

## Attenuation of lung injury using simvastatin in a rat sepsis model

Redução de lesões pulmonares usando sinvastatina em modelo de sepse em ratos

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### ABSTRACT

**Purpose:** Considering that statins have pleiotrophic effects, we hypothesized that simvastatin therapy could help in the setting of sepsis and lung injury. The aim of this study was to address the effect of simvastatin pretreatment on lung injury in rats with abdominal sepsis. **Methods:** Thirty male Wistar rats weighing  $235\pm 26$ g were used and distributed into the three groups: group 1, n=10 (sham), treated with oral injection of saline (10mL/Kg) 24 hs before and again immediately before surgery; group 2, n=10 (abdominal sepsis+saline), cecal ligation and puncture (CLP) and treatment with saline as group 1; group 3, n=10 (abdominal sepsis+simvastatin), CLP and treatment with oral injection (gavage) of 10mg/Kg of simvastatin suspension (10mg/ml) 24 hs before and again immediately before surgery. Commercial ELISA kits were used for measurement of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1) and interleukin-6 (IL-6). Lung tissue from all animals was studied with light microscopy to determine the distribution and amount of lung injury. **Results:** TNF- $\alpha$  plasma expression was significantly lower in rats treated with simvastatin ( $172.8\pm 25$  pg/mL) than in rats treated with 0.9% saline ( $298.5\pm 63$  pg/ml). IL-1 $\beta$  plasma levels showed a drastic decrease ( $53.3\pm 7$  pg/mL) in simvastatin treated rats, compared with the sepsis/saline group rats ( $127.6\pm 28$  pg/mL). The plasma levels of IL-6 in the sepsis/simvastatin treated rats ( $53.3\pm 7$  pg/mL) were lower than in sepsis/saline treated rats ( $134.6\pm 15$  mL). In control rats the plasma cytokines were significantly less expressive ( $28.4\pm 6$ ) than in the other groups. Representative lung histology demonstrated marked inflammation characterized by abundant interstitial neutrophils and edema in group sepsis/saline. Induced inflammation was greatly reduced by simvastatin pretreatment. **Conclusion:** In conclusion, our data suggest that simvastatin protects the lung against tissue injury in abdominal sepsis via inhibition of cytokines expression.

**Key words:** Lung injury. Simvastatin. Rat. Abdomen. Sepsis.

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## RESUMO

**Objetivo:** Considerando que as estatinas têm efeitos pleiotróficos, elaboramos a hipótese de que a terapia com sinvastatina pode atenuar a lesão pulmonar induzida pela sepse. O objetivo deste estudo foi abordar o efeito do pré-tratamento com sinvastatina sobre a lesão pulmonar provocada por sepse abdominal em ratos. **Métodos:** Trinta ratos Wistar foram utilizados e distribuídos em três grupos: grupo *sham* (n=10) tratados com injeção oral de salina (10ml/kg) 24 hs antes e imediatamente antes da cirurgia; no grupo sepse abdominal + solução salina (n=10) foi feita a ligadura e punção cecal (LPC) mais tratamento com solução salina como no grupo *sham*; no grupo sepse abdominal + sinvastatina (n=10) foi feita LPC, e tratamento com injeção por via oral (gavagem) de 10mg/kg de sinvastatina suspensão 24 hs antes e uma segunda dose imediatamente antes da cirurgia. Após 24 hs de observação, plasma foi separado de sangue colhido por punção cardíaca. Dosagem de TNF $\alpha$ , IL-1 $\beta$  e IL-6 pelo ELISA. Tecido pulmonar de todos os animais foi usado para estudo com microscopia óptica para quantificar lesões pulmonares. **Resultados:** A expressão de TNF- $\alpha$  foi significativamente menor nos ratos tratados com sinvastatina (172,8  $\pm$  25 pg / mL) do que nos ratos tratados com solução salina (298,5  $\pm$  63 pg / ml). IL-1 $\beta$  mostrou-se significativamente menor (53,3  $\pm$  7 pg / ml) em ratos tratados com sinvastatina, em comparação com o grupo sepse/salina (127,6  $\pm$  28 pg / mL). IL-6 nos ratos sepse/sinvastatina (53,3  $\pm$  7 pg / mL) foram significativamente (p<0,05) menores do que no grupo sepse/salina (134,6  $\pm$  15 ml). No grupo *sham* a expressão de citocinas foi significativamente menor (28,4 $\pm$ 6) do que nos outros grupos. A histopatologia pulmonar demonstrou marcada inflamação caracterizada por abundantes neutrófilos e edema intersticial nos animais do grupo sepse/salina. A inflamação foi significativamente reduzida após o pré-tratamento com sinvastatina. **Conclusão:** Em conclusão, nossos dados sugerem que a sinvastatina protege o pulmão contra a lesão tecidual na sepse abdominal através da inibição da expressão de citocinas.

**Descritores:** Lesão pulmonar. Sinvastatina. Rato. Abdome. Sepse.

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## INTRODUCTION

Statins, such as simvastatin, are potent inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (3HMG-CoA-reductase). These drugs reduce the serum levels of cholesterol and are successfully used to treat hypercholesterolemia and atherosclerosis<sup>1</sup>. Moreover, their effects in reducing the mortality and morbidity of cardiovascular diseases have been ascribed not only to their cholesterol-lowering actions but also to improve endothelial cell function, enhance fibrinolysis, and anti-inflammatory effects<sup>2-4</sup>. Statins have been shown to inhibit important immunomodulatory effects independent of lipid lowering<sup>5</sup>. Some publications have shown the inhibitory effects of statins on cytokine production<sup>4,6,7</sup>, decrease in inflammation-associated C-reactive protein<sup>8</sup>, but little is known about the effect of statins on lung injury in sepsis. If endotoxin and sepsis play an important role in the pathophysiology of the respiratory insufficiency<sup>9</sup>, and because statins improve prognosis in patients with severe sepsis admitted on intensive care units<sup>10</sup>, then it may be hypothesized that statin therapy could help in the setting of

sepsis and lung injury as well. The aim of this study was to address the effect of simvastatin treatment on lung injury in rats with abdominal sepsis.

## **METHODS**

The experimental protocol was approved by the Institutional Research Ethics Committee. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, Brazil (Law 11794), 2009.

### **Animals**

Thirty male Wistar rats weighing  $235\pm 26$ g were used. Rats were housed in polypropylene cages and maintained under controlled temperature conditions on a 12h light-dark cycle and allowed *ad libitum* access to commercially available rat chow (Labina, Purina®) and water.

### **Experimental design**

A total of 30 Wistar rats were randomly distributed into the following three groups: group 1, n=10 (sham), treated with oral injection of saline (10mL/Kg) 24 hs before and again immediately before surgery; group 2, n=10 (abdominal sepsis+saline), cecal ligation and puncture (CLP) and treatment with saline as group 1; group 3, n=10 (abdominal sepsis+simvastatin), CLP and treatment with oral injection (gavage) of 10mg/Kg of simvastatin suspension (10mg/ml) 24 hs before and again immediately before surgery.

### **Surgical models**

Animals were fasted 12 hr before the experiment and anesthetized with intramuscular injection of 0.1 mL/100g weight, of a solution prepared with 1.0 mL of ketamine (50mg/mL) and 1.0 mL of xilazine (20mg/mL). They breathed spontaneously throughout the procedures. After shaving, the abdominal skin was disinfected with 70% alcohol. All procedures were performed under sterile conditions. Midline laparotomy (3 cm) and gentle manipulation of cecum was performed. The abdomen was closed with 4-0 nylon sutures.

After anesthesia, the cecum was exposed, ligated with silk 2-0, one cm distally to the ileocecal valve to avoid intestinal obstruction, four punctures were performed with a 22-gauge needle, squeezed gently to force out a small amount of feces, and then it was returned to the abdominal cavity. The abdomen was closed with 4-0 nylon sutures. All animals were weighed again on the first postoperative day, when relaparotomy was performed under sterile conditions with ketamine anesthesia intramuscular (50 mg/kg).

Samples of peritoneal fluid were taken for aerobic cultures. Thorax was opened, blood was collected by cardiac puncture for cytokine assay and the lungs were resected for histological exam.

### **Cytokine assays**

Blood samples were collected from cardiac puncture. Serum was separated by centrifugation and was quantified by ELISA. Commercial ELISA kits (all from PeproTech, Rocky Hill, NJ, USA), were used for measurement of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1) and interleukin-6 (IL-6), according to the manufacturer's recommended protocols. All serum samples for each rat were stored at  $-40^{\circ}\text{C}$  and were measured at the same time by the same kit to avoid variation of assay conditions.

### **Histopathological study**

Lung tissue from all animals was studied with light microscopy to determine the distribution and amount of lung injury. At the end of the exposures, the left and the right lung were inflation-fixed via the endotracheal tube for 30 min at a fixative pressure of 30 cm formalin 10%. For histologic analysis, defined sections were fixed with 10% buffered formalin (pH 6.9) and embedded in paraffin. The lungs were sliced (5 $\mu\text{m}$ ) and stained with hematoxylin and eosin. These slices were evaluated for the presence of injury by a pathologist who was blinded to the nature of treatment. The damage of specimens was assessed in a blinded manner by an experienced pathologist according to the following microscopic criteria: measurements were made for epithelial volume density, including alveolar cells; endothelial volume density; inflammatory-cell volume densities in intravascular, interstitial, and alveolar compartments; and interstitial volume density. The interstitial space was analyzed with cell counts.

### **Statistical analysis**

Data are reported as mean $\pm$  SEM. Statistical analyses were conducted with the software BioEstat 6.0. Values of  $p < 0.05$  were considered significant. Data from animals subjected to bacterial challenge were compared using ANOVA with Tukey's *post hoc* test.

## RESULTS

### Cytokines analysis

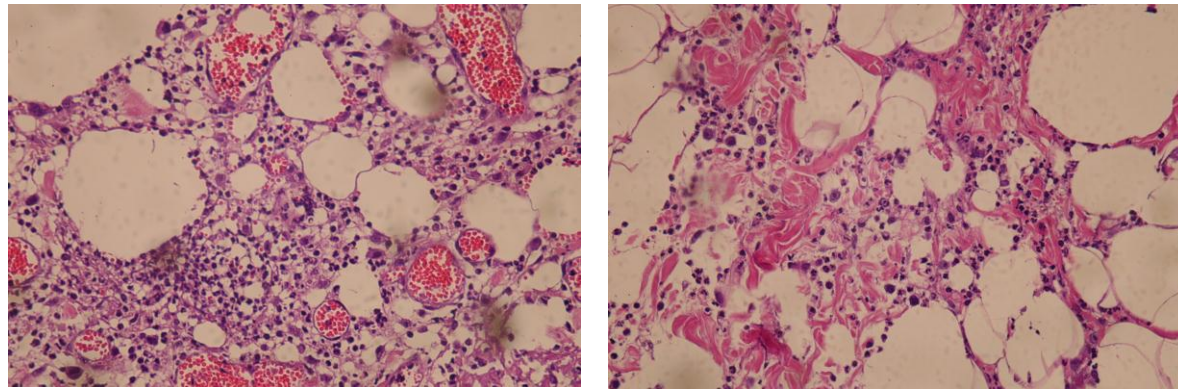
The cytokines assays were detected in the plasma of saline and simvastatin-treated rats, as well as in sham. TNF- $\alpha$  plasma expression was significantly lower in rats treated with simvastatin ( $172.8 \pm 25$  pg/mL) than in rats treated with 0.9% saline ( $298.5 \pm 63$  pg/ml). IL-1 $\beta$  plasma levels showed a drastic decrease ( $53.3 \pm 7$  pg/mL) in simvastatin treated rats, compared with the sepsis/saline group rats ( $127.6 \pm 28$  pg/mL). The plasma levels of IL-6 in the sepsis/simvastatin treated rats ( $53.3 \pm 7$  pg/mL) were lower than in sepsis/saline treated rats ( $134.6 \pm 15$  mL). In control rats the plasma cytokines were significantly less expressive ( $28.4 \pm 6$ ) than in the other groups. These data are summarized in Table 1.

**TABLE 1** – Plasma cytokine values from rats treated with simvastatin, saline 0.9% and sham.

Cytokines \ Groups	Sham	Sepsis/saline	Sepsis/simvastatin
TNF- $\alpha$ (pg/mL)	$32.8 \pm 6$	$298.5 \pm 63$	$172.8 \pm 25^*$
IL-1 $\beta$ (pg/mL)	$23.7 \pm 5$	$127.6 \pm 28$	$53.3 \pm 7^*$
IL-6 (pg/mL)	$28.4 \pm 6$	$134.6 \pm 15$	$54.6 \pm 12^*$

\*  $p < 0.05$  comparing the plasma cytokines from sepsis/saline treated rats and sham, by using the Tukey test.

Light microscopic analysis of lung tissue from animals with *E. coli* sepsis showed thickened alveolar septum with increased cellularity, primarily due to increased numbers of neutrophils in the intravascular compartment. Some intra-alveolar edema was present. In contrast, animals that were primed with heat-killed bacteria prior to live *E. coli* sepsis developed thickened alveolar septum with both increased neutrophils and a prominent increase in mononuclear and interstitial cells. Alveolar edema was also present in this group, and many inflammatory cells were present in the alveolar spaces. Open-lung biopsy, done in one animal at 24 h after treatment with simvastatin, showed minor changes in lung architecture by light microscopy. There was no increase in neutrophils in alveolar septum, and no cells or edema in the alveolar spaces (Figure 1).



**Figure 1** – Effect of simvastatin on CLP- induced inflammation. Representative lung histology demonstrates marked inflammation characterized by abundant interstitial neutrophils and edema in group sepsis/saline. (Left). Induced inflammation was greatly reduced by simvastatin pretreatment (Right). HE, 200x.

## DISCUSSION

The mortality rate of septic patients has remained high (30–70%) despite substantial investigative efforts<sup>11</sup>. Treatment of sepsis is largely limited to supportive care, except recombinant-activated protein C<sup>12</sup>. Nonetheless, recent studies have reported that simvastatin intake reduces hospital mortality<sup>13</sup> and decreases progression of bacterial infections<sup>10</sup> in septic patients. Moreover, animal experiments have shown that statin treatment improves survival in sepsis by improving cardiovascular function<sup>14,15</sup>. Clinical use of statins is mainly restricted to control cholesterol synthesis in patients with increased risk of cardiovascular complications via inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase. Beside their well-known, cholesterol-lowering effect, important studies of literature indicates that statins also exert so-called pleiotropic properties, such as inhibition of cytokine formation, adhesion molecule expression, and NO production<sup>16,17</sup>. However, the potential anti-inflammatory effects and mechanisms of statins in sepsis remain elusive.

Abdominal sepsis and septic shock is one of the most common, life-threatening medical conditions and is frequently complicated by organ failures, especially acute lung injury. Development of acute lung injury is associated with short and long term morbidity, mortality, prolonged hospitalization, and high costs<sup>18</sup>.

While septic shock has long been recognized as a trigger of acute respiratory distress syndrome<sup>19,20</sup>, the treatment and prophylaxis of acute lung injury in patients with septic shock has been limited.

Our previous study revealed a potent effect of simvastatin on inflammation of diabetic rats with abdominal sepsis model<sup>6</sup>. These *in vivo* findings led us to investigate the potential role of this drug in attenuating inflammation in lungs of septic rats by using the same model. We now report that simvastatin significantly decreased CLP-induced murine lung inflammation. We chose to administer simvastatin before CLP to establish a

proof of principle. In fact, the combination of simvastatin pretreatment (24 h) and before surgery treatment resulted in significant lung protection against inflammation. Although a strict extrapolation of our model to the clinical setting may not be realistic given the difficulty of accurately predicting the development of sepsis to allow for early simvastatin treatment, our findings have significant clinical relevance given the natural course of this disease. Specifically, early intervention to attenuate lung injury could have a marked impact on outcome, even if initiated after the onset of injury, since a prolonged clinical course is not uncommon in abdominal sepsis, and patients who fare poorly often progress to acute respiratory distress syndrome over the course of several days, mainly in intensive care units<sup>21</sup>.

In contrast to our results, negative effects of lovastatin was showed on pulmonary antibacterial host defense after intratracheal instillation of *Klebsiella pneumoniae*. These divergent results may have been caused by using different models of bacterial infection. Whereas we induced sepsis and lung infection by CLP, Fessler et al<sup>22</sup>. used a direct intratracheal instillation of *K. pneumoniae*. Moreover, they used lovastatin, a different statin inhibitor of HMG-CoA reductase that may have also displayed effects with different intensity.

These results may have clinical implications, but this have to be considered cautiously. However, the majority of the clinical studies revealed increasing evidence that statins have a beneficial effect on the outcome of infection in humans without disturbed host defense<sup>23</sup>.

In conclusion, our data suggest that simvastatin protects the lung against tissue injury in abdominal sepsis via inhibition of cytokines expression.

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