The Effect of Simvastatin on Pulmonary Damage in Experimental Peritonitis in Rats

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Abstract Statins are widely used in the treatment of hyperlipidemia, as they inhibit cholesterol synthesis. They also have anti-inflammatory, antioxidant, immunomodulatory, and positive endothelial–functional effects. It is hypothesized that simvastatin ameliorates pulmonary damage secondary to peritonitis in rats. Forty Wistar albino rats were divided into four

groups. In sham group, laparotomy was the standard procedure. In simvastatin group, simvastatin was given perorally before laparotomy. In sepsis group, peritoneal sepsis was constituted by cecal ligation and puncture technique. In sepsis+simvastatin group, the procedures of simvastatin and sepsis groups were applied together. After sacrifice at the 72nd hour, tissue samples from lungs were harvested for histopathological examination, wet and dry weight measurements, and tissue culture, tissue malondialdehyde, and nitric oxide tests. Blood samples were taken for C-reactive protein and whole blood count. While the malondialdehyde levels were found to be significantly higher in sepsis group, nitric oxide levels were found to be significantly lower in simvastatin+sepsis group. Alveolar hemorrhage was highest in simvastatin+sepsis group. There was no difference for C-reactive protein, leukocyte levels, and histopathological examination between any groups. The ratios of wet and dry lung weights were higher in simvastatin-given groups. Simvastatin has no positive effect in terms of lung dysfunction on experimental sepsis model. For a better understanding of the effects of simvastatin on lung injury in peritoneal sepsis, experimental models of longer duration that enable to search the effects of simvastatin beyond 3 days will be more useful.

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Introduction

Sepsis can be defined as a set of responses generated by the body against the toxins and microorganisms [1]. Today, although the pathophysiology of sepsis is not fully revealed, according to the most commonly accepted opinion, it is defined as a systemic inflammatory response syndrome occurring in the presence of uncontrolled infection or inflammatory response.

One of the reasons of sepsis is peritonitis, and in these cases, the most important step is successful management of infection resource [2, 3]. As a result of sepsis cascade, several-organ dysfunction can be seen, and among these, lungs are the most affected organs. Lungs play important roles in sepsis in two aspects. First, it could be the source of infection, and the second, it is the first affected remote organ in the other causes of sepsis. Acute lung injury was detected at the rate of 40–60 % in gram-negative sepsis [4, 5]. Endotoxins play a key role in the existence of gram-negative bacterial infection and inflammatory process [6, 7].

Statins are widely used in the treatment of hyperlipidemia as they inhibit cholesterol synthesis. Apart from their lipid-reducing effect, they also have several positive influences known as pleiotropic effect. Statins inhibit thrombocyte aggregation and thrombus synthesis and reduce vascular inflammation. They also have anti-inflammatory, antioxidant, immunomodulatory, and positive endothelial–functional effects [8].

Lungs play an important role in sepsis as a source. Besides, they are the first affected remote organ in the other causes of sepsis especially in gram-negative bacteria [5, 9]. Alveolocapillary membrane injury occurs. Type I cellular damage causes increases in alveolar permeability, fibrin, and plasma leakage. Type II cellular damage reduces surfactant synthesis and pulmonary compliance [10–13]. With their anti-inflammatory, antioxidative, and immunomodulatory effects, statins may play a positive role in the management of lung injury secondary to sepsis [14].

In the present study, the effect of simvastatin on lung tissue injury due to sepsis was investigated in rats with experimental peritoneal sepsis model.

Methods

The study was conducted in Kırıkkale University, Faculty of Veterinary Medicine after the ethical committee's permission.

Forty Wistar albino rats weighing 180–200 g were randomized into four groups ($n=10$).

Group A (sham): Laparotomy was the standard procedure. Group B (sepsis): Peritoneal sepsis was constituted by cecal ligation and puncture (CLP), following laparotomy. Group C (simvastatin): 10 mg/kg microemulsion form of simvastatin was given perorally 18 and 2 h before laparotomy.

Group D (simvastatin–sepsis): 10 mg/kg microemulsion form of simvastatin was given perorally 18 and 2 h before laparotomy. Peritoneal sepsis was also constituted.

Preparation and Administration of Simvastatin Microemulsion

A 40-mg tablet of simvastatin (Zocor® Fort Tablet, Merck & Co., Inc., Whitehouse Station, NJ, USA) was dissolved in 20 ml of 0.9 % NaCl. Simvastatin microemulsion dose of 10 mg/kg for every rat weighing 200 g was calculated as 1 ml. The microemulsion was given 18 and 2 h prior to surgery orogastrically. A dose of 1 ml of 0.9 % NaCl was administered afterwards.

Surgical Procedure

All rats received intramuscular injection of 50 mg/kg ketamine (Ketalar®, Pfizer Pharma GmbH, Germany) and 20 mg/kg xylazine (Alfazyne® 2 %, Alfasan International, Woerden, the Netherlands) as standard anesthetic procedure, with spontaneous respiration. After fixation to operation table, abdomen was shaved, and povidone iodine was used to achieve asepsis. Intra-abdominal cultures were collected following midline laparotomy.

For conducting sepsis, cecum was ligated 1 cm distally to the ileocecal valve with 2-0 silk suture. Two punctures were made with a 21-gauge needle to the antimesenteric side of the cecum. Rats in all the groups received 100 ml/kg physiologic saline intraperitoneally for fluid resuscitation, before closure of abdomen. The wound was closed with 4-0 polypropylene suture. Rats were sacrificed with a high-dose intramuscular ketamine anesthesia 72 h postoperatively. Intra-abdominal cultures were obtained. Blood samples of 1 ml were taken for C-reactive protein (CRP) analysis and leukocyte count from the right ventricle after sternotomy. The upper lobe of the right lung was resected for nitric oxide (NO) and malondialdehyde (MDA) analyses and stored at -80°C . Middle and inferior lobes of the right lung were preserved in 10 % formalin solution for histopathological examination. The examiners in the pathology and infectious disease laboratory were blinded according to the study protocol.

Leukocyte Count

Blood samples were preserved in ethylenediaminetetraacetic acid-containing tubes. Complete blood count analysis was made using Coulter Hematology Analyzer (Coulter Beckman HMX, Miami, USA). The staff in the testing laboratory was blinded to the study.

CRP Analysis

Blood samples (1 ml) were transferred to the laboratory. After separating the serum, they were preserved at -80°C . C-reactive protein analysis was made with turbidimetric method at the Olympus AU600 Chemistry Immuno Analyzer.

Wet/Dry Lung Weight

After sacrifice, left lungs were weighed with a precision balance (Precisa Moisture Balance 310M HA300, Precisa Gravimetrics AG, Dietikon, Switzerland), and the data were collected as wet lung weight. After weighing, wet lungs were preserved in the dry air sterilizer (Nuve, FN400, Ankara, Turkey) at 90 °C for 24 h and weighed again for collecting the data of dry lung weight.

Malondialdehyde Level

To evaluate the tissue-related level of lipid peroxidation, MDA levels of lung tissues were analyzed. The measurement which was defined by Mihara and Uchiyama [15] depends on spectrophotometric analysis of thiobarbituric acid (TBA) forming color complexes with MDA. Further, 3 ml of phosphoric acid and 1 ml of 0.67 % TBA solutions were mixed with 0.5 ml of homogenized tissue sample. The mixture was boiled in hot water for 1 h and allowed to cool. After adding 4 ml of 1-butanol, the mixture was stirred thoroughly. Butanol phase disappeared after centrifugation. MDA standard was prepared using 1,1,3,3-tetramethoxypropane. MDA concentration was calculated by measuring the absorbance of supernatants at 532 nm. MDA concentrations of tissue homogenates were indexed to the protein concentration, the method defined by Lowry et al. [16]. Results were given as nanomoles per milligram of protein.

Nitric Oxide Level

Nitrite and nitrate measurements depended on Griess reaction. The samples were deproteinized and, to assess total nitrite and nitrate, treated with copperized cadmium at pH9.7 in glycerin. After the cleaning process, samples were mixed with fresh

reagent, and the total nitrite concentrations were calculated by a spectrometer. With serial dilutions of sodium nitrite, standard curve was measured. To solve the equation, the sum of unknown sample concentrations was used. Results were given as nanomoles per milligram of protein of lung tissue.

Histopathological Study of Lung Tissue

The pathologist was blinded to the study. Tissues were fixed in 10% neutral formalin. After fixing period, they were dehydrated and embedded in paraffin. Tissue was sliced at 5-m thickness, and the sectors were stained with hematoxylin–eosin dye. The samples were examined under light microscope (Nikon, Eclipse E600, Japan), and digital copy of the images were saved.

Evaluation of Alveolar Hemorrhage [17]

Grade 0: no hemorrhage

Grade 1: a few erythrocytes within the alveoli

Grade 2: erythrocyte clusters that do not fill the alveoli completely

Grade 3: erythrocyte clusters that fill the alveoli completely

A blinded pathologist also evaluated the lung tissues in the respect of focal inflammation, perivascular edema, interstitial edema, vascular congestion, vascular inflammation, and thrombosis.

Statistical Analysis

SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Difference between groups was analyzed with nonparametric Kruskal–Wallis test. Difference between the two groups was analyzed with Mann–Whitney

Table 1 Results

	Sample size	Leukocyte	CRP (mg/l)	MDA (nmol/mg protein)	NO (nmol/mg protein)	Wet/dry lung
Sham	10	5,660±1,438	0.020±0.004	3.70±1.40	16.25±18.07	2.87±0.51*
Sepsis	10	5,540±1,327	0.021±0.007	5.36±1.92**	10.53±13.14***	3.52±0.66****
Simvastatin	10	5,920±1,888	0.019±0.007	3.83±1.87	3.92±7.69	7.43±2.23*****
Simvastatin+sepsis	10	5,980±1,088	0.024±0.006	3.87±1.23	1.15±1.03*****	5.51±0.49***** *****

* $P=0.027$, sham vs sepsis group (95 % CI, −1.20 to −0.08)

** $P=0.042$, sepsis vs sham group (95 % CI, −3.23 to −0.07)

*** $P=0.037$, sepsis vs simvastatin+sepsis group (95 % CI, 0.62 to 18.14)

**** $P=0.00$, sepsis vs simvastatin group (95 % CI, −5.46 to −2.36)

***** $P=0.00$, sham vs simvastatin group (95 % CI, −6.08 to −3.03)

***** $P=0.017$, sham vs simvastatin+sepsis group (95 % CI, 3.07 to 27.12)

***** $P=0.00$, sham vs simvastatin+sepsis group (95 % CI, −3.11 to −2.16)

***** $P=0.00$, sepsis vs simvastatin+sepsis group (95 % CI, −2.54 to −1.43)

Fig. 1 Normal alveolar image

U test for nonparametric variables and Student's *t* test for parametric variables. ANOVA, post hoc test, and Bonferroni correction were used for the comparison of multiple groups. Significance boundary was accepted at $P < 0.05$. The results were expressed as mean and standard error of the mean or median and range.

Results

All rats were sacrificed at the 3rd day postoperatively. There was no mortality, wound infection, and anesthesia-related complication ($n=40$). The results are summarized in Table 1.

While the MDA levels were found to be significantly higher in the sepsis group as compared with the sham group ($P=0.042$), there was no difference between the other groups ($P > 0.05$). NO levels were found to be significantly lower in the simvastatin+sepsis group as compared with the sepsis group ($P=0.037$) and the sham group ($P=0.017$). The ratios of wet and dry lung weights were higher in simvastatin-given groups. Normal alveolar image, alveolar hemorrhage, and inflammation can be seen in Figs. 1 and 2. Alveolar hemorrhage was found to be significantly higher in the simvastatin+sepsis group as compared with the sham group ($P=0.038$), and there was no difference between the other groups ($P > 0.05$) (Table 2).

There was no difference for CRP, leukocyte levels, and histopathological examination in terms of focal inflammation,

Fig. 2 Alveolar hemorrhage and inflammation

perivascular–peribronchial edema, vascular congestion and inflammation, interstitial edema, and thrombosis between any group.

Discussion

Lungs are one of the most affected organs in sepsis and systemic inflammatory response syndrome. As a result of impaired inflammatory system and uncontrolled immune system, alveolar injury was an expected result [18]. In the present study, an increase in alveolar hemorrhage in the sepsis group could have been expected compared with the sham group, but we could not demonstrate this phenomenon. Liao and Laufs [8] demonstrated the pleiotropic effects of statins besides their hypolipidemic effect. In this model, although alveolar hemorrhage was expected to decrease in simvastatin-added sepsis groups, inversely, an increase was observed. This is not thoroughly understood in this model; intervention of simvastatin seems to have no positive effect at this point. Furthermore, there was no statistically significant histopathological difference in terms of focal inflammation, perivascular–peribronchial edema, vascular congestion and inflammation, interstitial edema, and thrombosis between all the groups.

There was a statistically significant increase in dry/wet lung weight in the simvastatin–sepsis group, compared with both sepsis and sham groups. Dry/wet lung weight is indirect marker of lung damage. Wet lung weight is an indicator of lung tissue damage such as pulmonary edema, congestion, and alveolar hemorrhage resulting from parenchymal inflammation and immune system disorders. Carraway et al. [19] found an increase in wet/dry lung weight in their experimental CLP-sepsis model. The results were similar in our study. This was probably secondary to sepsis-induced intra-alveolar and interstitial fluid accumulation. The increase of wet/dry lung weight in simvastatin-given sepsis group in the present study is unclear.

High rates of focal inflammation, perivascular–peribronchial edema, vascular congestion and inflammation, interstitial edema, and thrombosis were expected in histopathological analysis of lung tissues in this model. Inversely, with an unclear mechanism, there was no difference between the

Table 2 Alveolar hemorrhage

Alveolar hemorrhage	Sample size	Grade 0	Grade I	Grade II	Grade III
Sham	10	1	5	4	0
Sepsis	10	1	5	4	0
Simvastatin	10	2	2	6	0
Simvastatin+sepsis*	10	0	2	6	2

* $P=0.038$, sham vs simvastatin+sepsis group

groups in terms of histopathological findings. These findings suggest that the cause of alveolar hemorrhage and pulmonary edema cannot be explained by inflammation and thrombosis.

Jacobson et al. [20] showed that simvastatin is protective against lung tissue damage with their pleiotropic effect. Conversely, simvastatin had no protective effect on wet/dry lung weight and alveolar hemorrhage. Because of anti-inflammatory, immunomodulatory, and antioxidant effects, lower values of wet/dry lung weight were expected. There is also a controversy with the results in the literature. In their cohort study, Thomsen et al. [21] reported lower mortality rates in patients with pneumonia using simvastatin during prehospital period compared with the nonusers [21]. Inversely, Majumdar et al. [22] reported no such positive effect in their study. Furthermore, in a review about interstitial lung disease and effect of statins, it was pointed out that statins may cause interstitial lung disease [23].

MDA is the end product of lipid peroxidation and an important precursor of oxidative injury due to peroxidation. Aydin et al. [24] reported in their experimental study on rats that atorvastatin, one of the HMG-CoA reductase inhibitors, reduced the MDA level in the liver [24]. The increase of MDA in lung tissue represents lipid peroxidation and, indirectly, lung tissue damage. In the study of Leipnitz et al., increase in MDA level secondary to cellular injury was demonstrated [24, 25]. In this study, secondary to cecal ligation and puncture-induced sepsis model, lung tissues were investigated for remote organ dysfunction, and MDA levels were calculated for the severity of injury. MDA levels were found to be increased significantly in the sepsis group compared with the sham group. There was no statistically significant difference among other groups ($P>0.05$). Due to uncontrolled inflammation, immune system-induced injury, inadequate working of antioxidant system, hypoxia, and defective coagulation system, lung tissue MDA levels are expected to increase. In this study, in sepsis-constituted groups, an increase in MDA levels was shown. To demonstrate the pleiotropic effect of simvastatin, it was given 18 and 2 h preoperatively in one of the sepsis groups. But lung tissue studies show no significant decrease in the simvastatin-given sepsis group compared with the sepsis group. Besides, there was no statistically significant difference in MDA levels between simvastatin and simvastatin+sepsis groups. It is concluded that simvastatin had no preventive effect on the increase in MDA levels of lung tissue, which is the precursor of remote organ dysfunction in CLP-sepsis model.

In conditions such as trauma, sepsis, shock, and systemic inflammatory response syndrome, NO level is expected to increase secondary to induction of iNOS. Trocha et al. [26] showed increased levels of eNOS secondary to ischemia reperfusion in their study. In our study, NO levels of lung tissue in CLP-sepsis model were investigated. NO levels of lung tissue were found to be lower in the simvastatin+sepsis

group compared with the sepsis group. The antioxidant effect of simvastatin, which is one of its pleiotropic effects, occurs with the help of NO. The reason of the increase in wet/dry lung weight in the simvastatin-given group could be simvastatin-induced increase in NO level and decreased antioxidant activity.

CRP is an acute phase reactant increasing in various inflammatory conditions and is used for monitoring various inflammatory conditions and accompanying diseases [27]. In this study, there was no statistically significant difference between groups regarding CRP level and leukocyte count.

To assess the effect of simvastatin, an inhibitor of HMG-CoA reductase, on remote lung injury in experimental CLP-sepsis model, more extended and long-term studies are required.

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