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Polymyxin B immobilized column is effective for hydrochloric acid-induced lung injury in rats

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Abstract

The purpose of this study was to examine whether hemoperfusion using polymyxin B (PMX) immobilized column improves hydrochloric acid (HCl)-induced lung injury. Rats weighing 350 g underwent hemoperfusion for 30 min at a flow rate of 120 ml/h after the intratracheal instillation of hydrochloric acid or water. Rats were divided into those that underwent hemoperfusion, with or without polymyxin B column, 30 min after HCl instillation ("HCl+PMX" and "HCl"), and hemoperfusion with or without polymyxin B column 30 min after water instillation ("Aqua+PMX" and "Aqua"). Systolic blood pressure, arterial blood gas analysis and white blood cell count and neutrophils were measured before, 1 and 3 h after hemoperfusion. Plasma and bronchoalveolar lavage fluid concentrations of growth-related oncogene/cytokine-induced neutrophils chemoattractant-1 (GRO/CINC-1) were measured at 1 and 3 h after hemoperfusion. Arterial oxygen concentrations were higher in the "HCl+PMX" group than in the "HCl" group. The total numbers of cells and neutrophils in the bronchoalveolar lavage fluid were significantly higher in the "HCl" group than in the others. The GRO/CINC-1 concentrations in the plasma and bronchoalveolar lavage fluid and the albumin ratio in the "HCl+PMX" group were significantly lower than in the "HCl" group. Direct hemoperfusion using polymyxin B immobilized column treatment affects the recruitment of circulating neutrophils to the lungs due to the decrease in mediators for non-endotoxic lung injury.

Keywords: Direct hemoperfusion using polymyxin B immobilized column; Hydrochloric acid-induced lung injury; Anandamide; Growth-related oncogene/cytokine-induced neutrophils chemoattractant-1; Neutrophil recruitment

1. Introduction

Sepsis is a major cause of irreversible hypotension and multiple organ failure, especially acute respiratory distress syndrome (ARDS) (Kreger et al., 1980). Endotoxin, a lipopolysaccharide, is one of the important initiators of sepsis or ARDS (van Deventer et al., 1982; Ziegler et al., 1982). Various approaches have been attempted for the treatment of ARDS based on its pathophysiology (Wheeler et al., 1990; Carraway et al., 1998). However, these therapeutic approaches showed either insufficient improvement in the clinical outcome or mortality in the clinical trials (Bone et al., 1995; Fisher et al., 1994; Cohen and Carlet, 1996). The removal or detoxification of circulating endotoxin using an extracorporeal perfusion system has emerged as a new treatment for sepsis or ARDS. Polymyxin B (PMX) is a cyclic cationic polypeptide that detoxifies endotoxin (Rifkind and Palmer, 1996). Direct hemoperfusion using a polymyxin B immobilized column (PMX-DHP) has been tested intravenously in dogs and has been found to neutralize Gram-negative bacteria-induced hemodynamic effects on systemic pressure and vascular resistance (Palmer and Rifkind, 1974). Furthermore, PMX-DHP treatment improved the survival of animals subjected to lethal doses of intravenous endotoxin (Tani et al., 1992) or live *Escherichia coli* in vitro (Rifkind and Palmer, 1996). Hanasawa et al. (1989) also showed that PMX-DHP treatment increased the survival rate of septic dogs. In clinical trials, Tani et al. (1992, 1998), Hanasawa et al. (1989) and Shoji et al. (1998) reported that PMX-DHP treatment of patients with sepsis-related multiple organ failure resulted in decreased plasma endotoxin levels and improved systemic blood pressure. The removal of endotoxin can produce a positive outcome in endotoxemia.

Endotoxin was detected in 64% of plasma samples obtained from patients with ARDS (Pittet et al., 1997). The wall materials

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of Gram-positive cocci bacteria formed teichoic acid and peptidoglycan. Teichoic acid activates monocytes via toll-like receptor-4, and peptidoglycan activates monocytes via the toll-like receptor-2 (Takeuchi et al., 1999). Therefore, septic ARDS can be caused not only by Gram-negative rod bacteria, but also by Gram-positive cocci bacteria or fungi. Systemic inflammation progresses through these receptors, and inflammatory cytokines produced by activated monocytes are released into the blood circulation. These inflammatory cells, especially neutrophils, are recruited to the inflammatory lesions. We previously reported that PMX-DHP treatment was effective against cases with septic ARDS that were not caused by Gram-negative rod bacteria (Tsushima et al., 2002). Iwama and Komatsu (1998) reported that PMX-DHP treatment was effective for a patient with septic shock caused by Gram-positive infection. Endotoxic shock or non-endotoxic shock may be also induced by something mediators other than endotoxin. PMX-DHP treatment cannot remove inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or IL-8. Direct hemoperfusion using polymyxin B immobilized column, however, was able to adsorb not only endotoxin, but also mediators, such as anandamide, in the peripheral blood (Wang et al., 2000), and may suppress the production of inflammatory cytokines. Anandamide, an endogenous cannabinoid, can be identified in activated macrophages during endotoxic shock and is thought to be a paracrine contributor to hypotension. Patients with endotoxic shock were found to have a large amount of anandamide (Maccarrone et al., 2002). Inflammatory cytokines, such as TNF-α, IL-1β and IL-8 are produced with anandamide by activated macrophages. The purpose of this study was to examine whether PMX-DHP treatment improves the circulatory and respiratory disturbances associated with non-endotoxic lung injury, and to clarify the efficacy of direct hemoperfusion using a polymyxin B immobilized column for non-endotoxic lung injury according to the dynamics of those cytokines.

2. Materials and methods

2.1. Ethical considerations

The study protocol was approved by the Institutional Review Board for the care of animals at Shinshu University. The care and handling of animals was in accordance with the guidelines of the National Institute of Health. The animals had free access to commercial rodent food and were given free access to drinking water.

2.2. Animal study

All experiments were performed in the laboratory room of the animal institution at Shinshu University. Adult Sprague–Dawley male rats weighing 350–400 g were kept on a 12-h light/dark cycle. Sprague–Dawley male rats were injected intraperitoneally with sodium pentobarbital and intubated. Ventilation was set at a 6 ml/kg tidal volume with 0.2 l/min 100% oxygen and at 60 breaths/min using a respirator (model SN-480-7; Rodent Ventilator; Shinano, Tokyo, Japan). The positive endo-expiratory pressure was set to 0 cm of water. A 3-Fr silicon tube was passed from the left internal carotid artery and returned to the left external carotid vein. Arterial blood pressure was monitored via a silicon tube inserted into the left internal carotid artery and connected to a pressure transducer. Direct hemoperfusion using the polymyxin B immobilized column (Toray Med Co. Tokyo) and infusion pump were placed between the left internal carotid artery and the left external carotid vein (Fig. 1). The total extracorporeal volume containing the polymyxin B column was

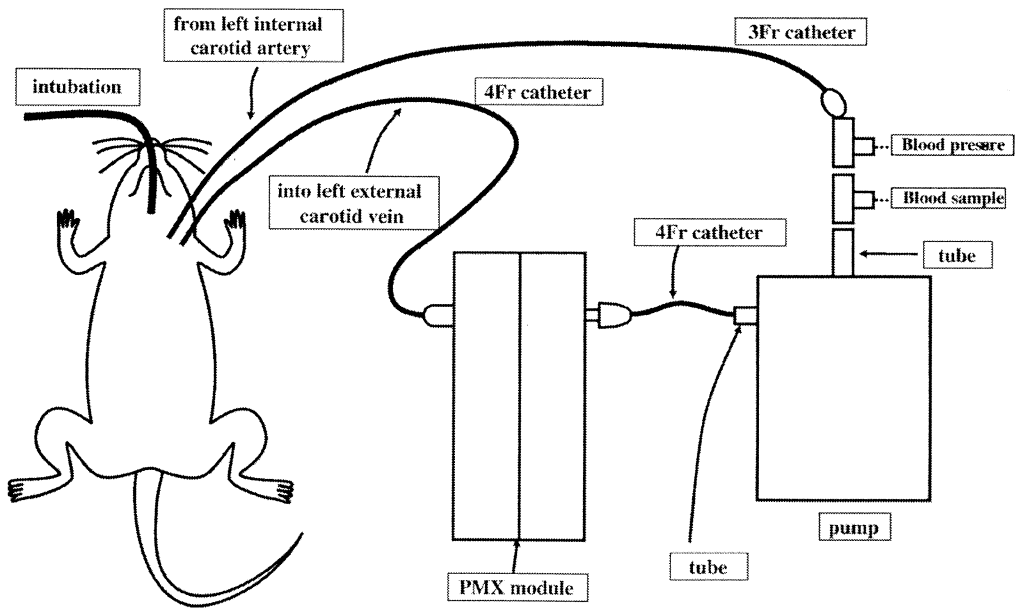

Fig. 1. PMX-DHP system. The anesthetized rat is intubated under intratracheal ventilation. A 3-Fr catheter is inserted into the left carotid artery and another one is inserted into the left external carotid vein. Blood pressure, arterial oxygen concentration and blood samples were measured through the carotid artery line. Direct hemoperfusion was performed at a flow rate of 120 ml/h for 30 min after 30 min of hydrochloric acid instillation.

about 0.8 ml. For the start of the experiment, we waited for 15 min after insertion of the catheter. After tracheal instillation, the rats were subjected to experimentation under a heat lamp to maintain a constant body temperature.

2.3. PMX-DHP treatment and experimental protocols

A specially prepared miniature polymyxin B column was used and hemoperfusion was carried out for 30 min at a flow rate of 120 ml/h under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally). Through an intratracheal tube, 0.1 N hydrochloric acid (HCl) or water was instilled in the anesthetized rats (1 ml/kg intratracheally). After the instillation of HCl or water, the rat was infused with a bolus of heparin sodium as an anticoagulant (125 U/kg intravenously). During the experience, the rats received a continuous infusion of saline via the left external carotid vein (1 ml/kg/h intravenously) to compensate for the extracorporeal volume and the maintenance of blood pressure. After hemoperfusion, observation was continued for 3 h. At the end of the experience, the rats were sacrificed and blood samples were taken from the abdominal artery. Complete bronchoalveolar lavage was subsequently performed.

Male Sprague–Dawley rats were divided into four groups. The "HCl+PMX" group (open triangle) ($n=8$) underwent hemoperfusion with a polymyxin B column 30 min after HCl instillation. The "HCl" group (closed triangle) ($n=8$) received hemoperfusion without the polymyxin B column 30 min after HCl instillation. The "Aqua+PMX" group (open circle) ($n=8$) underwent hemoperfusion with the polymyxin B column 30 min after water instillation. The "Aqua" group (closed circle) ($n=8$) underwent hemoperfusion without the polymyxin B column 30 min after water instillation. To examine the histological changes, six more animals from each group were instilled with 0.1 N HCl or water into the lung through an intratracheal tube and were sacrificed at 3 h after hemoperfusion and their lungs were removed for conventional histology.

2.4. Data collection

We recorded the hemodynamic parameters, such as the systolic blood pressure, arterial oxygen concentration (PaO^2), total leukocyte count and differential leukocyte count to evaluate the effects of PMX-DHP treatment. Heparinized blood samples for the total leukocyte count and arterial blood gases were obtained at baseline, after circulation, and 1 and 3 h after hemoperfusion, and for anandamide they were obtained at baseline, and 1 and 3 h after hemoperfusion. Ten microliters of each blood sample was diluted in 90 μl of 3% acetic acid and a total cell count was conducted. Cytocentrifuge preparations were prepared from each blood sample and stained with Giemsa to provide differential cell counts. All data are the mean ± standard deviation (S.D.).

2.5. Bronchoalveolar lavage

The chest cavity was carefully opened to allow the lungs to fully expand at 3 h after hemoperfusion. The catheter for intubation was left in the trachea and tied in place, and saline was instilled in 5 × 4 ml aliquots. The lavage fluid was recovered and placed on ice. Ten microliters of each bronchoalveolar lavage sample was diluted in 90 μl of 3% acetic acid and the total number of cells was counted. Cytocentrifuge preparations were prepared from each bronchoalveolar lavage sample and stained with Giemsa to provide differential cell counts. The bronchoalveolar lavage fluid was then centrifuged (300×g for 5 min) and the supernatant was removed. The supernatant was stored at −70 °C for subsequent experiments. The blood endotoxin level was measured at the Special Reference Lab. Inc. without the provision of any information.

2.6. Enzyme-linked immunosorbent assay for growth-related oncogene/cytokine-induced neutrophils, chemoattractant-1 (GRO/CINC-1) in the blood and bronchoalveolar lavage fluid

The levels of rat GRO/CINC-1 (IBL Co., Gunma, Japan) were measured in the blood samples obtained 1 and 3 h after hemoperfusion and albumin (ICN Pharmaceuticals, Inc., Aurora, OH) was measured in the blood and bronchoalveolar lavage fluid samples obtained 3 h after hemoperfusion, using a rat GRO/CINC-1 enzyme-linked immunosorbent assay (ELISA) kit, and albumin ELISA kit, respectively.

2.7. Endogeneous cannabinoids (arachidonyl ethanolamide (anandamide; ANA)

Aliquots of 0.2-ml of plasma were injected into 10 times the volume of acetonitrile. Deuterated endocannabinoid (d8-ANA [10 ng]) was added as an internal standard. After the removal of denatured protein with centrifugation (1710×g, 15 min), the supernatant was evaporated to dryness in vacuo at less than 40 °C. The resulting precipitate was resolved with 0.5 ml of acetonitrile for liquid chromatography-mass spectrometers (LCMS) analysis. The endocannabinoids were chromatographed with an octyl-silica (C-8) column (2 mm D, 5 cm L; GL Sciences) using High Performanced Liquid Chromatography (Agilent 1100 series, Agilent) using a gradient elution of H_2O (containing 0.1% (v/v) formic acid) and acetonitrile from 90–10% to 5–95 %, respectively. The endocannabinoids were assayed using MS/MS analysis with a Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS) system (Q-trap, Applied Biosystems). The parent and product ion were chosen (ANA, 346.3/62.1 amu; d8-ANA, 356.3/62.1 amu), and optimized for analysis under Atmospheric Pressure Chemical Ionization.

Endocannabinoids were assayed with LC/MS/MS and the stable-isotope dilution method. The assay was calibrated with authentic ANA to 10 ng d8-ANA as the internal standard, from 100 ng to 10 pg. The generating activities of endocannabinoids were expressed as "pg/ml plasma" and/or "ng/ml plasma". Authentic ANA and deuterated compounds were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (acetonitrile; LCMS grade) and other chemicals (SG grade) were purchased from Wako Pure Chemical Indust. (Osaka, Japan). The Octyl-Silica (C-8; 2 mm D, 5 cm L) column was purchased from GL Sciences.

2.8. Neutrophil accumulation in lung tissue

Lung specimens were obtained before tracheal injection and 3 h after hemoperfusion. Six rats were sacrificed by cervical dislocation under deep intraperitoneal anesthesia with sodium pentobarbital, and then the chest was opened and the lung vascular bed was flushed with a 15-ml cold phosphate buffered saline (PBS) injection through the right ventricle. The lungs were gently inflated through the trachea with 10% buffered formaldehyde, and were then removed en bloc and fixed with 10 % buffered formaldehyde. Tissue sections were then embedded in paraffin, and 5 μm sections were stained with hematoxylin–eosin and the periodic acid Schiff reaction. Two pathologists who had no knowledge of the animal groupings analyzed the samples. The number of neutrophils in the peripheral lung tissues was quantitated and the mean values were calculated under ×400 magnification.

2.9. Survival analysis

To investigate the survival rate, 48 rats received an intratracheal injection of HCl with PMX-DHP treatment ("HCl+PMX") ($n=12$), an intratracheal injection of HCl without PMX-DHP treatment ("HCl") ($n=12$), an intratracheal injection of water with PMX-DHP treatment ("Aqua+PMX") ($n=12$) or an intratracheal injection of water without PMX-DHP treatment ("Aqua") ($n=12$). Survival was estimated from the hour of hemoperfusion to the death of the rat or 5 h after hemoperfusion.

2.10. Statistical analysis

Data are presented as the mean±S.D. for each experimental group. The data among the different treatment groups were compared using the Mann–Whitney *U*-test. Baseline and pre- and post-treatment data of the same group were compared using repeated measure one-way analysis of variance (Fisher's protected least significant difference test). The survival curve was established with Kaplan–Meier survival analysis. In comparing the numbers of neutrophils/hyper-power field, Student's *t*-test was used. A P value less than 0.05 was used as the cut-off point for significance.

3. Results

The blood endotoxin level was within a normal range (<10 pg/ml) for all 4 groups in this experiment. A normal endotoxin level from the start of circulation to the completion of the experiment did not affect the therapeutic effects of each treatment.

As shown in Fig. 2, at 3 h after hemoperfusion, the systolic blood pressure of each group showed a significant decrease compared with the previous values. At 1 h after hemoperfusion, in the "HCl+PMX" group (open triangle), the systolic blood pressure had significantly increased compared with the previous value. It was changed from 83 to 105 and from 103 to 92 mm Hg in the "HCl+PMX" and "HCl" groups (closed triangle), respectively.

As shown in Fig. 3, after the HCl tracheal injection, the PaO^2 had significantly decreased compared with the "Aqua" group (closed circle). In the "HCl+PMX" group (open triangle), the PaO^2 remained at a significantly higher level compared with the "HCl" group (closed triangle) at the 3 h period ($P<0.05$).

As shown in Fig. 4, in the "HCl+PMX" (open triangle) and "HCl" group (closed triangle), the number of neutrophils changed from 1100 to 1050 and from 2050 to 1900/μl after hemoperfusion, respectively. The PMX-DHP treatment did not have any direct effect on the number of circulating neutrophils. At 3 h after hemoperfusion, the mean number of neutrophils was significantly increased in the "HCl+PMX" (8200/μl) as compared with the "HCl" group (6490/μl) ($P<0.05$).

As shown in Fig. 5, the total numbers (a) and neutrophils (b) in the bronchoalveolar lavage fluid were significantly higher in the "HCl" group compared with the other groups. The "HCl +PMX" group resulted in a significant suppression of

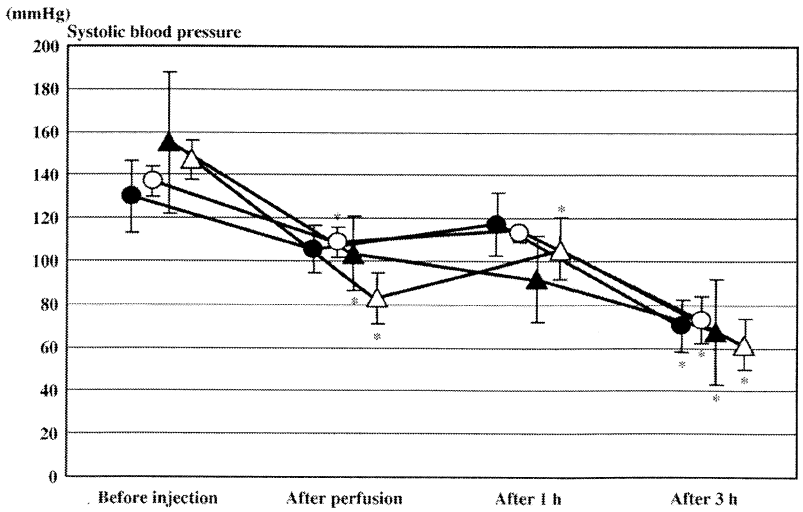

Fig. 2. Changes in systolic blood pressure after hemoperfusion. *$P<0.05$, compared with previous values, Fisher's protected least significant difference test. ●-●: Aqua, ○-○: Aqua+PMX, ▲-▲: HCl, △-△: HCl+PMX.

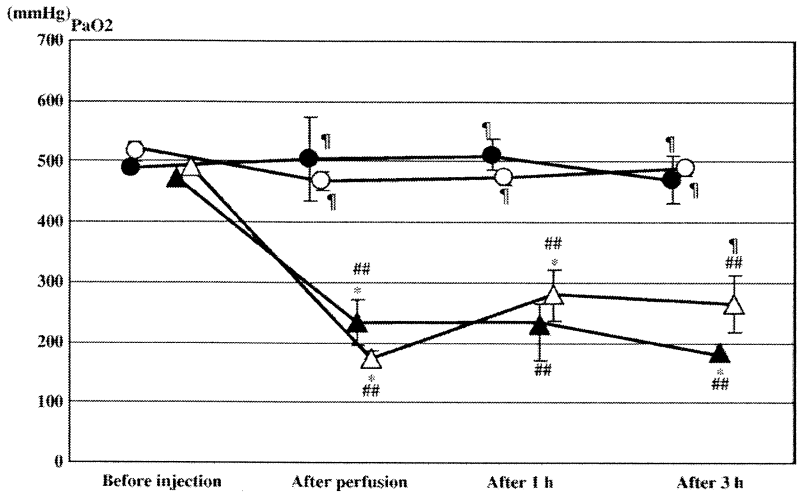

Fig. 3. Changes in PaO^2 after hemoperfusion. *$P<0.05$, compared with previous values, Fisher's protected least significant difference test. ##$P<0.01$, compared with the Aqua value over the same period, ¶$P<0.05$, compared with HCl value over the same period, Mann–Whitney U-test. ●-●: Aqua, ○-○: Aqua+PMX, ▲-▲: HCl, △-△: HCl+PMX.

neutrophil infiltration compared with the "HCl" group, but the pathohistological changes included pulmonary edema with mild infiltrated neutrophils at 3 h after hemoperfusion (c). The albumin ratio was significantly lower in the "HCl+PMX" than that in the "HCl" group (d).

As shown in Fig. 6, the light microscopic appearance of lung tissue in the groups at 3 h after hemoperfusion is shown as follows: (a) "Aqua" group, (b) "Aqua+PMX" group, (c) "HCl" group and (d) "HCl+PMX" group. Sections were stained with hematoxylin and eosin. The magnification was ×400. In the "Aqua+PMX" group, slight alveolar edema and infiltration of neutrophils were observed. In the "HCl" group, severe alveolar edema and alveolar wall disruption were observed. Numerous neutrophils and red blood cells were present in the alveolar space compared with the "HCl+PMX" group.

As shown in Fig. 7, the changes in the GRO/CINC-1 concentrations at 1 h (Fig. 7A), and 3 h after hemoperfusion (Fig. 7B) in the plasma and bronchoalveolar lavage fluid (Fig. 7C) were revealed. The plasma and bronchoalveolar lavage fluid level of this cytokine was significantly lower in the "HCl+PMX" group than in the "HCl" group, and was similar to those of the "Aqua" groups. The concentrations of GRO/CINC-1 in the bronchoalveolar lavage fluid correlated with the number of invading neutrophils in the bronchoalveolar lavage fluid ($R=0.70$, $P<0.001$).

As shown in Fig. 8, the plasma concentration of anandamide before instillation was 179±104 pg/ml. The plasma concentration of anandamide revealed significant differences between the "HCl" and the other group at 1 h after hemoperfusion and did not reveal any significant differences at 3 h after

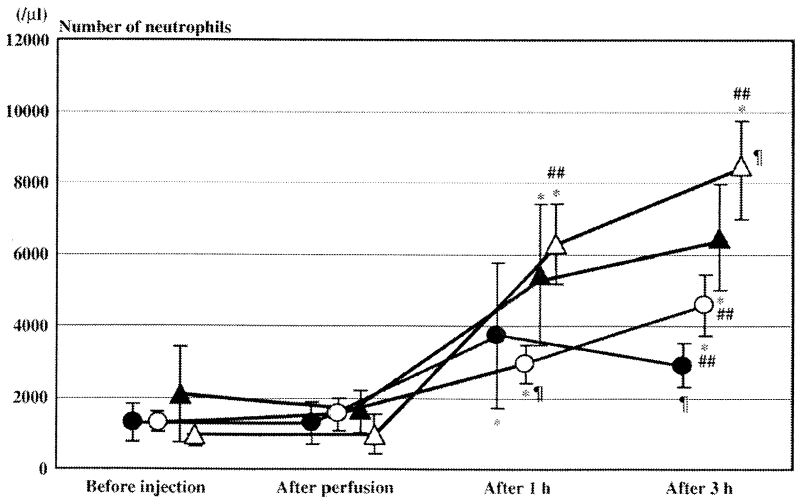

Fig. 4. Changes in the number of neutrophils in the peripheral blood of the four experimental groups after hemoperfusion. *$P<0.05$, compared with the previous value; Fisher's protected least significant difference test, ##$P<0.01$; compared with the Aqua value in the same period, ¶$P<0.05$, compared with the HCl value over the same period. Mann–Whitney U-test. ●-●: Aqua, ○-○: Aqua+PMX, ▲-▲: HCl, △-△: HCl+PMX.

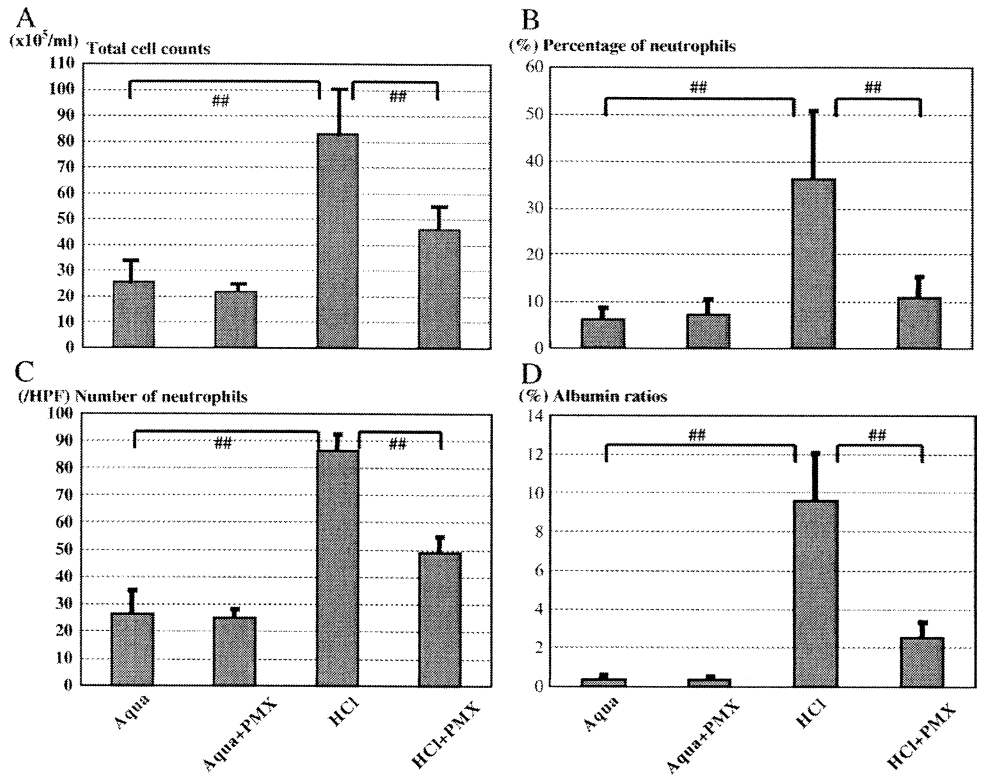

Fig. 5. Changes in total cell counts, neutrophils in the bronchoalveolar lavage fluid, pathohistological findings and albumin ratio after hemoperfusion. Changes in total cell counts (A) and percentage of neutrophils (B) in the bronchoalveolar lavage fluid samples obtained from the four experimental groups. Differences in the number of neutrophils per high power field in the alveoli of the lung (C) of each of the four experimental groups. The plasma to bronchoalveolar lavage fluid albumin ratios (D) of the four experimental groups. ##$P<0.01$; compared with the Aqua and HCl+PMX value, Fisher's protected least significant difference test.

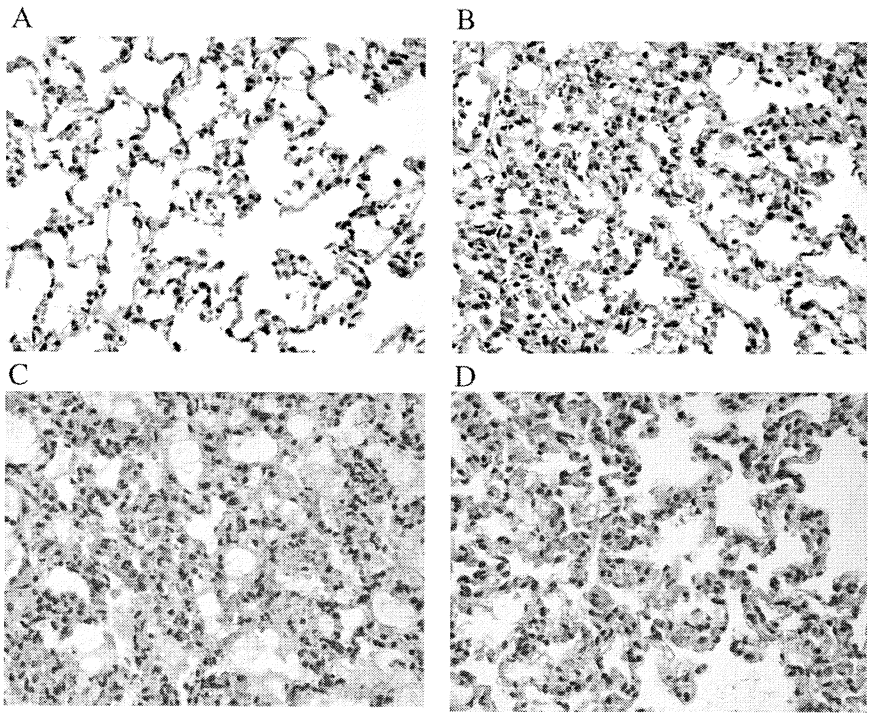

Fig. 6. Photomicrographs of the light microscopic appearance of lung tissue in the groups: (A) Aqua group, (B) Aqua + PMX group, (C) HCl group and (D) HCl + PMX group. Sections were stained with hematoxylin and eosin. The magnification was ×400. In the HCl group, alveolar edema and alveolar wall disruption were observed. Numerous neutrophils and red blood cells were present in the alveolar space compared with the HCl+PMX group.

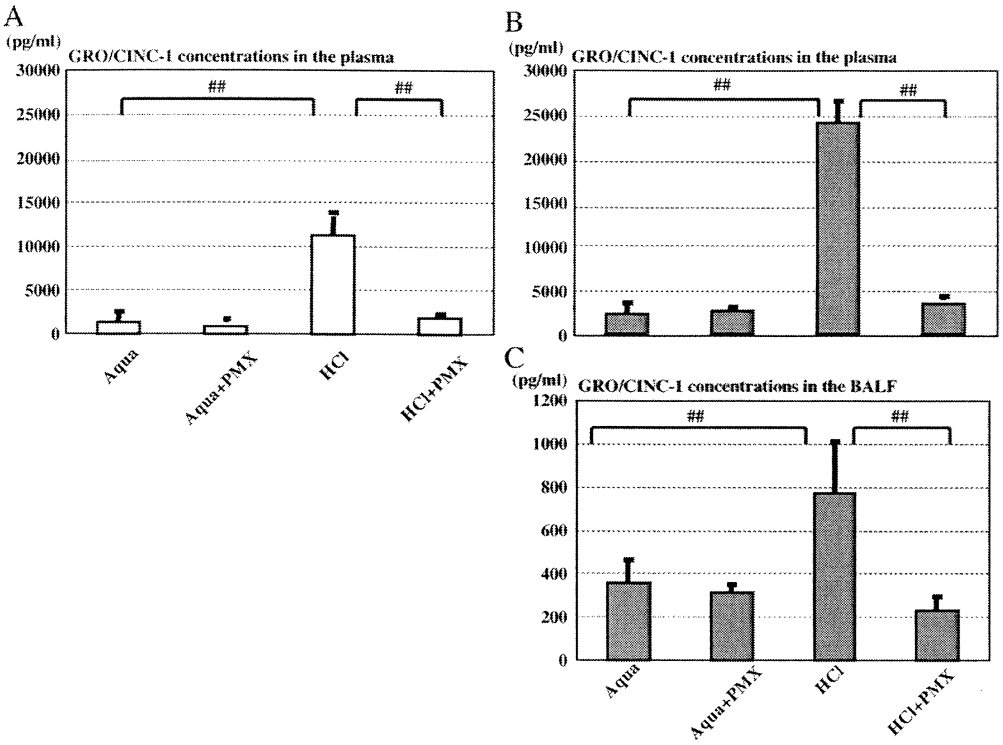

Fig. 7. GRO/CINC-1 concentrations in the plasma and bronchoalveolar lavage fluid. GRO/CINC-1 concentrations at 1 h after hemoperfusion in the plasma (A), at 3 h after hemoperfusion in the plasma (B) and bronchoalveolar lavage fluid (C) of the four experimental groups. ##$P<0.01$, compared with the Aqua and HCl+PMX value. Fisher's protected least significant difference test.

hemoperfusion among the groups. Anandamide in the plasma again increased 3 h prior to the increase of plasma GRO/CINC-1. However, each group with anandamide at 3 h after hemoperfusion showed significantly higher levels compared with those of the groups before HCl instillation. The correlation between anandamide and GRO/CINC-1 was shown to be significant at 1 h ($R=0.56$, $P<0.05$), but was not significant at 3 h after hemoperfusion.

As shown in Fig. 9, the survival analysis of the "HCl+PMX" group at 5 h after hemoperfusion showed significant differences between that of the "HCl" group ($P<0.05$).

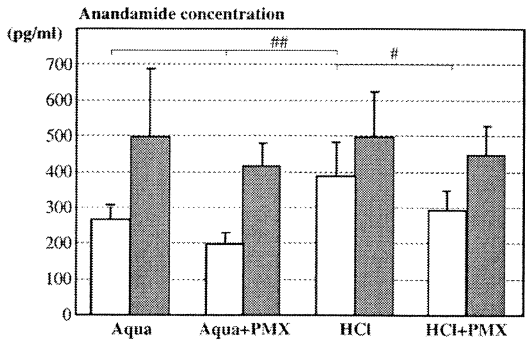

Fig. 8. Anandamide concentration in the plasma. The changes in anandamide at 1 (white bar) and 3 h (black bar) in the plasma after hemoperfusion. #$P<0.05$, ##$P<0.01$, compared with the Aqua and HCl+PMX value. Fisher's protected least significant difference test.

4. Discussion

We previously reported that PMX-DHP treatment was effective against cases with septic ARDS that were not caused by Gram-negative rod bacteria and unknown etiology (Tsushima et al., 2002). The inhalation of lipopolysaccharide is the direct lung injury model, but we used the direct induced ARDS model, which is similar to aspiration pneumonia clinically, to investigate this effect of PMX-DHP treatment.

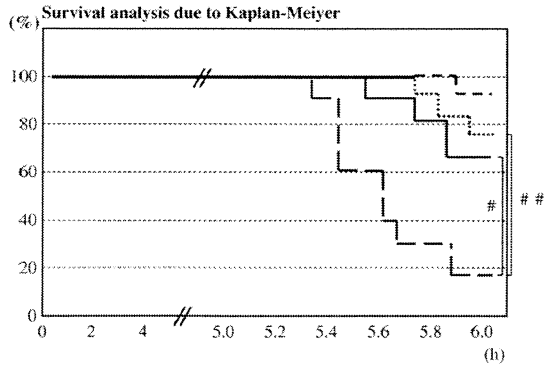

Fig. 9. Survival analysis of a survival curve using Kaplan–Meier methods. ····: Aqua group, —: Aqua+PMX group, —: HCl+PMX group, —·—: HCl group. The survival analysis of the "HCl+PMX" group at 5 h after hemoperfusion showed significant differences between that of the "HCl" group ($P<0.05$). The survival analysis of the "HCl+PMX" group at 5 h after hemoperfusion showed significant differences between that of the "Aqua" group ($P<0.01$).

Yamamoto et al. (2002) examined the effect of direct hemoperfusion using a polymyxin B immobilized column on endotoxemia-induced cardiopulmonary disorders in sheep. The most novel findings in their study were: PMX-DHP treatment significantly improved the systemic hypotension and hypoxemia during endotoxemia, and direct hemoperfusion using polymyxin B column significantly suppressed the nitric oxide production in response to endotoxin. With endotoxic lung injury in sheep, PMX-DHP treatment improved the endotoxic shock and hypoxemia independent of the leukocytes and/or polymorphonuclear leukocyte-related pathways. There is a discrepancy between our results and Yamamoto's report. They speculated that PMX-DHP treatment suppressed the production of mediators, including nitric oxide, which are related to the development of shock and acute lung injury. Nitric oxide is one of the major mediators suspected of precipitating the cardiopulmonary vascular collapse associated with septic shock (Kilbourn, 1997; Sittipunt et al., 2001). In many cases, septic shock is a complication associated with the development of ARDS caused by inflammatory cytokines. However, direct pulmonary injury results from causes as diverse as pneumonia, the aspiration of gastric contents or smoke inhalation, to indirect pulmonary injury from bacteremia, endotoxemia and hemorrhage/trauma (Ware and Matthay, 2000). Yamamoto et al. reported the latter, and our study reported the former. After injury, activated inflammatory cells, including peripheral monocytes and alveolar macrophages, leukocytes and polymorphonuclear neutrophils, are thought to play an essential role in the pathogenesis of ARDS. As shown in Fig. 4, the numbers of neutrophils did not change after instillation of HCl. However, in the endotoxemia model described in Yamamoto's report, the numbers of circulating leukocytes showed a marked decrease after the injection of endotoxin. We suggest that PMX-DHP treatment affects circulating leukocytes, and suppose that infiltrating neutrophils are recruited to the alveoli more quickly in the endotoxemia model than in the HCl-induced lung injury as described in Yamamoto's report. We suppose that the start of PMX-DHP treatment is responsible for the delay in the endotoxemia model. Therefore, PMX-DHP treatment improved endotoxic shock and hypoxemia independent of the leukocyte pathway. However, we propose that the PMX-DHP treatment affects the circulating leukocytes. Several studies suggested that neutrophils play an important role in the development of acute lung injury (Snapper et al., 1983). Aspiration or bronchial instillation of HCl results in a biphasic lung injury (Kennedy et al., 1989). In the HCl induced lung injury model, in the first phase a direct physicochemical injury to the alveolocapillary membranes occurs, and in the second phase (after approximately 2 h) activated neutrophils are recruited from extrapulmonary sites into the lungs. This activation of neutrophils takes approximately 2 h. Therefore, the most effective adaptation would involve PMX-DHP treatment before the infiltration of neutrophils into the lung. The PMX-DHP treatment can afford to affect to the circulating neutrophils. In the present study, the percentage of neutrophils in the bronchoalveolar lavage fluid in the "HCl+PMX" group was significantly lower, and the number of neutrophils in the peripheral blood in the "HCl+PMX" group was significantly higher than that of the "HCl" group at 3 h after hemoperfusion. Specimens of the "HCl+PMX" group showed a slight suppression in the neutrophil invasion compared with the "HCl" group. We suggest that neutrophils in the peripheral blood were not recruited into the lung, and remained in the peripheral blood. However, because the direct lung injury due to HCl was very severe, finally, in the "HCl+PMX" and "HCl" groups, neutrophils were recruited into the lung. These results suggest that PMX-DHP treatment has affects on the systemic circulation through its affects on neutrophils and causes the suppression of the lung infiltration of neutrophils. We could explain our results by the preponderant effect of neutrophil recruitment after PMX-DHP treatment.

Rat GRO/CINC is a well known chemoattractant protein and is homologous to human IL-8 (Iida et al., 1992). Alveolar macrophages and circulating monocytes produce GRO/CINC-1 in rats. Inflammatory initiators, including endotoxin, first cause the release of TNF-α, as well as GRO/CINC-1, from macrophages (Iida et al., 1992). GRO/CINC-1 recruits neutrophils to inflammatory sites where they become activated, release myeloperoxidase and cause tissue damage. The GRO/CINC-1 concentration in the bronchoalveolar lavage fluid and plasma of the "HCl+PMX" group were significantly lower than those in the "HCl" group. As shown in Fig. 5D, the albumin ratio was significantly lower in the "HCl+PMX" than that in the "HCl" group. These data were related to the inflammation with neutrophils in the alveoli. The suppression of GRO/CINC-1 reduces the vascular permeability in the alveoli, and hence the albumin concentrations in the parenchyma were low in the "HCl+PMX" group, according to the suppression of neutrophil recruitment. These results suggest that the "HCl+PMX" group showed a higher level of oxygen concentration compared with the "HCl" group. The local production of GRO/CINC-1 is associated with activated macrophages and monocytes in the alveoli or peripheral blood. These results suggest that PMX-DHP treatment has systemic effects on GRO/CINC-1 production, and PMX-DHP treatment suppresses neutrophil recruitment to injured lungs; hence, improvements due to PMX-DHP treatment are dependent on circulatory neutrophils in rats with HCl-induced lung injury. These results for the GRO/CINC-1 concentrations and histopathological findings indicate that PMX-DHP treatment was able to suppress the development of lung injury due to activated neutrophils and macrophages. PMX-DHP treatment suppresses neutrophil activation and recruitment to the lung due to GRO/CINC-1, because PMX-DHP treatment can not remove GRO/CINC-1 directly.

The well-known neurobehavioral modulators, cannabinoids, including the endogenous ligand anandamide (Devane et al., 1992), can elicit hypotension mediated via the peripherally located CB1 cannabinoid receptors (Varga et al., 1995). Endotoxins and non-endotoxins (teichoic acid and peptidoglycan) stimulate the production of anandamide in macrophages. Wagner et al. (1997) reported that the activation of peripheral CB1 receptors in rats by macrophage- and platelet-derived substances contributes to the hypotension of hemorrhagic

shock, and that anandamide is generated by activated circulating macrophages, but not by platelets. Direct hemoperfusion using a polymyxin B immobilized column binds directly to anandamide, resulting in the neutralization of the biological functions of the anandamide (Wang et al., 2000). Although anandamide was instilled through an intratracheal tube on the preliminary study, direct lung injury was not caused by anandamide. However, anandamide enhanced the production and release of proinflammatory cytokines, including IL-1β, IL-6, TNF-α and nitric oxide (Luo et al., 1992). As shown in Fig. 7, the plasma concentrations of anandamide were observed to be significantly different between the "HCl+PMX" and "HCl" group at 1 h after hemoperfusion, and were associated with the improved ratio of blood pressure. The correlation between anandamide and GRO/CINC-1 was shown to be significant at 1 h, but was not significant at 3 h after hemoperfusion. Anandamide was not significantly different between the "HCl +PMX" and "HCl" group at 3 h, because PMX-DHP treatment could not maintain a continuous effect. Anandamide in the plasma again increased at 3 h prior to the increase of plasma GRO/CINC-1. These increases in anandamide might have been associated with hypotension at 3 h after PMX-DHP treatment. When seen in one longitudinal period, PMX-DHP treatment affects the circulating leukocytes, systolic blood pressure, and PaO_2. Therefore, we suggested that the final effect of PMX-DHP treatment showed discrepancies between the hematological and circulatory parameters.

Hypoxemia or hypotension cannot explain the mortality at 5 h after hemoperfusion. Although the systolic blood pressure was lower after perfusion in the "HCl+PMX" group, we supposed that the resistance of flow to the right ventricle decreased venous return to return through the polymyxin B filter in rats with HCl instillation. There is a potential mechanism of direct hemoperfusion using polymyxin B immobilized column action. The groups that finally died showed metabolic and respiratory acidosis and severe lung edema with the infiltration of neutrophils. The PMX-DHP treatment is effective to reduce neutrophil recruitment, but is not effective for repair of direct lung injury caused by HCl instillation. HCl instillation causes severe direct alveolar damage and finally brings about metabolic and respiratory acidosis. We suggest that shock and anuria complicated with this acidosis resulted in death. The difference of mortality at 5 h after hemoperfusion is due to this pathological difference, such as pulmonary edema. We suggest that the main potential mechanism of direct hemoperfusion using a polymyxin B immobilized column is the suppression of neutrophil recruitment into the alveoli. The death rate of the "HCl+PMX" group is 33% at 5 h after hemoperfusion. This data is better compared with the "HCl" group. Direct alveolar damage, including the infiltration of neutrophils, induced by HCl instillation remains in the alveoli after hemoperfusion. Moreover, we suspect that the differences in the amount of anandamide at 1 h after hemoperfusion are associated with the differences in the GRO/CINC-1 production at 3 h after hemoperfusion, and the differences of GRO/CINC-1 concentration at 3 h after hemoperfusion are associated with the mortality 5 h after hemoperfusion. Therefore, we suspect that the adsorption of anandamide at the early phase leads to the suppression of inflammatory cytokine production, and the suppression of neutrophil recruitment in the alveoli due to PMX-DHP treatment.

In conclusion, the use of PMX-DHP treatment improved the oxygenation associated with HCl lung injury. This therapy may suppress neutrophil recruitment to the lungs. While further studies are needed to elucidate the other mechanism by which PMX-DHP treatment affects the recruitment of circulating neutrophils due to the decrease in mediators, our results showed that PMX-DHP treatment has positive effects for lung injury associated with non-endotoxic lung injury.

References

Bone, R.C., Balk, R.A., Fein, A.M., Perl, T.M., Wenzel, R.P., Reines, H.D., Quenzer, R.W., Iberti, T.J., Macintyre, N., Schein, R.M., 1995. A second large controlled clinical study of E5, a monoclonal antibody to endotoxin: results of a prospective, multicenter, randomized, control trial. The E5 Sepsis Study group. Crit. Care Med. 23, 994–1006.

Carraway, M.S., Welty-Wolf, K.E., Kantarow, S.P., Simonson, Y.C., Que, L.G., Kishimoto, T.K., Piantadosi, C.A., 1998. Antibody to E- and L-selectin does not prevent lung injury or mortality in septic baboons. Am. J. Respir. Crit. Care Med. 157, 938–949.

Cohen, J., Carlet, J., 1996. INTERSEPT, an international, multicenter, placebo-controlled trial of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis. International Sepsis Trial Study group. Crit. Care Med. 24, 1431–1440.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Fisher Jr., C.J., Slotman, G.J., Opal, S.M., Pribble, J.P., Bone, R.C., Emmanuel, G., Ng, D., Bloedow, D.C., Catalano, M.A., 1994. IL-1 RA Sepsis Syndrome Study Group. Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of sepsis syndrome: a randomized, Open label, placebo-controlled multicenter trial. The IL-1 RA Sepsis Syndrome Study group. Crit. Care Med. 22, 12–22.

Hanasawa, K., Tani, T., Kodama, M., 1989. New approach to endotoxic and septic shock by means of polymyxin B immobilized fiber. Surg. Gynecol. Obstet. 168, 323–331.

Iida, M., Watanabe, K., Tsurufumi, M., Takaishi, K., Iizuka, Y., Tsurufui, S., 1992. Level of neutrophil chemotactic factor CINC/gro, a member of the interleukin-8 family, associated with lipopolysaccharide-induced inflammation in rats. Infect. Immun. 60, 1268–1272.

Iwama, H., Komatsu, T., 1998. Effect of an endotoxin-removing column containing immobilized polymyxin B fiber in a patient with septic shock from gram-positive infection. Acta Anaesthesiol. Scand. 42, 590–593.

Kennedy, T.P., Johnson, K.J., Kunkel, R.G., Ward, P.A., Knight, P.R., Finch, J. S., 1989. Acute acid aspiration lung injury in the rat: biphasic pathogenesis. Anesth. Analg. 69, 87–92.

Kilbourn, R., 1997. Nitric oxide synthase inhibitors—a mechanism-based treatment of septic shock. Crit. Care Med. 27, 857–858.

Kreger, B.E., Craven, D.E., McCabe, W.R., 1980. Gram-negative bacteremia: IV. Re-evaluation of clinical features and treatment in 612 patients. Am. J. Med. 68, 344–355.

Luo, Y.D., Patel, M.K., Wiederhold, M.D., Ou, D.W., 1992. Effects of cannabinoids and cocaine on the mitogen-induced transformations of lymphocytes of human and mouse origins. Int. J. Immunopharmacol. 14, 49–56.

Maccarrone, M., Bari, M., Battista, N., Finazzi-Agro, A., 2002. Endocannabinoid degradation, endotoxic shock and inflammation. Curr. Drug Targets Inflamm. Allergy 1, 53–63.

Palmer, J.D., Rifkind, D., 1974. Neutralization of the hemodynamic effects of endotoxin by polymyxin B. Surg. Gynecol. Obstet. 138, 755–759.

Pittet, J.F., Mackersie, R.C., Martin, T.R., Matthay, M.A., 1997. Biological markers of acute lung injury: prognostic and pathogenetic significance. Am. J. Respir. Crit. Care Med. 155, 1187–1205.

Rifkind, D., Palmer, J.D., 1996. Neutralization of endotoxin toxicity in chick embryos by antibiotics. J. Bacteriol. 92, 815–819.

Shoji, H., Tani, T., Hanasawa, K., Kodama, M., 1998. Extracorporeal endotoxin removal by polymyxin B immobilized fiber cartridge: designing and anti-endotoxin efficacy in the clinical application. Therap. Apher. 2, 3–12.

Sittipunt, C., Steinberg, K.P., Ruzinski, J.T., Myles, C., Zhu, S., Goodman, R.B., Hudson, L.D., Matalon, S., Martin, T.R., 2001. Nitric oxide and nitrosine in the lungs of patients with acute respiratory distress syndrome. Am. J. Respir. Crit. Care Med. 163, 503–510.

Snapper, J.R., Bernard, G.R., Hinson Jr., J.M., Hutchison, M., Loyd, J.E., Ogletree, M.L., Brigham, K.L., 1983. Endotoxemia-induced leukopenia in sheep: correlation with lung vascular permeability and hypoxemia but not with pulmonary hypertension. Am. Rev. Respir. Dis. 127, 306–309.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., Akira, S., 1999. Differential roles of TLR 2 and TLR 4 in recognition of gram-negative and gram-positive bacterial cell components. Immunity 11, 443–451.

Tani, T., Chang, T.M., Kodama, M., Tsuchiya, M., 1992. Endotoxin removed from hemoglobin solution using polymyxin-B immobilized fiber (PMX-F) followed by a new turbidometric endotoxin assay. Biomater. Artif. Cells Immobil. Biotechnol. 20, 457–462.

Tani, T., Hanasawa, K., Endo, Y., Yoshioka, T., Kodama, M., Kaneko, M., Uchiyama, Y., Akizawa, T., Takahasi, K., Sugai, K., 1998. Therapeutic apheresis for septic patients with organ dysfunction: hemoperfusion using a polymyxin B immobilized column. Artif. Organs 22, 1038–1044.

Tsushima, K., Kubo, K., Koizumi, T., Fujimoto, K., Hora, K., 2002. Direct hemoperfusion using a polymyxin B immobilized column improves acute respiratory distress syndrome. J. Clin. Apher. 17, 97–102.

van Deventer, S.J., Buller, H.R., ten Cate, J.W., Sturk, A., Pauw, W., 1982. Endotoxemia: an early predictor of septicaemia in febrile patients. Lancet 2, 605–609.

Varga, K., Lake, K., Martin, B.R., Kunos, G., 1995. Novel antagonist implicates the CB1 cannabinoid receptor in the hypotensive action of anandamide. Eur. J. Pharmacol. 278, 279–283.

Wagner, J.A., Varga, K., Ellis, E.F., Rzigalinski, B.A., Martin, B.R., Kunos, G., 1997. Activation of peripheral CB1 cannabinoid receptors in hemorrhagic shock. Nature 322, 671–677.

Wang, Y., Liu, Y., Sarker, K.P., Nakashima, M., Serizawa, T., Kishida, A., Akashi, M., Nakata, M., Kitajima, I., Maruyama, I., 2000. Polymyxin B binds to anandamide and inhibits its cytotoxic effect. FEBS Lett. 470, 151–155.

Ware, L.B., Matthay, M.A., 2000. The acute respiratory distress syndrome. N. Engl. J. Med. 342, 1334–1349.

Wheeler, A.P., Hardie, W.D., Bernard, G., 1990. Studies of anti-endotoxin antibody in preventing the physiologic changes of endotoxemia in awake sheep. Am. Rev. Respir. Dis. 142, 775–781.

Yamamoto, H., Koizumi, T., Kaneki, T., Fujimoto, K., Kubo, K., Honda, T., 2002. Direct hemoperfusion with polymyxin B-immobilized fiber improves shock and hypoxemia during endotoxemia in anesthetized sheep. J. Endotoxin Res. 8, 419–426.

Ziegler, E.J., McCutchan, J.A., Fierer, J., Grauser, M.P., Adoff, J.C., Douglas, H., Braude, A.I., 1982. Treatment of Gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. N. Engl. J. Med. 307, 1225–1230.