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Label-Free Assessment of Vericiguat Therapy on Mitochondrial Redox States in Septic Mice by Resonance Raman Spectroscopy

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ABSTRACT: Sepsis is a life-threatening infectious disease. Mitochondrial dysfunction is widespread in severe sepsis. The myocardium contains a large number of mitochondria, and the survival rate of sepsis decreases sharply when cardiac dysfunction is involved. Vericiguat (BAY 1021189) is a novel drug for the prevention of heart failure. In this study, we evaluated the mitochondrial function of septic mice and drug-treated mice by resonance Raman spectroscopy (RRS). RRS is a non-invasive and label-free technique that can identify molecular vibrations by their unique fingerprints, making it ideal for quantitative studies. By choosing 532 nm as the excitation wavelength, which is close to the absorption peak of cytochrome, we can greatly enhance the Raman signal of mitochondrial redox state. RRS can accurately identify the Raman characteristic peak at 750 cm^{-1}, 1128 cm^{-1} and 1585 cm^{-1} attributed to the reduced cytochrome in septic mice. We found that the intensity of the characteristic peak was significantly decreased in septic mice, indicating an imbalance of mitochondrial redox function, while the function was improved in the drug-treated group. It proves that BAY has the potential as a novel treatment for mitochondrial dysfunction in sepsis.

1. INTRODUCTION

Sepsis is a life-threatening multi-organ dysfunction syndrome caused by infection, which is attributed to the virulence and drug resistance of pathogenic microorganisms and immune dysfunction. In severe sepsis, there is widespread mitochondrial dysfunction. Mitochondria are vital organelles for energy conversion, biosynthesis and signal transduction, sensing and integrating molecular signals, and participate in the regulation of cell growth, metabolism and death [1]. Mitochondrial damage is one of the key factors involved in the pathogenesis of sepsis. Mitochondria regulate intracellular Ca^{2+} homeostasis, reactive oxygen species (ROS) production, and cytochrome C release. The survival rates of critical diseases is related to mitochondrial function [2–5]. Cardiomyocytes contain a large number of mitochondria. Sepsis mortality increases significantly when cardiac function degrades.

So far clinical treatment to sepsis has not significantly reduced sepsis mortality. Protecting heart function and mitochondrial function may be two essential starting points for improving sepsis survival. Vericiguat (BAY 1021189) is a novel oral cardiac protectant for the prevention of heart failure deterioration, which targets activation of intracellular soluble guanylate cyclase and induces cyclic guanosine monophosphate (cGMP) generation [6, 7]. The effect of cardiac protective agent BAY on mitochondrial function in sepsis remains unclear. In this paper we evaluate the effects of BAY on the mitochondrial damage in sepsis using lipopolysaccharide (LPS) mice models by resonance Raman spectroscopy (RRS).

Most of the current methods for detecting mitochondrial function either require isolation and purification of mitochondria or rely on fluorescent probes, which may affect the normal function of mitochondria [8–10]. Raman scattering is an optical process where monochromatic light, typically from a laser, interacts with a sample [11]. This interaction results in inelastically scattered light that has shifted in energy due to the vibrational modes of the sample's chemical bonds. This shift provides information about these vibrational modes. In Raman spectra, lines with frequencies less than the incident light are called Stokes lines, while those with frequencies greater are called anti-Stokes lines. Stokes lines, which represent scattered photons that have lost energy (redshifted), are typically more intense as most electrons in molecules at room temperature primarily occupy the vibrational ground state. Hence, Stokes scattering is commonly detected in the Raman analysis [12]. The detection of Raman signal requires a much higher spectral resolution as compared to hyperspectral detection [13, 14], RRS is a specific technique that increases the intensity of Raman scattering when the incident photons match an electronic transition's energy. RRS is non-invasive and label-free, producing

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sharp spectral peaks that can identify molecular vibrations by their unique fingerprints, making it ideal for quantitative studies. The excitation wavelength of RRS is usually located in the ultraviolet-visible absorption region, making the laser wavelength equal or close to the absorption peak of the material, which will greatly increase the intensity of Raman signal, and thus lower the limit of detection and reduce the measurement time, making it suitable for the detection of some biological samples. Several studies have reported that RRS could be used to detect mitochondrial function in both isolated cells and tissues [15, 16].

In this study, we use a label-free RRS method to evaluate the mitochondrial redox state in blood and myocardium of septic mice. To investigate the improvement effect of BAY on mitochondrial function in sepsis, we treat septic mice with this novel drug. We hypothesize that BAY can improve the mitochondrial redox state and cardiac function of septic mice by activating sGC. The objectives of this study are to, (1) measure the Raman characteristic peaks of reduced cytochrome in septic mice and drug-treated mice; (2) compare the intensity and ratio of these peaks to reflect the changes of mitochondrial redox state; (3) evaluate with RRS the cardiac mitochondrial function in septic mice and drug-treated mice.

2. MATERIALS AND METHODS

2.1. Animals

Male C57BL/6 mice (7–9 weeks old, weight 20–28 g; from Shanghai Slac Laboratory Animal Co., Ltd, and Hangzhou Qizhen Laboratory Animal Technology Co., Ltd., China) were used in the Laboratory Animal Center of Enze Medical Center (Group) in Taizhou, Zhejiang Province. The mice were kept in a pathogen-free barrier environment under standardized conditions, with a 12-hour light/dark cycle (lights on from 8 a.m. to 8 p.m.) and free access to food and water. All animal experiments were approved by the Experimental Animal Care and Use Committee of Enze Medical Center (Group) (ID, tzy2023218, ID, tzy-2022146).

2.2. Lipopolysaccharide (LPS) Mice Model

LPS administration is one of the most widely used methods of sepsis models in animal studies, which can induce severe sepsis and acute multiple organ failure. Human is also sensitive to LPS. We make choices based on operability and repeatability of LPS-induced sepsis. LPS is an important virulence factor in multidrug-resistant Gram-negative bacteria. The binding of LPS with Toll-like receptor 4 (TLR4), myeloid differentiation factor 2 (MD-2), cluster of differentiation 14 (CD14) and lipopolysaccharide binding protein (LBP), triggered the inflammatory cascade signal (including expression of cytokines and inflammatory molecules), and consequently multiple pathways of systemic inflammatory responses, leading to LPS dose-dependent multi-organ damage and acute organ dysfunction. Downstream signaling pathways activated by TLR4 are highly conserved in all animal species (including humans). Advantages of being an inducer include ease of operation, repeatability and ease of standardization. Disadvantages include the limited range of pathogenic microorganisms carrying LPS, which is more consistent with sepsis whose pathogenic organism is Gram-negative bacteria.

Intraperitoneal (IP) injection of 18 mg/kg body weight LPS (O55:B5, L2880, Sigma) induces excessive immune inflammation with organ dysfunction in mice [17, 18]. According to our experimental results, the C57BL/6 mice septic model requires one to three doses of LPS administration. LPS induced myocardial mitochondrial damage requires two injections of LPS (IP.), once per day.

2.3. Therapeutic Agents

For the BAY treatment experiment, our mice were administrated with BAY (Vericiguat, BAY 1021189, Bayer.) 12.5 mg/kg body weight (BAY is always orally administrated throughout this paper), once per day for two days prior to the LPS injection. Our mice were randomly divided into groups, 6 mice in each group, which were labeled as control group, LPS group, LPS+ BAY group. In the experiment of RRS monitoring for five days, 3 doses of LPS were injected in total, and 4 doses of BAY were orally administrated (2 doses before LPS and 2 after the 1st LPS, one dose per day).

2.4. RRS System

A single-mode fiber-coupled 532 nm laser (FC-D-785-400mW, Changchun) and a fiber-type Raman spectrometer (QE Pro-Raman, Ocean Insight) was connected to the excitation and collection fibers of a Raman fiber probe (RPB-532, Oceanhood, China), respectively. The laser output power and the spectrometer integration time were adjusted to optimize the signal-to-noise ratio. The probe tip was brought close to or in contact with the sample surface, keeping a certain working distance to avoid damaging the probe or the sample. The laser and the spectrometer were turned on and Raman scattering signals were collected, observed and recorded. If different positions or samples were measured, the above steps were repeated until all measurements were completed. In this study, 10 Raman spectra were measured and averaged for each sample under 532 nm excitation wavelength, with a collection time of 15 s and a laser power of 25 mW.

2.5. RRS Spectra of Blood

The real-time blood samples of the mice were obtained by tail vein bleeding and Raman signals of the blood at different time points were recorded. Raman spectral measurements were performed on at least 10 random points for each sample. The normal groups, the LPS groups, and the drug treatment groups ($n = 6$ for each group), totaling 3 groups, were represented by the average spectra of each type of groups.

2.6. RRS Spectra of Myocardium

The mice were euthanized by cervical dislocation under anesthesia. Then, the cardiac tissue slices were obtained and the Raman spectra of the myocardium were recorded. Raman spectral measurements were performed on at least 10 random points for

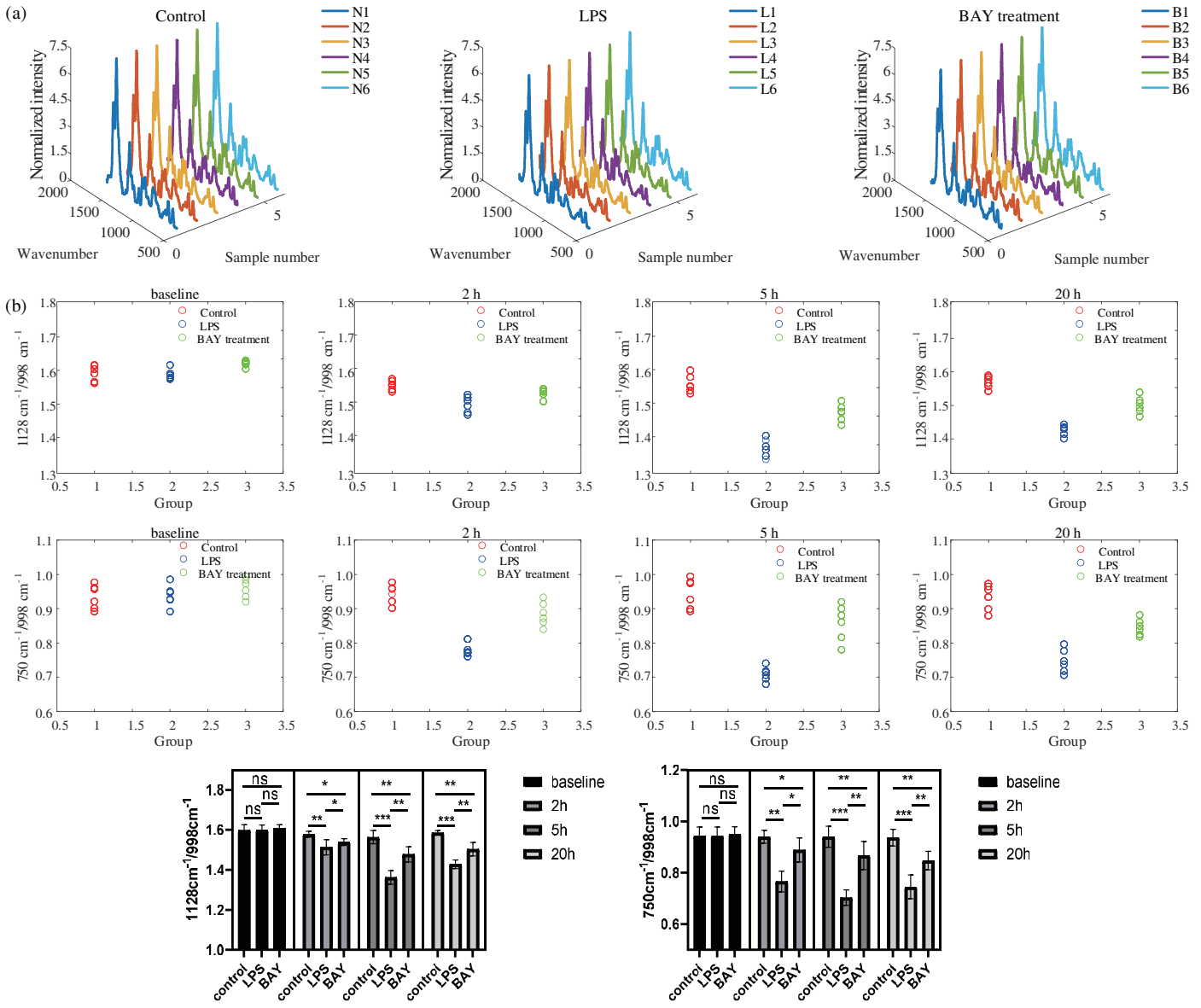

FIGURE 1. Raman spectra of mouse blood responded to redox reaction in mitochondrial during several hours. (a) Comparison of the Raman spectra of the control group, LPS model group, and BAY treatment group at 5 h after LPS injection; (b) Comparison of the normalized intensity at 1128 cm^{-1} and 750 cm^{-1} of the control group, LPS model group, and BAY treatment group at baseline, then 2 h, 5 h, and 20 h after LPS injection. $p < 0.0001$ (one way ANOVA analysis). The data were also presented as the mean $\pm$ SE. P values are indicated by *, ** and *** for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. ns means no significant differences.

each sample. The normal groups, the LPS groups, and the drug treatment groups ($n = 3$ for each group), totaling 3 groups, were represented by the average spectra of each type of groups.

3. RESULTS AND DISCUSSION

3.1. Analysis of BAY Therapy on Mitochondrial Redox States of Mice Blood by RRS

We used a 532 nm laser to excite the Raman signal of mouse blood, and the characteristic peaks were accurately identified at 750 cm^{-1}, 1128 cm^{-1} and 1585 cm^{-1}, which are the symmetric vibrations of porphyrin, the vibrations of C_b-CH_3 side radicals and methyl bridges (C_aC_m, C_aC_mH bonds), respectively. They are all sensitive to the reduced cytochrome Raman bands, mainly involving cytochrome (cyt) c and cyt b (Fe^{2+}) [19–21]. Oxidative stress can decrease the level of reduced cytochrome, which means the electron transport chain (ETC) on the mitochondrial inner membrane is damaged or inhibited, thereby resulting in the accumulation of the levels of oxidized nicotinamide adenine dinucleotide (NADH) and the decreased amounts of reduced nicotinamide adenine dinucleotide (NAD^+), which reflects the mitochondrial redox dysfunction. This can impair the mitochondrial redox function and affect the cellular energy metabolism and signaling pathways. Using 532 nm excitation light, cyt c and cyt b could be selectively enhanced by such a resonance-enhancement effect and can be clearly visualized by Raman spectra. Due to the com-

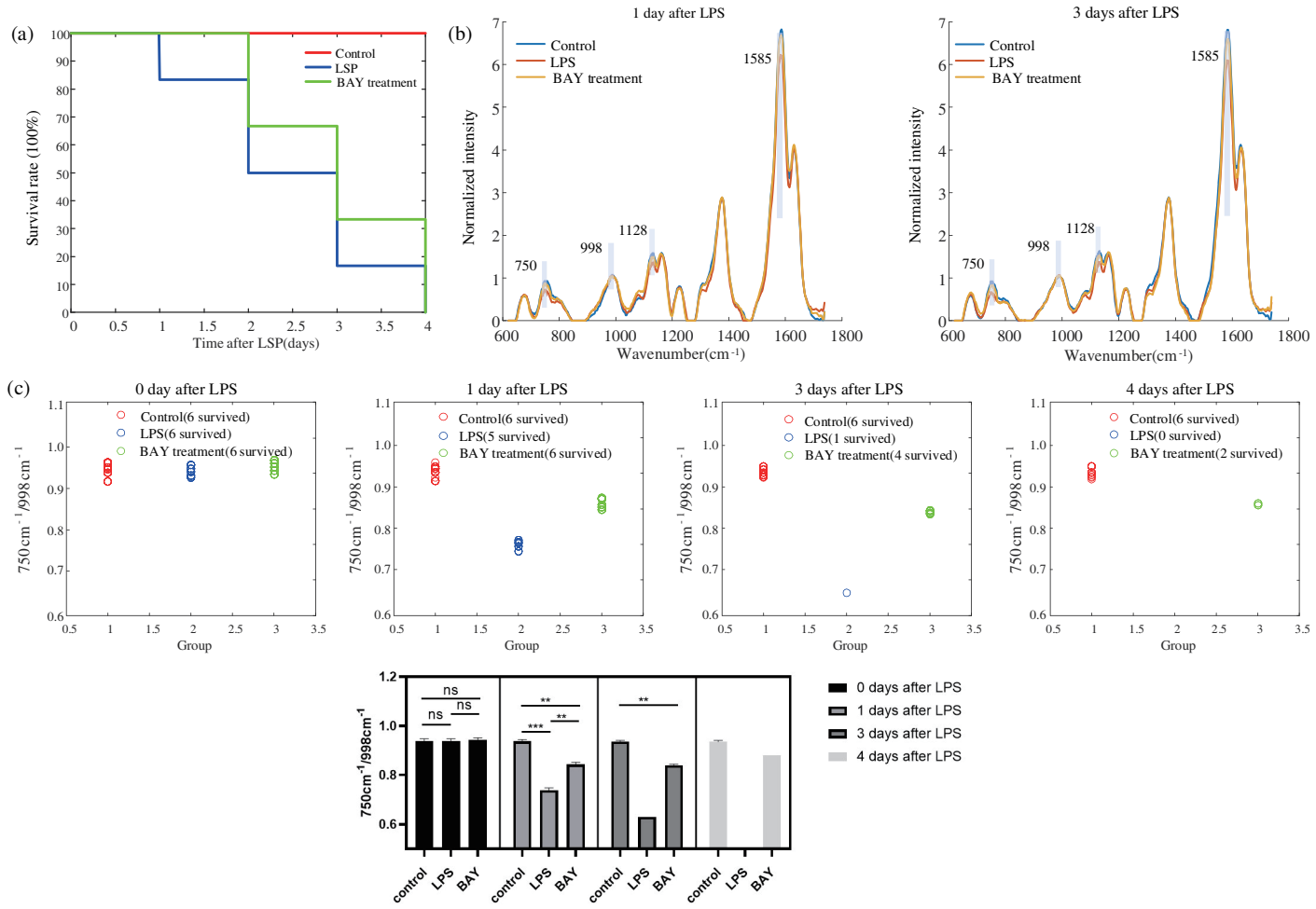

FIGURE 2. Raman spectra of mouse blood responded to redox reaction in mitochondrial during several days. (a) The survival rate of the control group, LPS model group, and BAY treatment group; (b) Comparison of the average Raman spectra of the control group, LPS model group, and BAY treatment group at 24 hours and 72 hours after LPS injection; (c) Comparison of the normalized intensity at 750 cm^{-1} of the control group, LPS model group, and BAY treatment group at 0 day, 1 day, 3 days and 4 days after LPS injection.

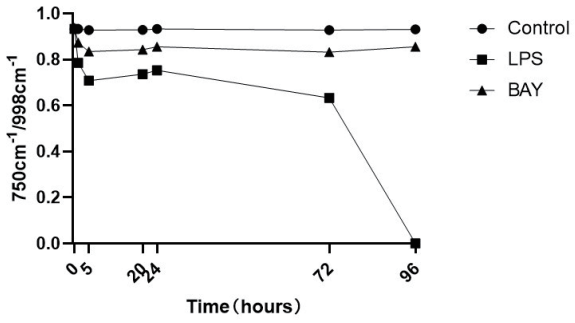

FIGURE 3. Comparison of the normalized intensity at 750 cm^{-1} of the control group, LPS model group, and BAY treatment group at different time points after LPS injection.

plexity, individuality and diversity of blood, an internal standard must be selected to ensure that the correct data are collected. The Raman band located at wavenumber shift 998 cm^{-1} corresponds to the breathing mode of phenylalanine [22]. Here, the 998 cm^{-1} peak was set as a reference to quantify mitochondrial redox states, as it shows the stable conformational changes of proteins to normalize the spectra.

We measured the blood RRS of the control group, LPS model group, and BAY treatment group 2 days before LPS injection (baseline, as 0 time point), 2 h, 5 h, and 20 h after LPS injection, and observed that the intensity of the characteristic redox peaks near 750 cm^{-1}, 1128 cm^{-1} and 1585 cm^{-1} in the LPS model group was significantly reduced compared with those of the control group, while the characteristic peaks of the BAY treatment group were significantly increased compared with those of the LPS model group, indicating that the BAY treatment can improve the mitochondrial function. However, the band 1585 cm^{-1} may overlap the band of other proteins [19]. Here, we used the peaks at 1128 cm^{-1} and 750 cm^{-1} to analyze mitochondrial redox states. At the 5th hour after LPS injection, the mitochondrial redox status of the three groups differed the most, but at the 20th hour after LPS injection, the mitochondrial function of the LPS model group and the BAY treatment group had a slight improvement (Figs. 1(a), (b)).

In addition, we continuously monitored for five days until all the mice in the LPS model group died. Here, we used the

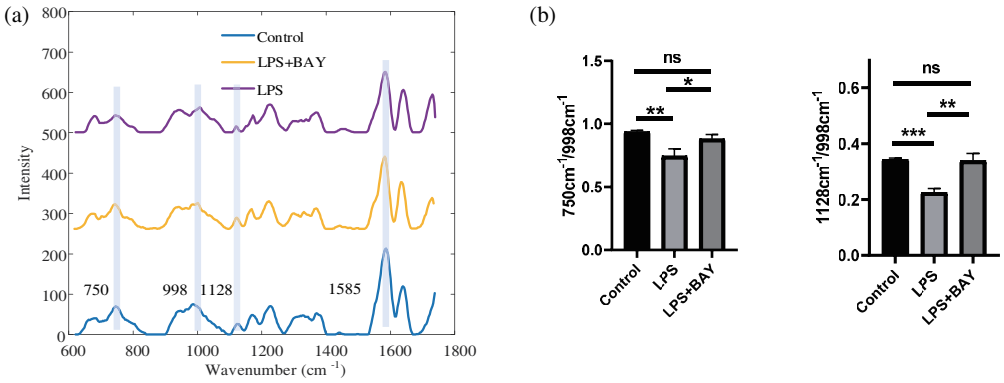

FIGURE 4. Raman spectra of mouse myocardium responded to redox reaction in mitochondria. (a) Comparison of the average Roman spectra of the myocardium of the three groups. (b) Comparison of the normalized intensity at 750 cm^{-1} and 1128 cm^{-1} of the myocardium of the three groups, $p < 0.0001$ (One way ANOVA analysis).

peak at 750 cm^{-1} to analyze mitochondrial redox states. Three days after LPS injection, only one mouse survived in the LPS model group, and all mice in the LPS model group died four days after LPS injection, while two mice survived in the BAY treatment group (Figs. 2(a), (b), (c)). During this period, the mitochondrial redox states of the BAY treatment group were always worse than those of the control group, but better than those of the LPS model group. This suggests that BAY treatment is effective (Fig. 3).

3.2. Analysis of BAY Therapy on Mitochondrial Redox States of Mice Cardiomyocytes by RRS

Mitochondria are the main organelles for energy supply in cardiomyocytes, and their homeostasis (structural and functional stability) is essential for maintaining cardiac perfusion. Sepsis can affect cardiac mitochondrial function through various pathways, leading to cardiomyocyte damage and cardiac dysfunction. Therefore, protecting mitochondrial function may be an important direction for treating septic cardiomyopathy.

To demonstrate whether our drugs also have protective effects on the myocardium, we measured the Raman signals of the myocardia in different groups of mice, and observed that the intensity of the characteristic redox peaks near 750 cm^{-1}, 1128 cm^{-1} and 1585 cm^{-1} in the LPS model group was significantly reduced compared with those of the control group, while the characteristic peaks of the BAY treatment group were significantly increased compared with the LPS model group (Fig. 4(a)).

Here, we used the peaks at 750 cm^{-1} and 1128 cm^{-1} to further analyze mitochondrial redox states and observed that the intensity of the characteristic redox peaks near 750 cm^{-1} and 1128 cm^{-1} in the drug treatment group was significantly improved compared with those in the LPS model group. The results showed that the BAY drug had the useful effect on repairing myocardial mitochondrial damage (Fig. 4(b)).

4. CONCLUSION AND OUTLOOK

In this study, we have evaluated the mitochondrial function of septic mice and drug-treated mice by RRS, and found that BAY drug gave the better results as a novel potential treatment for sepsis. Mitochondrial Raman detection at wavenumber shifts of 750 cm^{-1}, 1128 cm^{-1} and 1585 cm^{-1} has the advantage of convenient and reliable analysis of mitochondrial function. The mitochondrial redox imbalance of blood and myocardium in LPS mice were quantified by the reduction of Raman peak intensity at 750 cm^{-1} and 1128 cm^{-1}. RRS can be used not only to evaluate mitochondrial function, but also to analyze other types of biological tissues and diagnose diseases. For example, RRS can be used to detect tumor tissues, skeletal muscle tissues, etc., providing information about their structure and composition. The applications of RRS in different fields demonstrate its advantages and potential, providing a new idea and method for medical analysis.

We found that BAY can protect the redox capacity of mitochondria in myocardium and blood of septic mice. BAY can also improve the LPS-mediated reduction of myocardial mitochondrial redox and protect myocardial mitochondrial function. At the same time, BAY can restore damaged mitochondrial redox in blood samples from sepsis and has the potential to be an important part of sepsis treatment strategies.

Our study suggests that BAY treatment is effective, but the effect is limited and needs to be supplemented with other drugs or treatment. The present BAY treatment can be combined with other drugs to improve further the mitochondrial function of septic mice and this can be a future research direction.

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