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RESEARCH ARTICLE

Differential Profile of Cytokine TNF-A, MDA and SOD Level in Sepsis Induced By Cecal Ligation and Puncture Compared with Induced By LPS

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Abstract

Objective: The aim of this study is to analyse the differential of TNF-α cytokine profile, MDA, and SOD levels between sepsis induced in mice by cecal ligation and puncture (CLP) which were then compared with lipopolysaccharide (LPS) injection. Methods: This study made use of experimental animals, 28 mice divided into 4 groups: control (healthy mice), mice model of sepsis due to cecal ligation and puncture (CLP), a group of mice modelled sepsis due to LPS i.p injection of 103 (LPS1) and LPS 105 CFU i.p injection dose (LPS2). Afterwards, TNF-α levels were examined using sandwich enzyme immunoassay technique and calculation of MDA and SOD levels in blood, liver and kidneys. Results: The results showed that the level of TNF-α cytokines in mice model of sepsis with LPS induction of 105 CFU (LPS2) increased significantly ($p < 0.05$) compared to the sepsis model with CLP (LPS2: 1.11 ng/ml vs 0.34 ng/ml). MDA levels in serums, livers and kidneys of the experimental animals were also found to increase after LPS induction of 103 and 105 CFU doses. In contrast, SOD levels experienced a significant decrease due to LPS injection of dose 105 CFU in comparison with the CLP sepsis model. Conclusion: This study found that the best sepsis model was the one with LPS induction of 105 CFU doses which was characterized by a high production of TNFα cytokines, excessive MDA levels, and low levels of SOD antioxidant enzymes. By finding the best sepsis model, it can be used for therapeutic needs by targeting the inhibition of TNF-α proinflammatory cytokines and oxidative stress indicators MDA and SOD.

Keywords: *Cecal ligation and puncture (CLP), LPS, MDA, sepsis, SOD, TNF-α*

Introduction

Sepsis is the excessive body response to bacterial infections in blood, urine, lung, skin and other tissues [1, 2, 3]. It is characterized by fever, mental disorder, hypotension, decreased urinary excretion, and thrombocytopenia. Furthermore, this condition will develop into respiratory or kidney failure, coagulation abnormalities, and finally death [4, 5].If therapy is not received. 13 million people suffer from sepsis each year around the world, and 4 million die as a result [6]. Indonesia as a developing country, the mortality rate from sepsis is two to three times higher than that of The United States.

In 1996, there were 4774 patients admitted to Surabaya Teaching Hospital, with 504 of them being diagnosed with this life-threatening condition and a recorded mortality rate of 70.2% [7]. Sepsis can be caused by various microorganisms which include viruses, bacteria, fungi and protozoa. However, its main cause is lipopolysaccharide (LPS) exposure. LPS is a structural component of the outer membrane

of gram-negative bacteria, and it is a proinflammatory endotoxin [8]. It activates macrophages and leukocytes to □secrete proinflammatory cytokines, such as tumor

necrosis factor- α, interleukin-6 (IL-6), IL-1 and reactive oxygen compounds when it is released into circulation and TNF-□ will induce endotoxic shock. An increase in TNF-□ will be accompanied by the formation of reactive oxygen compounds in endotoxic shock [9]. Pathological conditions in sepsis (severe sepsis or septic shock) can affect almost every cell component, including endothelial cells, smooth muscle cells, leukocytes, erythrocytes, and tissues [10]. The treatment given to septic patients is immune system modulation therapy, with its target being the inflammatory mediator.

Targets for septic therapy such as anti-lipids, IL-1 receptor antagonists, platelet activating factor (PAF) inhibitors, anti-TNF-α monoclonal antibodies, immunoglobulins, high-dose corticosteroids, anti-endotoxin, and anti-thrombin III. Anti-TNF-α monoclonal antibodies, corticosteroids, and intravenous immunoglobulin administration bring about efficient clinical outcomes in patients suffering from severe sepsis and septic shock. Elimination of natural killer (NK) cells and T-cell activation can also serve as sepsis therapy [11, 12].

Thorough exposure to sepsis can be characterized by the secretion of TNF-α proinflammatory cytokines, and also high oxidative stress that can be monitored using the malondealdehyde indicator (MDA) and the superoxide dismutase (SOD) antioxidant enzyme. This study aims to determine differences between septic mice made with CLP and those with intraperitoneal LPS injection. It also aims to determine the better sepsis mice model among the 2 models through inflammatory parameters and oxidative stress.

Materials and Methods

Experimental Design

This study made use of experimental animals, 28 Rattus norvegicus rats which were divided into 4 groups namely: control group, group of mice model of sepsis due to cecal ligation and puncture (CLP), group of mice model sepsis due to LPS or Eschericia coli (E. coli) injection dose 103 CFU and a group of mice modelled sepsis due to LPS or E. coli injection dose 105 CFU.

Sepsis Mouse Model with CLP

Cecal ligation and puncture (CLP) measures were carried out in mice 24 hours prior to the serum collection. Before surgery, rats were treated with 20% urethane anesthesia (1 g/kgbw). Subsequently, abdominal incisions and caecal isolation were performed. The rat cecum was ligated with 3-0 silk at 0.5 mm from the end of the cecum, and the function on the opposite side of the 12 gauge needle was performed [13].

E. coli injection was administered intra-peritoneally in mice, as much as 1/5 ml (equivalent to 103 CFU of E.coli bacteria) and 1/10 ml (equivalent to 105 CFU of E.coli bacteria). They were then observed, and serum samples were taken after 24 hours.

Analysis of TNF-α Levels

The method used for TNF-□ examination was the quantitative principle of sandwich enzyme immunoassay technique. The principle is that monoclonal antibodies specific to TNF-□ are superimposed on micro plates, then the standard and sample pipetted to the well and the presence of TNF-□ will be bound by immobilized antibodies. After washing the unbound compounds, specific enzyme-linked monoclonal antibodies to TNF-□ are added to the well.

In the course of washing off compounds that are not bound to antibody-enzyme reagents, the substrate solution is added to the well and the colour will appear according to the proportion of TNF-□ that bounded in the initial stage. Colour development is stopped, and the intensity of the colour is measured. The inspection results will be read using a spectrophotometer (□= 450 nm). However, TNF-□ levels were calculated based on standard curves, and expressed in pg/ml.

Analysis of MDA and SOD levels in Serum, Liver and Renal

A total of 3-4 ml of blood from the animal without coagulant was centrifuged at 1000 rpm for 15 minutes. Blood serum obtained was divided into two, having a test serum and a control serum. For the first, hepatic and renal organs were homogenizated.

In the test and control serum supplemented with 100 µL of Thiobarbituric (TCA), vortex and 250 µL of HCl were added. After centrifuging at 500 rpm for 10 minutes, the supernatant was taken and then filtered with glass wool, after which 100 µL of Na-thiobarbiturate was added to the test serum. Vortex the test and control serum, then heated in a 100° C water bath for 20 minutes, and allowed to stand at room temperature.

The examination of serum MDA, liver and kidney is carried out using a spectrophotometer and reads absorbance at 529 nm. The SOD examination was conducted using a standard method developed in the Pharmacology Laboratory of the Medical Faculty of Brawijaya University. These methods were modification procedure from Bannister & Calabrese [14].

Statistical Analysis

Analysis of TNF-α, MDA and SOD levels was performed by ANOVA test using SPSS 17 for Windows 1.6 software with a significance level of 0.05 and 95% confidence level (α = 0.05).

Ethical Clearance

All procedures have been ethically feasible through the ethics commission of the Faculty of Medicine, Wijaya Kusuma University of Surabaya, Indonesia number 46/ SLE /FK/ UWKS/ VI/2013.

Results

The TNF-α proinflammatory cytokine levels

The TNF-α is one of the pro-inflammatory cytokines produced during sepsis. The results showed that LPS induced by doses 1 (103 CFU E. coli) and 2 (105 CFU E. coli) significantly increased TNF-α cytokines levels in the blood ($p < 0.05$) compared to controls (LPS2: 1.11 ng/ml, LPS1: 0.81 ng/ml, control: 0.52 ng/ml).

The amount of TNF-α in septic mice with LPS induction of 103 and 105 CFU doses was higher than the induction of caecal ligation and puncture (CLP) ($p < 0.05$) with TNF-α levels of 0.34 ng/ml. The results show that the induction of sepsis with LPS can trigger the production of TNF-α proinflammatory cytokines in experimental animal serum.

E. coli bacterial product, lipopolysaccharide (LPS) triggers NF-κB activation which plays a central role in the induction of cytokines and inflammatory mediators that cause sepsis.

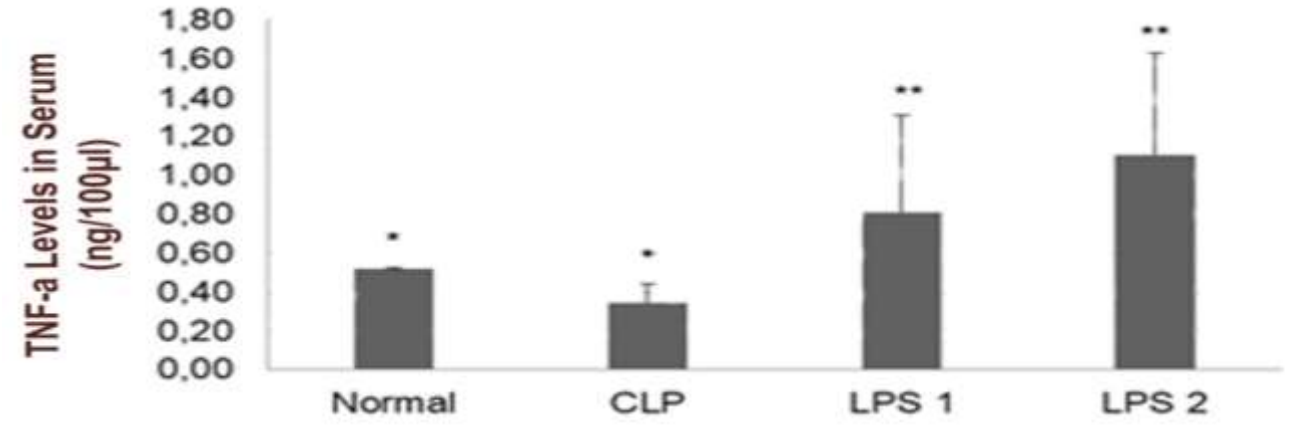

Figure 1: The TNF-α levels in experimental animal serum

MDA levels in Serum, Liver and Kidneys

This study also calculates the MDA levels in the blood serum of experimental animals in each treatment to determine the level of oxidative stress via the MDA indicator. LPS induction of doses 1 (103 CFU E. coli) and 2 (105 CFU E. coli) proved to be capable of significantly increasing MDA serum levels ($p < 0.05$) compared to controls (Figure 2A).

The sepsis induction model with LPS also had higher MDA levels compared to that with CLP. Successively, MDA levels in each treatment were 0.15 ng/100 µl (LPS2), 0.13 ng/100 µl (LPS1), and 0.06 ng/100µl (CLP). Furthermore, the same increase was found in MDA levels of the kidneys and liver of experimental animals. Sepsis induction using LPS dose 1, dose 2 and CLP showed a significant increase in MDA levels ($p < 0.05$)

in kidney organs compared to controls, respectively 0.27ng/100µl (LPS1), 0.27 ng/100 µl (LPS2) , 0.25 ng/100 µl (CLP) and 0.12 ng/100 µl (Normal) (Figure 2B).

The liver organ was found to have significantly increased MDA levels in the sepsis model with LPS dose 1 or 2 compared to that with CLP (LPS2: 0.21ng/100µl; LPS1: 0.18 ng/ 100µl; CLP: 0.13 ng/100 µl; Normal: 0.04 ng/100 µl) (Figure 2C).

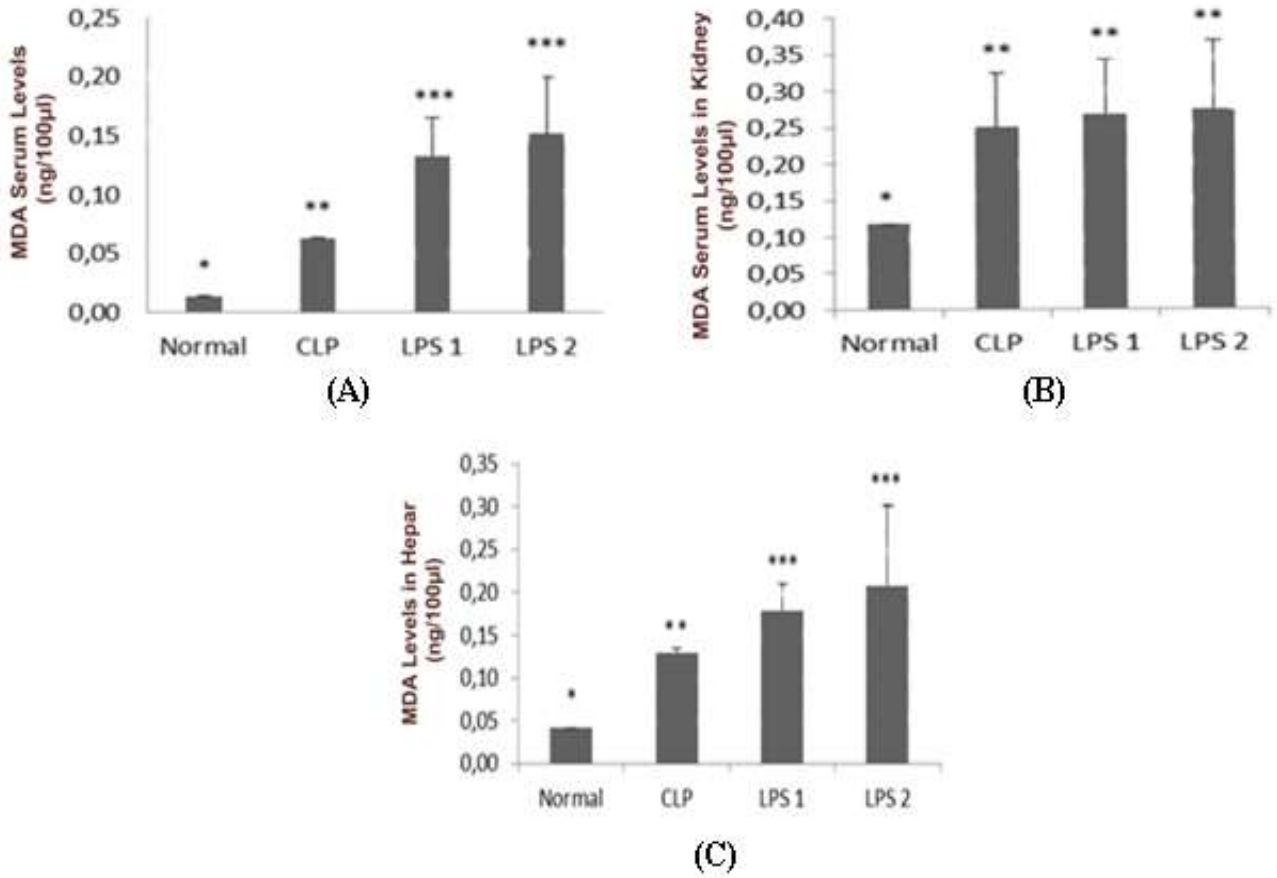

Figure 2: The result of (A) MDA levels in experimental animal serum; (B) MDA levels in the kidneys of experimental animals; (3) MDA levels in the liver of experimental animals

The SOD Levels in Kidneys and Liver Animals

Sepsis disease often causes septic shock and organ damage. One of the most common complications of this disease is acute kidney damage caused by oxidative stress. Hence, this study also calculated the levels of antioxidant enzymes that can regulate the presence of oxidative stress in cells. Based on the results of this study, the presence of this stress is characterized by high serum MDA levels in experimental animals with LPS induction.

It was also observed that the level of SOD in renal animal models of sepsis with a dose of LPS2 (105 CFU E. coli) 8.19 ng/100 µl showed a significant decrease ($p < 0.05$) compared to normal rats 14.17 ng/100 µl. This result shows that a higher oxidative stress brings about a decrease in the SOD antioxidant enzyme level. As regards the SOD level in the animals, the CLP model was higher than the LPS induction of dose 1 or 2 (Figure 3A). The SOD levels were found in the liver of the sepsis model with dose-induced LPS induction, where LPS induction of 105 CFU E. coli 4.94 ng/100 µl also decreased significantly compared to the normal mice with no treatment 12.49 ng/100 µl ($p < 0.05$) (Figure 3B). In comparison with the CLP model, the SOD level in the LPS dose 2 is still lower (CLP: 9.76 ng/100 µl).

It shows that the greater oxidative stress shown by MDA levels, the lower level of the antioxidant enzyme Superoxide Dismutase (SOD). Sepsis model with LPS dose 2 or 105 CFU E. coli is possibly the best sepsis model which is characterized by high production of TNF-α proinflammatory cytokines, high serum MDA levels and low SOD antioxidant enzymes in the kidneys and liver.

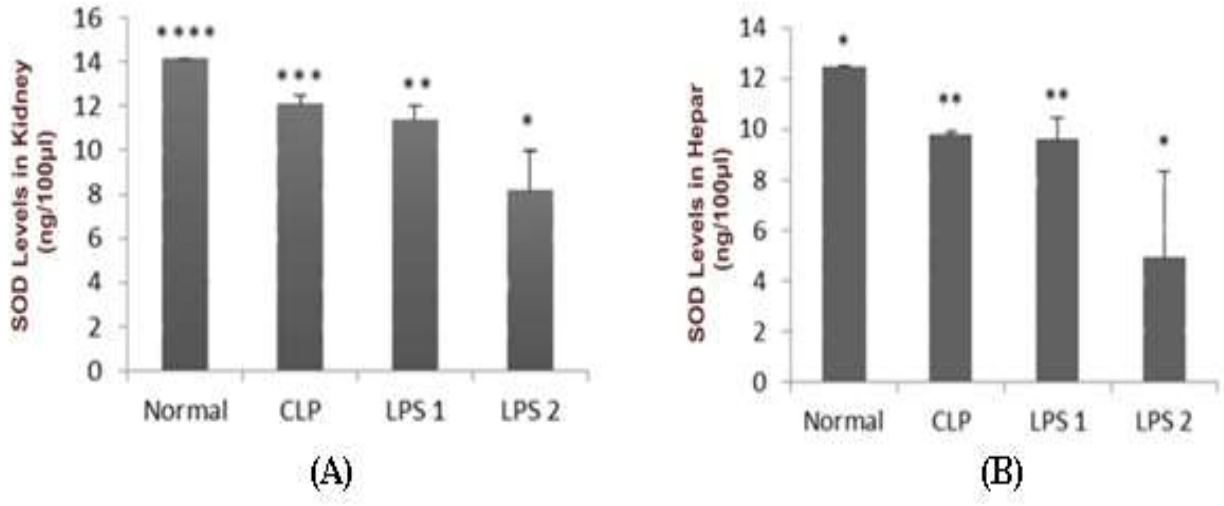

Figure 3: The results of (A) SOD levels in the kidneys of experimental animals, and (B) SOD levels in the liver of experimental animals

Discussion

Sepsis is one of the highest causes of death with mortality rates ranging from 30-70%. It is a chronic disease caused by endotoxin exposure to bacteria. Bacterial products such as lipopolysaccharide (LPS) play a crucial role in the septic shock. LPS is a major component of the outer membrane of gram-negative bacteria which can activate a systemic inflammatory response or Systemic Inflamatory Response Syndrome (SIRS).

SIRS can cause septic shock and Multiple Organ Failure (MOF) [15]. The existence of LPS can directly induce cellular and humoral immune systems and lipopolysaccharide antibody production (abLPS). T-lymphocytes secrete Th1 cytokines, namely: IFN-γ, IL-2, and colony stimulating factor macrophage (M-CSF0) as an effort to protect against sepsis. Th2 lymphocytes secrete cytokines IL-4, IL-5, IL-6, and IL-10.

IFN-γ stimulates macrophages to excrete IL-1ß and TNF-α. In sepsis, IL-2 and TNF-α can damage the vascular endothelium. IL-1ß also plays a role in the formation of prostaglandin E2 (PG-E) and stimulates intercellular adhesion molecule-1 (ICAM-1) expression. ICAM-1 plays a role in the neutrophil adhesion process with endothelial. These neutrophils will release lysozyme which causes endothelial wall lysis. They also carry free radical antioxidants which will affect mitochondrial oxygenation and then results in vascular endothelial damage. This damage will cause vascular disorders resulting in multiple organ damage [16, 17]. In recent times, cytokines have been intensively studied in several cases of sepsis. Research by Eskandari et al. has examined the role of TNF-α cytokines in the CLP sepsis model where cytokine levels increase after 12 hours of CLP induction.

In addition, the study also found TNF-α level to significantly increase after 90 minutes of LPS induction in comparison with the CLP model. In line with our study, it was found that TNF-α level in the sepsis-induced model with LPS were higher than the CLP model. This suggests that the sepsis induction model using LPS is more optimal in triggering the production of TNF-α cytokines compared to the model with CLP [18].

Villa et al. stated that there was no biological activity of TNF-α in the plasma of CLP animal model at some point in time [19]. However, an increase in TNF-α level occurs at the inflammatory site and peritoneum. Unlike LPS or endotoxin models that show rapid increases in TNF-α within 2 hours, the CLP model does not show its activation characterized by high mortality.

In this study, the comparison of cytokine activity and levels of oxidative stress was carried out on two sepsis models namely, lipopolysaccharide induction (LPS) and cecal ligation and puncture (CLP) models. Based on the results, the best sepsis model is by LPS induction of 105 CFU doses which is characterized by a high production of TNF-α cytokines.

Remick et al. proved that both models of sepsis had the same mortality and morbidity rate of 85%. Levels of TNF cytokines, IL-1, IL-6 and chemokines KC and MIP-2 in plasma and peritoneum sepsis of experimental animal with LPS were higher than those in septic models with CLP. The number of cytokines increased 1.5 hours and 4 hours after LPS induction, and decreased 8 hours after.

In contrast, cytokine levels in the CLP model increased after 8 hours, exceeding the number in LPS induction [20]. Oxidative stress is an imbalance between antioxidant defences and the production of reactive oxygen compounds. The latter can cause severe tissue damage from its reaction with lipids, proteins and DNA. They are produced by endogenous cellular substances which include mitochondria, cytochrom P450, peroxisomes and exogenous from environmental sources such as ultraviolet light, ionizing radiation and pollution.

Cell membranes are the most vulnerable sites for damage to reactive oxygen compounds. Free radicals can react with fatty acid cell membranes and form lipid peroxides. Cell membrane damage via lipid peroxide can cause interference with the fluidity and elasticity of the membrane, which triggers cell rupture.

This change will affect the life of the cell, while lipid peroxides are known to measure MDA levels [21]. The MDA is an indicator that can be used as a sign of fat peroxidation. The presence of this MDA compound can show the activity of free radicals in cells which makes it capable of being used as an indicator of oxidative stress due to free radicals [22, 23, 24]. Lipopolysaccharide (LPS) in E. coli bacteria triggers NF-kB activation which plays a central role in the induction of cytokines and inflammatory mediators that cause the pathophysiology of sepsis.

During inflammatory stimulation, translocation of NF-κB from the cytosol into the cell nucleus induces the expression of a large number of genes involved in the inflammation, and they include encoding cytokines (IL-1, IL-6 and TNF-α), molecules adhesion, acute phase proteins, and enzymes such as nitric oxide syntethase (NOS) [21].

High MDA levels in plasma are caused by tissue damage due to oxidative stress. The results of this study indicate that MDA levels in serum, kidney and liver increased significantly in mice model of sepsis due to 105 of LPS induction doses.

This indicates that the sepsis model with LPS shows greater tissue damage compared to the CLP model. A response to septic infection is a change in the immune system both humoral and cellular to eliminate infectious microbes through the formation of antibodies, complement and secretion of inflammatory mediators. However, systemic inflammatory responses to bacterial infection are the secretion of proinflammatory cytokines, adhesion molecules, vasoactive mediators and formation of SOR.

Furthermore, loss of balance between proinflammatory and anti-inflammatory cytokines in the body underlies the occurrence of sepsis which results in SOR formation and increased levels of MDA [25].Decreased levels of the antioxidant enzyme Superoxide dismutase (SOD) in the sepsis group induced by LPS indicates the occurrence of lipid peroxidation [26].

It can be concluded from this study that the greater the degrees of sepsis, the more free radicals are released and the decrease in antioxidants which will lead to an increase in oxidative stress. This increase in oxidative stress can significantly increase MDA levels in serum, kidney and liver in sepsis rats LPS induction model.

Conclusion

LPS inductions by 105 CFU doses were better used as septic mice model than the CLP-induced mice. This is characterized by the high production of TNF-α cytokines, excessive MDA levels, and low levels of SOD antioxidant enzymes in serum, kidneys and liver. By identifying the best sepsis model, it can be used for therapeutic needs with the target of inhibiting proinflammatory cytokines and oxidative stress.

Abbreviation

CFU: Colony Forming Unit

CLP: Cecal Ligation and Puncture

MDA: Malondialdehyde

SOD: Superoxide Dismutase

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