

Efficacy of linezolid against PVL-positive MRSA in a mouse model of hematogenous pulmonary infection

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Abstract

Background: Many strains of community-acquired methicillin-resistant *Staphylococcus aureus* have a pore-forming leukotoxin, referred to as Panton-Valentine leukocidin (PVL), which can cause severe necrotizing pneumonia. Linezolid (LZD) is a new antibacterial agent that has potent antibacterial activity against MRSA.

Methods: We used a mouse model of hematogenous pulmonary infection to compare the efficacies of LZD and vancomycin (VCM) against pulmonary infection caused by PVL-positive *S. aureus*.

Results: After antibiotics were administered for 3 days, the numbers of viable bacteria in the control, VCM, and LZD groups were 6.77 ± 0.14 , 5.29 ± 0.27 , and 4.25 ± 0.33 log cfu/lung (mean \pm SEM), respectively. LZD significantly decreased the number of viable bacteria in the lungs as compared with the control group and the VCM group ($p < 0.05$). The survival rate at day 7 post-inoculation was higher in the LZD group (100%) than in the VCM group (50%) or control group (0%). Histopathological examination and cytokine analysis also showed beneficial efficacy of LZD in comparison with VCM.

Conclusion: LZD significantly reduced bacterial numbers and inflammation in a mouse model of PVL-positive *S. aureus* hematogenous infection and improved the survival rate of infected mice as compared with VCM. LZD is clinically effective against PVL-positive *S. aureus*.

Introduction

Community-acquired MRSA infection has been increasing worldwide¹. The many strains of community-acquired MRSA have a phage harboring the Pantan-Valentine leukocidin (PVL) genes.²⁻⁴ PVL is a pore-forming leukotoxin,^{5,6} and PVL-positive *S. aureus* can cause primary skin and soft-tissue infections,⁷ as well as severe necrotizing pneumonia in young immunocompetent patients.^{6,7} The mortality rate is 75%,⁴ and autopsy reveals extensive necrotic and hemorrhagic lesions of the trachea, bronchi, alveolar septa, and parenchyma. Thus, new treatment strategies are needed for PVL-positive *S. aureus*.

Linezolid (LZD) has potent antibacterial activity against Gram-positive cocci, vancomycin-resistant enterococci, and MRSA. LZD was reported to be more effective than VCM in achieving microbiological eradication for the treatment of MRSA infections.⁹

We established a mouse model of pulmonary infection with *S. aureus* by intravenous injection of bacteria enmeshed in agar beads.¹⁰⁻¹² The aim of the present study was to compare the activity and efficacy of LZD and VCM against PVL-positive *S. aureus* hematogenous pulmonary infection in a model mouse.

Materials and Methods

Bacterial strains and culture conditions

PVL-positive *S. aureus* was kindly provided by Prof. T. Yamamoto (Niigata University, Niigata, Japan). This strain was classified as staphylococcal cassette chromosome mec (SCCmec) type IV⁶. The bacteria were stored at -80°C in Microbank (Pro-Lab Diagnostics, Ontario, Canada) until use. Bacteria were grown at 37°C on Mueller Hinton II Agar (Becton Dickinson and Company, Sparks, MD, USA) or in brain-heart infusion (BHI) broth (BBL Microbiology System, Cockeysville, MD, USA).

Antibiotics

LZD (Pfizer Japan Inc., Tokyo, Japan) and VCM (Shionogi Pharmaceutical Co., Osaka, Japan) were dissolved in sterile water immediately before use.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) of each agent was determined by the microplate dilution technique. Mueller-Hinton II medium (Becton Dickinson and Company) and an inoculum size of 5×10^5 cfu/ml was used. MIC was defined as the lowest concentration of the test agent that inhibited visible growth of bacteria after 18 hours of incubation at 37°C.

Laboratory animals

Six-week-old, male, ddY, specific pathogen-free mice were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Nagasaki University Graduate School of Biomedical Sciences. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University.

Inoculum

Inoculation was performed as previously described.¹⁰⁻¹² Briefly, PVL-positive *S. aureus* was cultured overnight on Mueller Hinton II agar plates at 37°C. Bacteria were suspended in endotoxin-free sterile saline and harvested by centrifugation (3,000 rpm, 4°C, 10 min). Organisms were resuspended in cold sterile saline and diluted to between 2×10^{10} and 4×10^{10} cfu/ml, or 2×10^{11} and 4×10^{11} cfu/ml, as estimated by turbidimetry. The suspension was warmed to 42°C, and 10 ml of the suspension was then mixed with 10 ml of 4% (wt/vol) molten noble agar (Difco Laboratories, Detroit, MI, USA) at 42°C. The agar-bacterium suspension (1.0 ml) was placed into a 1.0-ml syringe, and the suspension was rapidly injected via a 26-gauge needle into 49 ml of rapidly stirred ice-cooled sterile saline. This procedure resulted in solidification of the

agar droplets into beads of approximately 200 μm in diameter. The final concentration of agar was 0.04% (wt/vol), and the final number of bacteria was 2×10^8 to 4×10^8 cfu/ml for cytokine, bacteriological, and histopathological studies or 2×10^9 to 4×10^9 cfu/ml for survival studies.

Animal model of hematogenous pneumonia

A total of 0.25 ml of the suspension containing agar beads with bacteria was injected into the tail vein of each mouse (10 ml/kg of body weight). The method used for inducing infection has been described in detail elsewhere.¹⁰⁻¹² Treatment commenced a day after inoculation by intraperitoneal administration. Animals were allocated into 3 groups: LZD (100 mg/kg/dose), VCM (100 mg/kg/dose) or control. Each drug was administered twice daily for 1 day or 3 days for bacteriological study (n = 10 in each group), 3 days for histopathological and cytokine studies (n = 10 in each group), or 7 days for survival studies (n = 6 in each group).

Bacteriological, histopathological and survival examinations

Mice were sacrificed by cervical dislocation 12h after administration of antibiotics.

After exsanguinations, the lungs were dissected and removed under aseptic conditions.

Organs used for bacteriological analysis were homogenized and cultured quantitatively

by serial dilutions on Mueller Hinton II agar plates. Lung tissue for histological examination was fixed in 10% buffered formalin and stained with hematoxylin-eosin. Specimens were examined under a microscope, and total abscesses were counted. The lung area was calculated by using cross-section paper.

Enzyme-linked immunosorbent assays (ELISA)

Concentrations of TNF- α , MIP-2, and IL-1 β in the lung homogenates were assayed by using mouse cytokine ELISA kits (R & D Systems, Minneapolis, MN, USA).

Statistical analysis

Bacteriological and cytokine data were expressed as means \pm SEM, and survival data were expressed by Kaplan-Meyer curves. Differences between groups were examined with the unpaired *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

MIC of each antibiotic for PVL-positive S. aureus

The MIC of LZD and VCM for PVL-positive *S. aureus* was 2 μ g/ml and 1 μ g/ml, respectively.

Bacteriological effects of antibacterial agents

When antibiotics were administered for 1 day, the number of bacteria in the lungs in the control group, VCM group, and LZD group were 6.69 ± 0.16 , 6.52 ± 0.21 , and 6.30 ± 0.22 log cfu/ml, respectively (No statistically significant differences) (Fig. 1, panel a).

When antibiotics were administered for 3 days, the number of bacteria in the lungs in the control group was 6.77 ± 0.14 (log cfu/ml). In contrast, the numbers in the VCM and LZD groups were 5.29 ± 0.27 and 4.25 ± 0.33 (log cfu/ml), respectively. The treatment with VCM significantly decreased the number of viable bacteria as compared with the control group ($p = 0.0004$). Furthermore, LZD administration resulted in a significant decrease in the number of viable bacteria when compared with that in the control group ($p < 0.0001$) or VCM group ($p = 0.0088$) (Fig. 1, panel b).

Histopathological examination

Lung specimens from mice infected with PVL-positive *S. aureus* were microscopically examined 3 days after treatment, and lung abscesses consisting of bacterial colony with infiltrating acute inflammatory cells were found (Fig. 2). The control (panel a) and VCM (panel b) groups exhibited numerous abscesses and inflammation. In contrast, the LZD group (panel c) exhibited fewer abscesses and less inflammation than the control and VCM groups.

In whole lung specimens, the number of abscesses in control, VCM, and LZD groups were 15.6 ± 4.25 , 10.2 ± 3.31 , and 2.6 ± 0.93 , respectively (Fig. 3, panel a). The number of abscesses per cm^2 also showed similar consequents (control, VCM, and LZD groups were 10.6 ± 3.08 , 5.89 ± 1.91 , and 1.58 ± 0.53 count per cm^2 , respectively) (Fig. 3, panel b). The numbers of abscesses in whole lungs and per lung area were significantly decreased in the LZD group, as compared with those in the control group ($p = 0.013$ and 0.011 , respectively). There were no significant differences in the number of abscesses in whole lungs and per lung area between the control and VCM groups and between the VCM and LZD groups.

Cytokine concentrations in lung extracts

When antibiotics were administrated for 3 days, the concentrations of proinflammatory cytokines, TNF- α , MIP-2, and IL-1 β , were significantly lower in the VCM and LZD groups as compared with the concentrations in the control group. In addition, all of these cytokine concentrations were lower in the LZD group than in the VCM group; however, the difference was not statistically significant (Fig. 4).

Survival study

The survival rate at day 7 post-inoculation was higher in the LZD group (100% survival) when compared with the VCM group (50%) and the control group (all dead within 6 days) (Fig. 5).

Discussion

The present study is the first report to demonstrate the establishment of the mouse model of PVL-positive *S. aureus* hematogenous pulmonary infection. Moreover, this model was used to compare the efficacy of LZD and VCM. LZD showed beneficial efficacy on bacteriological and histopathological examinations, cytokine levels, and survival rates.

We previously reported the effect of LZD against a mouse model of hematogenous pulmonary infection with MRSA NUMR101, which was isolated from clinical samples at Nagasaki University Hospital.¹⁰ The results of that study are identical to those in the present study, with the exception of the cytokine analysis. The present cytokine analysis showed that proinflammatory cytokines, TNF- α , MIP-2, and IL-1 β , were obviously decreased in the LZD group. This finding supports that LZD significantly reduces the number of bacteria and level of inflammation in the lungs.

In the present study, although the MIC of LZD for PVL-positive *S. aureus* was higher than that of VCM, LZD was more effective than VCM against PVL-positive *S.*

aureus infection *in vivo*. There are several explanations for this discrepancy. LZD may inhibit PVL production, probably resulting in the reduced pathogenicity of PVL-positive *S. aureus*, because LZD markedly suppresses translation of the PVL gene,¹³ and a subinhibitory concentration of LZD (but not VCM) inhibits the PVL level in a concentration-dependent manner.¹⁴ Secondly, LZD inhibits other staphylococcal virulence factors, enterotoxin A and B,¹⁵ bifunctional autolysin,¹⁵ and autolysin, protein A,¹⁵ also indicating the reduced pathogenicity. A third reason is that the time above MIC of LZD was similar to that of VCM in the previous study¹⁰, implying the equal efficacy of antibiotics. Thus, LZD was more effective against PVL-positive *S. aureus* infection *in vivo*, compared with VCM. Although the sub-MIC effect of LZD against PVL production was not examined in the present study, it needs to be examined in the future.

In conclusion, LZD clearly reduced bacterial numbers and inflammation in a mouse model of PVL-positive *S. aureus* hematogenous, and LZD improved the survival rate, as compared with VCM. LZD is thus considered to be clinically effective against PVL-positive *S. aureus*.

FUNDING SECTION

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TRANSPARENCY DECLARATIONS

None to declare.

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Figure legends

Figure 1. Effects of LZD and VCM on the number of bacteria in the lungs of mice with PVL-positive *S. aureus* hematogenous infection. Mice were treated with each agent for 1 day (a) or 3 days (b)

Figure 2. Histopathological examination of lung specimens from mice sacrificed 3 days after treatment for PVL-positive *S. aureus*. Each specimen exhibited typical features of bronchopneumonia with accumulation of neutrophils inside the bronchial lumen, infiltration of acute inflammatory cells, and exudates in the alveolar spaces (hematoxylin and eosin stain; original magnification, $\times 50$). (a) control, (b)

VCM-treated mice, (c) LZD-treated mice. Note that the severity of the inflammatory process is lower in LZD group as compared with the other 2 groups.

Number of abscesses in whole lungs (d) and per lung area (e) in the PVL-positive *S. aureus* infection model. Specimens were examined under a microscope, and lung area was determined by using cross-section paper.

Figure 3. Effects of LZD and VCM on the concentrations of cytokines and the survival rate of mice. Concentrations of TNF- α (a), IL-1 β (b), and MIP-2 (c) in the lung homogenates of mice infected with PVL-positive *S. aureus*. Mice were treated with each agent for 3 days after infection with PVL-positive *S. aureus*. These cytokine concentrations were assessed by ELISA.

Effects of LZD and VCM on the survival rate of mice (d). Mice were treated with each agent after infection with PVL-positive *S. aureus*. The survival rate was determined daily over a 7-day period.

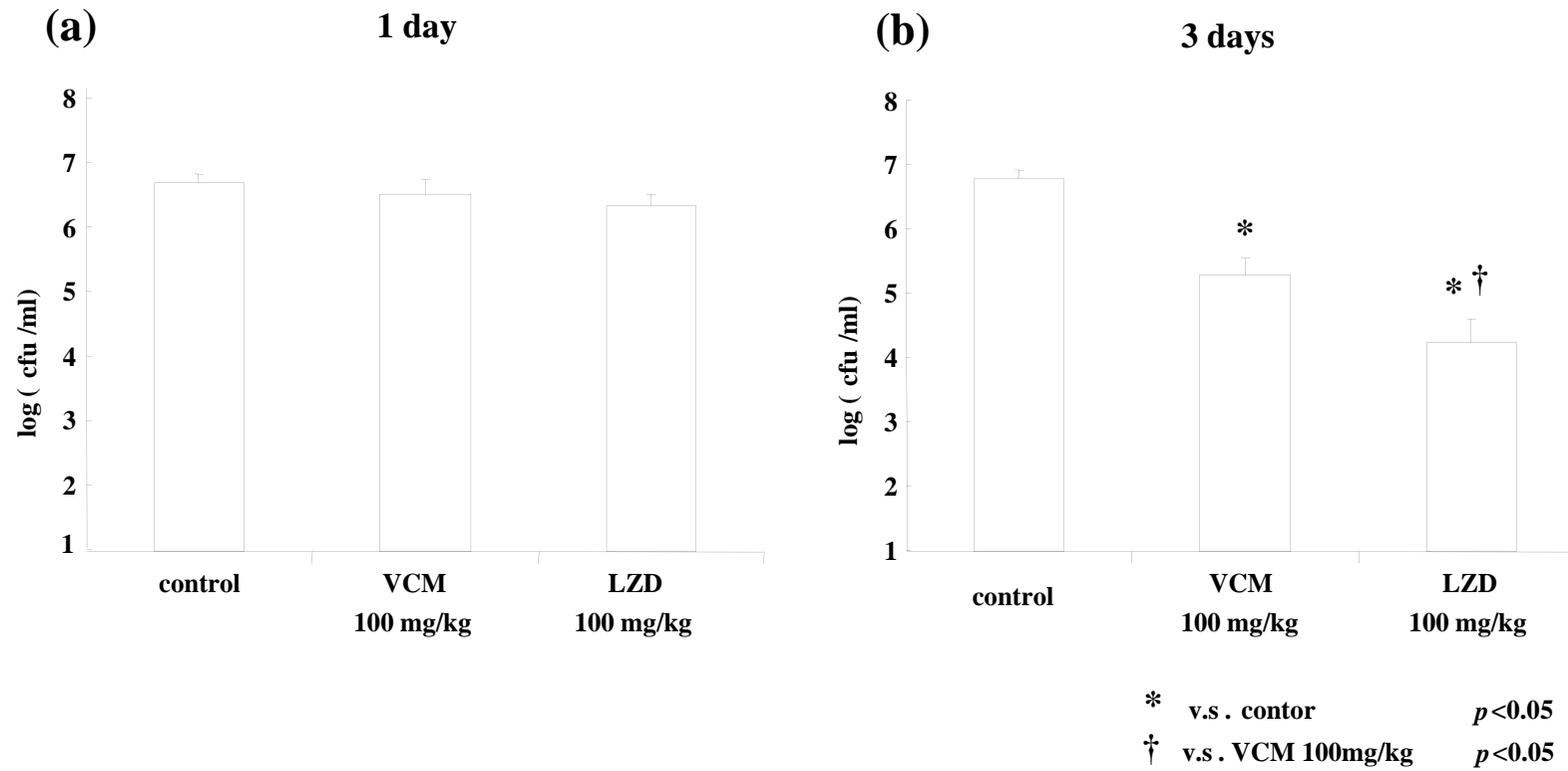
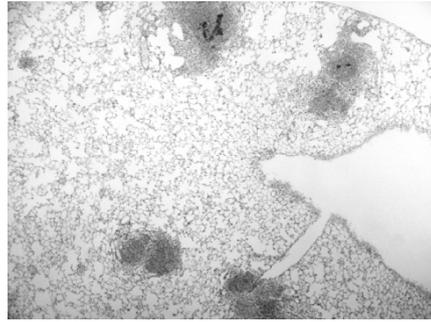
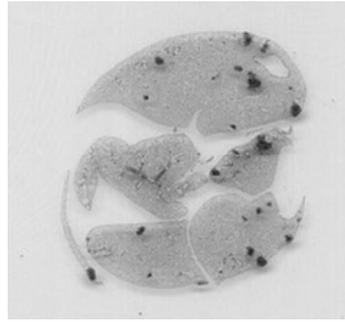
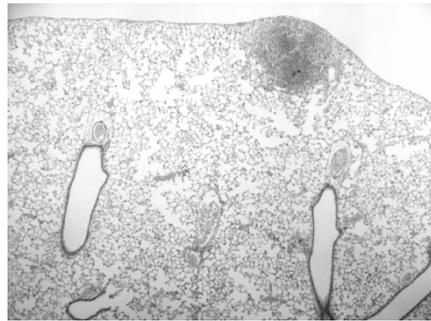
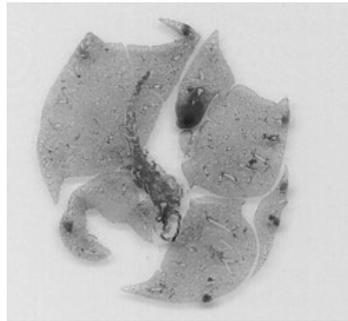


Figure 1.

(a) control



(b) VCM
100 mg/kg



(c) LZD
100 mg/kg

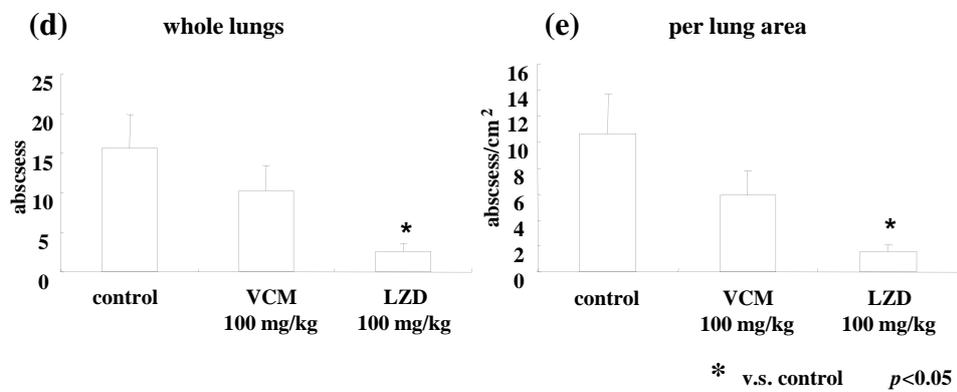
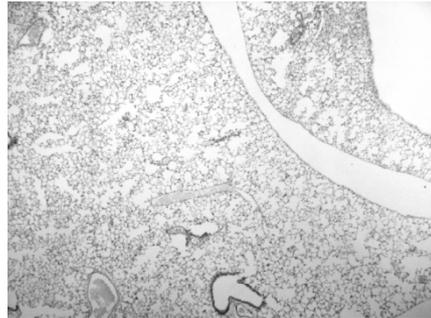
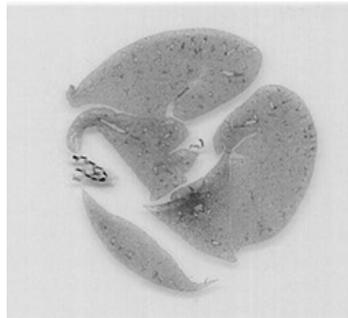


Figure 2.

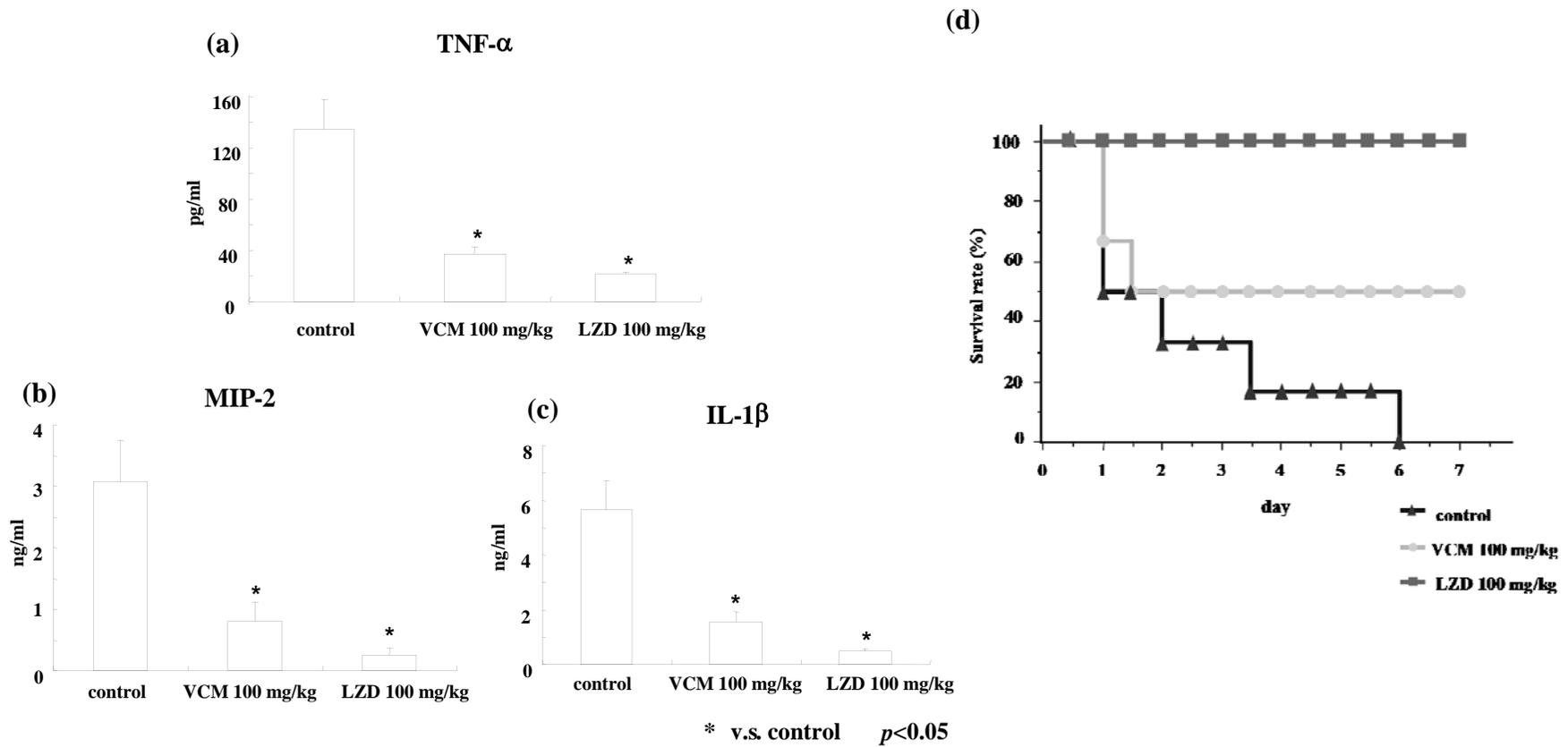


Figure 3.