Surfactant protein A is decreased in the lung of rats with hepatopulmonary syndrome

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ABSTRACT

PURPOSE: To evaluate surfactant protein A levels in an hepatopulmonary syndrome rat model. To date, there have been no studies aimed at evaluating surfactant levels in the setting of cirrhosis or hepatopulmonary syndrome.

METHODS: A total of 35 rats were divided into control, sham, and experimental HPS groups. We evaluated surfactant protein A levels in rats and the experimental model designed to induce hepatopulmonary syndrome was common bile duct ligation. Statistical analysis was performed using GraphPad Prism Software. Differences were considered statistically significant when p<0.05.

RESULTS: Lung homogenate surfactant protein A levels were lower in the experimental hepatopulmonary syndrome and sham groups in comparison to the control group (p<0.05). Serum SP-A levels were the same in experimental hepatopulmonary syndrome and control groups but decreased in the sham group compared with the experimental groups (p<0.05). Myeloperoxidase activity was higher in the experimental hepatopulmonary syndrome group than the other two groups (p<0.05).

CONCLUSION: Surfactant protein A is present in experimental hepatopulmonary syndrome and leads to an imbalance between serum and pulmonary levels due to systemic inflammatory response.

Key words: Hepatopulmonary Syndrome. Pulmonary Surfactant-Associated Protein A. Models, Animal. Rats.
Introduction

Hepatopulmonary syndrome (HPS) is formed by the clinical triad of chronic liver disease, intrapulmonary vascular dilatation, and hypoxemia. This condition is present in 4% to 32% of patients with cirrhosis. The pathogenesis of this condition is not well defined, but it is speculated that a combination of factors, such as an imbalance in the response of vascular endothelin receptors, pulmonary microvascular remodeling, and genetic predisposition, leads to intrapulmonary vascular dilatation and bacterial translocation1-4. The pathophysiology of hypoxemia in this syndrome is multifactorial, however, predominant mechanisms include pulmonary shunts, impairment of the ventilation-perfusion ratio, low diffusion, diffusion-perfusion mismatch, or changes in the alveolar-capillary oxygenation gradient5,6.

Hypoxemia in patients with this syndrome initially responds to low-flow supplemental oxygen, however oxygen supplementation requirements usually increase over time. Mild disease improvement has been reported with several treatments, such as sympathomimetics, almitrine bismesylate, estrogen blockers, indomethacin, octreotide, methylene blue, acetyl salicylic acid, and an inhibitor N-nitro-L-arginine methyl ester (L-NAME). Currently, no pharmacological intervention can readily improve arterial oxygenation and alter the course of HPS. Thus, liver transplantation is the only effective therapeutic option for the resolution of HPS1,2,6.

The pathophysiological features of experimental HPS induced by common bile duct ligation (CBDL) includes alterations in the pulmonary microvasculature, including vasodilation, intravascular monocyte accumulation, and angiogenesis2-4,7. Some authors have shown that an increase in the expression of inducible nitric oxide synthase (iNOS) in the lungs of CBDL animals can also contribute to local nitric oxide production during the progression of HPS2-4,8.

Pulmonary surfactant, which is secreted by alveolar type II pneumocytes, reduces surface tension at the air/liquid interface of the alveoli and stabilizes alveoli at low lung volumes9-11. Endogenous surfactant is a mixture of phospholipids (90%) and specific proteins (10%) such as SP-A (surfactant protein A), SP-B, SP-C and SP-D. SP-A, however, it is the most abundant protein and represents approximately about 5–6% of the dry weight12,13. The function of pulmonary surfactant is to increase lung compliance, reduce surface tension, and prevent atelectasis and lung collapse14. The absence or reduction of surfactant is associated with neonatal respiratory distress syndrome and congenital surfactant deficiency15,16. Conversely, there is an increase in pulmonary alveolar proteinosis, which is related to significant respiratory dysfunction. Exogenous surfactant preparations were initially developed to treat neonatal respiratory distress syndrome. Surfactant supplementation with SP-A and SP-D has been used as a therapy for lung disease in infancy and the inflammatory and immune protection benefits of this treatment have been proven15-17.

The pathophysiology of HPS is not completely understood and we hypothesized that arterial blood gas changes in the setting of systemic inflammation and pulmonary disease (HPS) may alter surfactant production. The aim of this study was to evaluate the presence or absence of SP-A using a HPS rat model.

Methods

This project was approved by the Ethics Committee for the Guidelines of Animal Experimentation of the University of Sao Paulo, School of Medicine. The study was designed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Council for international Organization of Medical Sciences (CIOMS).

Thirty five male Wistar rats (200–250g, LIM 37/USP, Sao Paulo, Brazil) were housed at 19°C ± 3°C with a 12-h (06:00–18:00 hours) artificial light cycle. Two or three animals from the same treatment group were housed per cage. The animals had free access to tap water and standard food during the entire experiment. Food intake was not measured.

Study design

The animals underwent a common bile duct ligation (CBDL group n=16) as previously described18,19. The sham group (n=8) underwent laparotomy and mobilization of the common bile duct. The control group (n=5) only underwent the experimental analysis for data collection. All procedures began with the intraperitoneal anesthesia using 5% ketamine hydrochloride 30 mg/kg (Ketalar®, Cristália). Animals were kept warm with a 45W, 127V halogen bulb and body temperature was monitored using a rectal digital thermometer (YSI 4000A Precision Thermometer, USA) and maintained between 35°C and 37°C.

Arterial and venous blood analysis

After 28 days, animals were anesthetized and underwent a laparotomy. We collected blood samples using abdominal aorta (arterial) and superior vena cava (venous) punctures. Then, the animals were sacrificed.
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Arterial blood gas analysis (oxygen saturation and PaO₂) was performed with an ABL 800 radiometer (Radiometer Copenhagen, Denmark) and results of oxygen saturation are expressed in % and PaO₂ in mmHg. The following laboratory (venous) biochemical parameters were measured: aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TB), direct bilirubin (DB), gamma glutamyl transferase (GGT), and alkaline phosphatase (AP). The quantification of AST, ALT, BT, GGT, and AP was performed using an ultraviolet optimized method (Cobas Mira, Roche) according to the International Federation of Clinical Chemistry. Results are expressed in units per liter (U/L).

### Inclusion criteria

For the evaluation of surfactant protein A, we adopted the following inclusion criteria: an increase in GGT (>30 U/L), TB (>5.0 U/L), and DB (>4.0 U/L), changes in arterial blood gas by true measurement of PO₂ <80 mmHg and decreased O₂ saturation <90%.

### Evaluation of surfactant protein A (SP-A) in rats

Initially, for establishing and detecting SP-A in rats, we purified lung porcine SP-A and made a standard curve to identify the best dilution of polyclonal rabbit antisera against porcine-SP-A to detect SP-A at an optical density (OD) above 0.5. Different concentrations of SP-A versus dilutions of the polyclonal rabbit antisera against porcine-SP-A (primary antibody) and dilution fixed anti-rabbit* IgG conjugated with peroxidase (Sigma® Chemical Co.) (secondary antibody). (*Product No. A-0545 Lot 074H4829 Anti-Rabbit IgG (whole molecule) Peroxidase Conjugate Antibody Developed in Goat Affinity Isolated Antigen Specific Antibody Adsorbed with Human IgG)²⁰.

We ultimately chose a dilution 1:1600 of polyclonal rabbit antisera against porcine-SP-A to detect μg/50μL 0.0625 or 1.25 mg/mL. Then we investigated SP-A in control rat serum and lung homogenates using polyclonal rabbit antisera against porcine-SP-A (indirect ELISA). The dilution selected was 1:5. Finally, the determination of SP-A in rat serum and lung homogenates of control, sham, and cirrhosis (CBDL) groups is shown in Table 1. The SP-A concentration is expressed in μg/ml¹⁰,¹².

### Myeloperoxidase activity

Lung myeloperoxidase (MPO) activity was used as an indicator of neutrophils in the lung²¹. Samples were homogenized in PBS containing 0.5% hexadecyl and 5 mM EDTA, pH 6.0, sonicated and then centrifuged at 3000xg for 30 min. The supernatant was measured spectrophotometrically for MPO activity based on optical density (460 nm) changes due to the decomposition of H₂O₂ in the presence of o-dianisidine. Results are expressed as OD at 460 nm.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism Software®. Differences were considered significant with the p-value was <0.05. Data are presented as the mean ± standard deviation (SD) for continuous variables. Comparisons between groups were made using one way analysis of variance Kruskal-Wallis test (nonparametric ANOVA) and the post-hoc Dunn-Bonferroni test was used to perform multiple comparisons.

### Results

To establish HPS, 20 rats were used in the CBDL group (three were excluded because HPS was not established and one died during anesthesia). For the sham group, 10 rats were used (one died during anesthesia and one died during the second post-operative period). The control group consisted of five rats. Macroscopic findings 28 days after surgical bile duct ligation showed evidence of cirrhosis in all experimental rats (CBDL group) including cirrhotic aspects of the liver, splenomegaly and impairment of lung tissue.

In the laboratory analysis, we observed a significant elevation of liver enzymes (AST and ALT), total and direct bilirubin, and GGT in the CBDL group in comparison with the control and sham groups. Only the AP value showed no significant differences as shown in Table 2. Assessment of the arterial blood gases in the CBDL group showed lower levels of PO₂ and O₂ saturation than other groups (Table 2).

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**Table 1** – SP-A serum and lung homogenate of control, sham and rats with experimental HPS (CBDL).

<table>
<thead>
<tr>
<th>Serum</th>
<th>Lung Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>sham</td>
</tr>
<tr>
<td>OD</td>
<td>0.136</td>
</tr>
<tr>
<td>Ug/ml</td>
<td>1.380</td>
</tr>
<tr>
<td>%</td>
<td>14</td>
</tr>
</tbody>
</table>

Note: surfactant protein A (SP-A); optical density (OD); Hepatopulmonary syndrome (HPS); common bile duct ligation (CBDL).
Levels of surfactant protein A (SP-A) were significantly decreased in the lung homogenate in sham and CBDL groups compared with the control group (Figure 1A). The serum SP-A levels in the sham group were significantly lower than the control group, however, levels measured in the CBDL group did not differ from the control group (Figure 1B). Myeloperoxidase (MPO) activity was significantly higher in the CBDL group than the other groups, however control and sham groups showed no significant differences (Figure 1C).

Macroscopic features of lungs and livers from the CBDL group showed evidence of damage, which was not present in the control group. A histological analysis was conducted in only a few samples and the results were not analyzed statistically. In the CBDL group, lung tissue showed alveolar edema (Figure 2A) and the liver showed fibrosis and inflammatory cells (Figure 2B).
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**Discussion**

This is the first study to evaluate surfactant protein A (SP-A) levels in hepatopulmonary syndrome in a rat model. Interestingly, it was observed that lung surfactant was consumed in not only experimental HPS but also in a systemic inflammatory process (surgical trauma). Moreover, an imbalance between the level of surfactant protein A in lung homogenate and serum was also observed in the setting of experimental HPS.

As demonstrated by some authors previously, the induction of biliary cirrhosis by common bile duct ligation (CBDL) in rats is an established experimental model for HPS. Our study is in accordance with the literature and HPS was established in this study in more than 85% of rats using a previously described model. SP-A and SP-D are members of a family of innate immune molecules called collectins. The most well defined function of the collectins is the ability to opsonize pathogens, including bacteria and viruses, regulate a variety of immune cell functions, and facilitate phagocytosis by innate immune cells, such as macrophages and monocytes. SP-A and SP-D have direct bactericidal activities in addition to their well-described opsonic activity and ability to regulate inflammatory mediator production.

Myeloperoxidase (MPO) levels were significantly increased in the CBDL group as compared with other groups. MPO activity in cirrhosis models with hepatopulmonary syndrome (HPS) was explored by other authors and it has been proposed that MPO may be involved in the regulation of iNOS expression. Additionally, previous studies have shown that SP-A stimulates the chemotactic activity of alveolar neutrophils and myeloperoxidase activity is increased in hepatopulmonary syndrome in rats.

The presence of SP-A in serum was reported in pulmonary fibrosis. In fact, the present study found surfactant protein A (SP-A) in the blood in all groups of animals. It was found that surgical trauma (sham group) resulted in a decrease in serum and pulmonary (lung homogenate) SP-A, which did not normalize after 28 days. In contrast, in the experimental HPS group (CBDL group), pulmonary SP-A was also consumed but an imbalance was observed between serum and pulmonary levels.

The alveolar cell in the lung produces surfactant (SP-A) and HPS starts with pulmonary inflammation that progresses to vascular shunts or microvascular lung changes with decrease in arterial blood-gas with PO$_2$<80 mmHg and decreased O$_2$ saturation<90%. We speculate that this alteration may explain the consumed pulmonary SP-A in experimental HPS (CBDL group), while serum level remains higher. As cited by Zhang and Fallon, the main features in experimental HPS are pulmonary microvascular dilation and angiogenesis that lead to abnormal pulmonary gas exchange. In acute pancreatitis, the expression of SP-A in rat lung was reported to be reduced. Based on the findings of this study, the hypothesis that SP-A may play a role in systemic inflammation is strengthened.

The above explanation may be simple, but the experimental HPS group also underwent laparotomy and the serum SP-A was not consumed. It was previously demonstrated that SP-A increases in the blood circulation of patients with acute lung injury/acute respiratory distress syndrome. Lung SP-A levels first underwent alteration most likely due to systemic inflammation and surgical trauma (Sham group). However, the later development of pulmonary inflammation or microvascular changes would impair the balance between the body compartments, and as expected, SP-A remained higher in the blood.

**Conclusion**

Surfactant protein A is present in experimental Hepatopulmonary Syndrome and leads to an imbalance between serum and pulmonary levels due to a systemic inflammatory response.

**References**


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