was determined by the method of Lowry et al.[25]

Histological Analysis of the Pancreas

Pancreas tissue was sliced in 5-µm sections and stained with hematoxylin/eosin. Histological assessment was performed by a pathologist unaware of the experimental design.
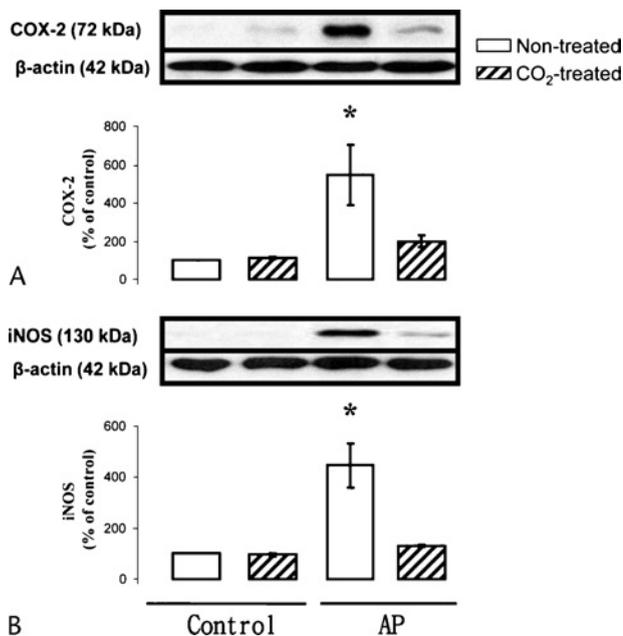
FIGURE 3. Effect of CO_2 on the expression of COX2 and iNOS and in the pancreas. Acute pancreatitis was induced in rats by intraductal instillation of 0.5 mL of 5% taurocholic acid. One group received abdominal insufflation with CO_2 30 minutes before induction of AP. The expression of COX2 and iNOS was assessed by Western blot analysis. Illustration of the Western blot represents 1 of 4 independent experiments. Expression of protein was quantified by the AlphaEase FC software. Values are mean and SEM of 4 animals per group. *$P < 0.001$.
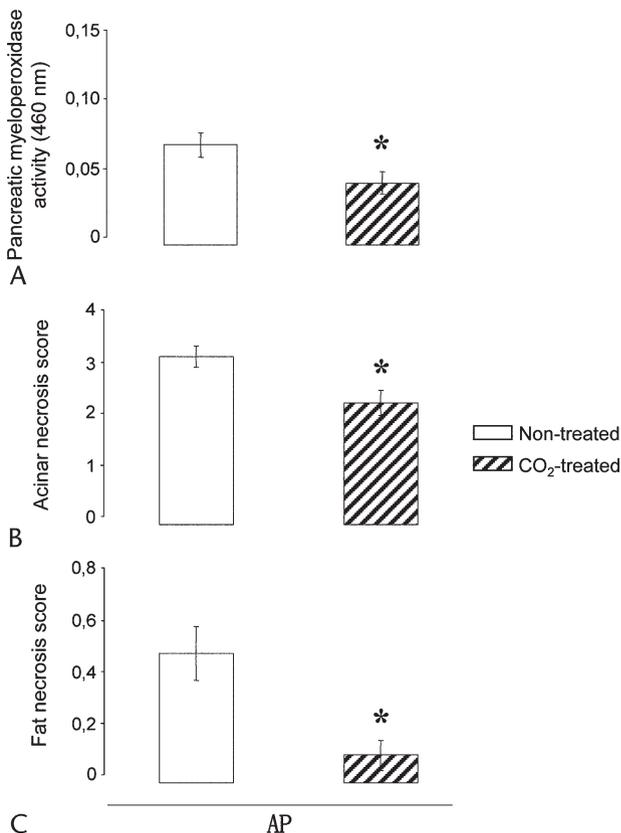
FIGURE 4. Effect of CO_2 on the pancreas. Acute pancreatitis was induced in rats by intraductal instillation of 0.5 mL of 5% taurocholic acid. One group received abdominal insufflation with CO_2 30 minutes before induction of AP: MPO activity measured in homogenates of pancreas samples (A), acinar pancreatic necrosis (B), and fat pancreatic necrosis (C). Data are expressed as mean ± SEM of 10 animals per group. *$P < 0.05$.

The severity of acinar and fat necrosis was analyzed in accordance with Schmidt et al.[26] A scale of 0 to 4 was used according to the following histological scoring criteria:

Acinar necrosis

0 Absent
0.5 Focal occurrence of 1 to 4 necrotic cells/high-power field (HPF)
1 Diffuse occurrence of 1 to 4 necrotic cells/HPF
1.5 Same as 1 + focal occurrence of 5 to 10 necrotic cells/HPF
2 Diffuse occurrence of 5 to 10 necrotic cells/HPF
2.5 Same as 2 + focal occurrence of 11 to 16 necrotic cells/HPF
3 Diffuse occurrence of 11 to 16 necrotic cells/HPF (foci of confluent necrosis)
3.5 Same as 3 + focal occurrence of more than 16 necrotic cells/ HPF
4 More than 16 necrotic cells/HPF (extensive confluent necrosis)

Fat necrosis

0 Absent
0.5 1 focus
1 2 foci
1.5 3 foci
2 4 foci

2.5 5 foci
3 6 foci
3.5 7 foci
4 8 foci or more

Statistical Analysis

Results are reported as mean values with SEM. Continuous variables (amylase, TNF-α, IL-6, IL-10, peritoneal cells, COX2 and iNOS expression, MPO activity, and oxidation and phosphorylation of liver mitochondria) between groups were compared by using unpaired Student t test. Histological analysis was determined using the Mann-Whitney U test.

Survival was evaluated using the Kaplan-Meier method and was analyzed for significance with the log-rank test (Cox-Mantel) and Wilcoxon test.
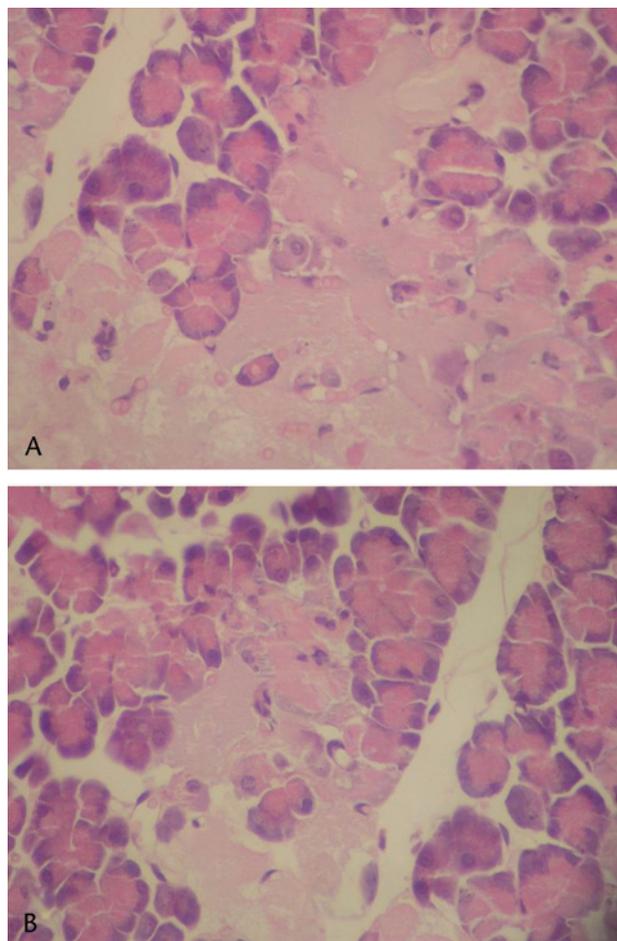
$P < 0.05$ was considered significant.

The GraphPad Prism Software (GraphPad Software, San Diego, Calif) was used for statistical analysis.

RESULTS

CO_2 Pneumoperitoneum Effect on the Peritoneal Inflammation

During AP, there is accumulation of ascitic fluid in the peritoneal cavity when compared with animals of the control

FIGURE 5. Reduction in acinar pancreatic necrosis 2 hours after AP. A, Group without pretreatment. B, Group pretreated with abdominal insufflation with CO_2. Hematoxylin/eosin staining.

nonmanipulated group. A significant decrease in the volume of ascitic fluid was observed in animals pretreated with abdominal insufflation of CO_2 when compared with animals without pretreatment ($P < 0.05$; Fig. 1A).

Figure 1C shows that, after 2 hours of AP induction, the number of cells in the peritoneum was not significantly different from that found in the control group. Carbon dioxide peritoneal insufflation significantly reduced the number of cells in both control and AP groups ($P < 0.05$).

In this model of AP, we found TNF-α in the ascites (Fig. 1B), and pretreatment with CO_2 significantly reduced the total amount of peritoneal TNF-α ($P < 0.05$).

CO_2 Pneumoperitoneum Effect on the Systemic Inflammation

We found TNF-α, IL-6, and IL-10 in the serum after 2 hours of AP induction. Treatment with CO_2 caused a significant decrease on serum levels of TNF-α (Fig. 2A) and IL-6 (Fig. 2B) when compared with the nontreated group ($P < 0.05$). There was no significant difference in serum levels of IL-10 (Fig. 2C).

However, a marked reduction of TNF-α/IL-10 ratio was observed after treatment with CO_2 (6-fold). These results indicate that there is a prevalence of anti-inflammatory activity in the CO_2-treated group compared with the nontreated group.

The elevated serum amylase levels found in the AP group (7.7 ± 0.2 vs. 17.5 ± 0.8 U/mL in control and AP groups, respectively) were not affected by CO_2 treatment.

CO_2 Pneumoperitoneum Effect on Pancreas Inflammation

The iNOS and COX2 enzymes were induced during inflammatory conditions and were used here as markers of inflammation. We found the expression of both enzymes in the pancreas after 2 hours of AP induction. Carbon dioxide treatment suppressed the expression of both enzymes (Fig. 3).

To evaluate neutrophils' infiltration into the pancreas, we measured the MPO activity in tissue samples. We found negligible MPO activity in the control group, whereas in the AP group, it was clearly detected. The group with CO_2 abdominal insufflation pretreatment had a significant reduction in pancreatic MPO activity compared with group without pretreatment (Fig. 4A).
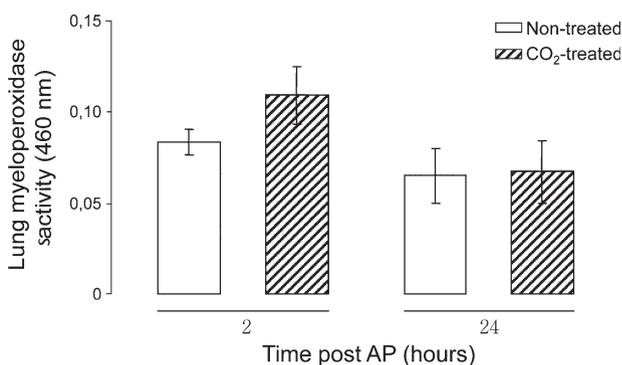
FIGURE 6. Effect of CO_2 on pulmonary MPO activity. Acute pancreatitis was induced in rats by intraductal instillation of 0.5 mL of 5% taurocholic acid. One group received abdominal insufflation with CO_2 30 minutes before induction of AP. The MPO activity measured in homogenates of lung samples 2 and 24 hours after AP induction. Data are expressed as mean ± SEM of 10 animals per group. $P > 0.05$.
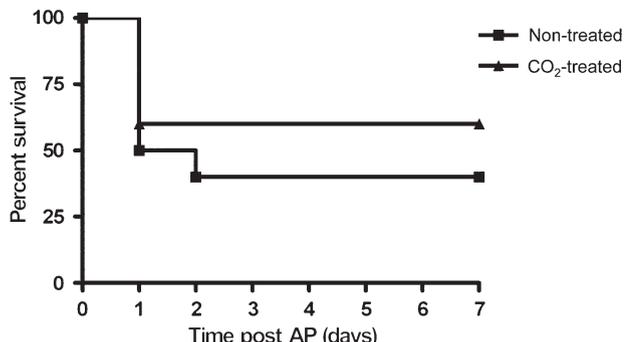
FIGURE 7. Effect of CO_2 on animals' survival. Acute pancreatitis was induced in rats by intraductal instillation of 0.5 mL of 5% taurocholic acid. One group received abdominal insufflation with CO_2 30 minutes before induction of AP. The Kaplan-Meier curve for the 2 groups is shown. Mortality was observed for 7 days after AP induction. $P > 0.05$.

Already at 2 hours of AP induction, we observed a significant reduction in acinar and fat necrosis in the group of animals pretreated with CO_2 abdominal insufflation (Figs. 4B and C). Figure 5 illustrates the reduction in acinar pancreatic necrosis 2 hours after AP in the group pretreated (Fig. 5B) with abdominal insufflation with CO_2 compared with the group without pretreatment (Fig. 5A). Fat necrosis was undetectable in the CO_2-treated group.

CO_2 Pneumoperitoneum Effect on Lung and Liver

After 2 hours of AP induction, we detected MPO activity in lung homogenates indicating that AP induced neutrophils' infiltration into the lung (0.018 ± 0.002 vs 0.084 ± 0.007 for control and AP groups, respectively). Treatment with CO_2 did not affect the lung MPO activity either after 2 hours or after 24 hours (Fig. 6).

Also, we found that AP affected the liver because decreased mitochondrial oxidation and phosphorylation were observed (RCR: 4.02 ± 0.14, S3: 99.41 ± 3.63, S4: 24.73 ± 0.90 nmol/mg protein per minute vs RCR: 2.82 ± 0.12, S3: 104.37 ± 8.58, S4: 37.01 ± 2.43 nmol/mg protein per minute for control and AP groups, respectively). Pretreatment with CO_2 did not significantly affect these parameters.

Mortality Study

Figure 7 shows decrease in mortality in CO_2-treated group (8/20, 40%) compared with the nontreated group (12/20, 60%); however, it did not reach statistical significance.

DISCUSSION

It became clear since the 1990s that AP is associated with the production of inflammatory cytokines that are implicated in the development of systemic inflammatory response.[27,28]

As has been previously shown, macrophages are an important source of IL-1β and TNF-α.[29] These cells are concentrated in the liver, lung, spleen, lymph nodes, and lining of the serosal membrane of the peritoneum and may be stimulated during AP by leakage of activated pancreatic enzymes.[12] It has also been described that depletion of the cellular component from the peritoneal cavity by lavage previous to induction of AP reduces the toxicity of ascitic fluid without changing the severity of the pancreatic lesions. The authors report that more than 90% of the depleted cells were macrophages.[30]

Recently, it has been demonstrated that CO_2 pneumoperitoneum could modify the inflammatory response to abdominal

injury. Indeed, recent reports have shown that CO_2 pneumoperitoneum attenuates the acute-phase response in sepsis produced by LPS injection or by cecal-ligation puncture.[31,32] Carbon dioxide abdominal insufflation pretreatment not only significantly suppressed the plasma levels of the proinflammatory cytokine IL-6 but also increased survival in animals with a LPS-contaminated laparotomy.[15] These effects explain why laparoscopic cholecystectomy is followed by reduced plasma levels of IL-6 when compared with cholecystectomy by laparotomy.[33] The effect of CO_2 peritoneal insufflation is probably more important than the size of incision.[15]

In the present study, CO_2 abdominal insufflation pretreatment decreased the systemic inflammatory response induced by AP (Figs. 1B, C and 2A, B). Serum levels of TNF-α and IL-6 were significantly reduced in the CO_2 pneumoperitoneum group when compared with the control group. However, the serum levels of the anti-inflammatory cytokine, IL-10, were similar between groups (Fig. 2C). When the ratio of TNF-α/IL-10 was calculated, it clearly showed prevalence of anti-inflammatory activity in the CO_2-treated group. This indicates that CO_2 pneumoperitoneum influences the SIRS consequent to AP.

Previous studies demonstrated that CO_2 pneumoperitoneum reduced TNF-α and IL-6 production, whereas it upregulated the anti-inflammatory cytokine IL-10 in animals whose peritoneal cavity was instilled with LPSs during laparotomy.[32] Further studies evaluating the influence of CO_2 pneumoperitoneum pretreatment in LPS-contaminated laparotomy model, however, did not demonstrate any significant changes in IL-10.[15] These findings could be explained by the timing that blood samples were harvested for cytokine determinations because IL-10 generally peaks earlier.[15]

The CO_2 treatment also markedly affected the peritoneal inflammation: it reduced the volume of ascitic fluid and the total peritoneal TNF-α. The number of cells in the peritoneum was similar in the AP and control groups at 2 hours, and the CO_2 insufflation reduced the cell number in both groups. This effect could be attributed to the adhesion of peritoneal cells to the serosal lining.

The inflammatory response in the pancreas was also reduced by CO_2 treatment. Inflammation involves activation of intracellular signaling pathways, which leads to increased expression of specific genes such as those for iNOS and COX2. Thus, these enzymes can be used as markers of inflammation. The results presented show that CO_2 insufflation before induction of AP suppressed the expression of these enzymes (Fig. 3). Neutrophils' infiltration into the pancreas is another feature of AP and was evaluated here by measuring the MPO activity in tissue samples. We found that CO_2 abdominal insufflation pretreatment induced a significant reduction of pancreatic MPO activity (Fig. 4A), suggesting a reduction on the number of infiltrated neutrophils. Moreover, we observed a significant reduction in acinar and fat necrosis in the group of animals pretreated with CO_2 abdominal insufflation (Figs. 4B and C). These results indicate that CO_2 pneumoperitoneum is affecting the inflammatory response in the pancreas after taurocholate instillation. We are unable to say at what level this occurs, but this procedure should necessarily interfere with signal transduction pathways or with gene transcription factors. Another interpretation for these results is that CO_2, by inhibiting the COX2 and iNOS expression, would inhibit the production of 2 proinflammatory molecules, NO and PGE2. This would reduce pancreatic inflammation and, consequently, the subsequent SIRS.

Lung and liver inflammation does not seem to be affected by CO_2 treatment, at least considering the parameters measured.

The lung MPO activity and the liver mitochondrial oxidation and phosphorylation were similar in the treated and nontreated groups.

We also observed a decrease in mortality in the CO_2-treated group (8/20, 40%) compared with the nontreated group (12/20, 60%); however, this difference did not reach statistical significance. Thus, there is a tendency of protection by CO_2 treatment. This effect might become more relevant in conditions of milder pancreatitis or perhaps in pancreatitis induced by different stimuli.

In a previous study, it has been shown that CO_2 abdominal insufflation pretreatment increased survival after an LPS-contaminated laparotomy.[15] In that study, the systemic inflammatory response was secondary to the stimulation mainly of peritoneal macrophages, whereas in AP, systemic inflammatory response is supposed to be due also to stimulation of macrophages from extra peritoneal sites by cytokines, enzymes, and other substances released in the circulation from damaged pancreatic tissue.[34,35] Indeed, we have previously demonstrated that in AP there is an up-regulation of NO production by nonparenchymal liver cells and an inhibition of Kupffer cell microbicidal activity by the platelet-activating factor released during AP.[36]

The effects of CO_2 pneumoperitoneum are durable but reversible,[14] producing a prophylactic effect against subsequent AP. Therefore, if an endoscopy for stone removal from common bile duct needs to be performed, it should better be done during the laparoscopic cholecystectomy to reduce the inflammatory response if an AP follows the procedure.

Whereas CO_2 pneumoperitoneum pretreatment reduces the systemic inflammatory response in AP, CO_2 pneumoperitoneum after the induction of AP did not result in decrease of cytokine release.[37,38]

The main causes of morbidity and mortality after pancreatic surgery are related to AP with consequent pancreatic fistula and systemic inflammation. Our data demonstrated that CO_2 abdominal insufflation attenuates systemic inflammatory response in AP with a reduction of proinflammatory cytokines TNF-α and IL-6 without a significant change in serum IL-10. It also reduces the inflammation both in the pancreas and in the peritoneal cavity. This article suggests that CO_2 pneumoperitoneum plays a critical role on the better outcome in patients undergoing laparoscopic pancreatic surgery.

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