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Anisodamine hydrobromide attenuates oxidative stress and proinflammatory cytokines in septic rats induced by cecal ligation and puncture

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ABSTRACT

We previously demonstrated that anisodamine hydrobromide (Ani HBr) ameliorates septic organ injury induced by lipopolysaccharide. The present study is aimed to explore the role of Ani HBr in protecting the organs against inflammation and oxidative stress in septic rats induced by cecal ligation and puncture. A total of forty-two rats were randomly divided into sham (sham operation), septic shock, Ani HBr, atropine, and racemic anisodamine (Rac Ani) groups. Ani HBr (1.8, 3.6, and 5.4 mg/kg), atropine (5.4 mg/kg), and Rac Ani (5.4 mg/kg) were administrated to septic rats. After 24 h, the plasma and organs including brain, heart, liver, lung, kidney, and intestine were obtained. Then, H&E staining and TUNEL staining were performed. The proinflammatory factors TNF-α and IL-6 and oxidative stress markers superoxide dismutase (SOD) and malondialdehyde (MDA)in plasma were detected by ELISA. H&E staining showed that the tissues in the brain, heart, liver, lung, kidney, and intestine in the septic shock group were seriously damaged. Consistently, TUNEL staining showed an increase of apoptotic cells in those tissues. Ani HBr treatment alleviated the injury and apoptotic cells in all those organs in septic rats. Ani HBr, atropine, and Rac Ani reduced the plasma TNF-α and IL-6 levels in septic rats, whereas 5.4 mg/kg Ani HBr reduced the cytokines more than Rac Ani. Ani HBr raised SOD activity and reduced plasma MDA levels in a concentration-dependent manner, which at 5.4 mg/kg were greater than atropine and Rac Ani. Therefore, anisodamine hydrobromide suppressed the proinflammatory cytokines and oxidative stress, thereby alleviating organ injury in rats with septic shock. Moreover, the therapeutic effect of Ani HBr is more powerful than that of atropine or Rac Ani, which suggests that Ani HBr is a preferred treatment for septic shock.

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Introduction

Sepsis is a life-threatening organ dysfunction (1) caused by dysregulation of the body's response to infection (2). Septic shock occurs when sepsis is combined with severe circulatory disorders and cell metabolism disorders, and the risk of death is higher than that of simple sepsis (3). The global incidence of septic shock and its high fatality rate motivate us to investigate its underlying mechanism and develop targeted medicines.

Septic shock has a high death rate due to tissue damage, organ circulation abnormalities, and cell metabolism issues caused by an intense inflammatory response. After the pathogen is recognized by immune cells in the early stages of septic shock, immune cells such as neutrophils and mononuclear macrophages are activated (4, 5), and proinflammatory cytokines such as TNF-α and IL-6 are generated and released (6, 7). In the late stage of infection, injured cells and immune cells bind to the Toll-like receptor 4 (TLR 4) and activate signal transduction pathways such as NF-κB (8), which further release inflammatory factors such as TNF-α and IL-6 and aggravate tissue damage. Pathophysiological mechanisms that lead to the development and progression of shock in septic patients and ultimately can lead to organ dysfunction are very complex and still not properly understood. One of the most crucial pathways of shock development leading to multiple organ failure appears to be oxidative stress (9, 10). Increased oxidative stress brought on by sepsis results in cell death and organ failure (11). Various inflammatory cells are activated during sepsis, resulting in an excess of reactive oxygen species (ROS) (12). Excessive ROS generation overwhelms the scavenger capacity of the antioxidant system, resulting in oxidative stress and oxidative damage to lipids, proteins, and DNA (13, 14). Furthermore, excessive ROS generation leads to mitochondrial dysfunction and ATP depletion (15), causing cytochrome c to leak from the mitochondria into the cytoplasm and ultimately initiating death (16). According to studies, patients with sepsis have higher blood levels of the oxidant malondialdehyde (MDA) (17), whereas their superoxide dismutase (SOD) activity is lower (18). Furthermore, MDA levels and SOD activity have also been connected to the severity and prognosis of sepsis (19, 20). Antioxidant protective advantages in animal sepsis models, on the other hand, have been documented (21). These findings suggest

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that approaches addressed the oxidant/antioxidant imbalance have significant potential in the treatment of sepsis.

Anisodamine hydrobromide (Ani HBr) is an active component derived from the root of *Scopolia tangutica* (maxim), a Chinese specialty plant. It is a one-of-a-kind medicine in China that is used to treat septic shock, acute gastroenteritis, renal colic, pulmonary edema, mycoplasma pneumonia in children, fundus disease, vertigo, diabetic foot, and other conditions (22, 23). Ruan et al. found that anisodamine was able to counteract endothelial cell activation through the NF-κB pathway (24). NF-κB plays an important role in inflammatory illnesses by regulating the production of pro-inflammatory cytokines (25). Ani HBr has been reported to reduce LPS-induced acute kidney injury by inhibiting lipopolysaccharide-induced inflammatory cytokines, mitochondrial dysfunction, and oxidative stress (26). These findings revealed that anisodamine's anti-shock action was partially associated with anti-inflammation (27). In addition to its role in septic shock, Ani HBr has been shown to protect the glycocalyx against LPS-induced increases in microvascular endothelial layer permeability and nitric oxide production (28). Cecal ligation perforation (CLP) was frequently employed in the development of rat septic shock models (29). We hypothesized that Ani HBr reduces CLP-induced tissue injury in important organs such as the brain, heart, liver, lung, kidneys, and intestines by attenuating proinflammatory cytokine and oxidative stress in the current study.

Materials and Methods

Animal

Forty-two male Specific Pathogen Free (SPF) Sprague Dawley (SD) rats weighing 200-250g were used. The rats were housed in a solid-bottom cage at constant temperature (25 ± 2°C) and humidity (55 ± 10%) in a temperature-controlled facility in a standard breeding environment, with 12:12 h light/dark cycles. All rats were allowed access to food and water ad libitum, but they fasted for 1 hour before the experiments.

Experimental design

The rats were divided into seven groups randomly: Sham group; CLP group, positive control group (5 mg/kg atropine, Suicheng Pharmaceutical Co., Ltd, China), racemic anisodamine group (5.4 mg/kg Rac Ani, Suicheng Pharmaceutical Co., Ltd, China), and Ani HBr treatment group (1.8 mg/kg, 3.6 mg/kg, and 5.4 mg/kg). The purity of Ani HBr is > 98.5% (Lot. 190501) that provided by Chengdu NO.1 Pharmaceutical Co., Ltd, China.

Cecal ligation and puncture (CLP)

CLP was performed essentially as described previously (30). Briefly, the rat was shaved and disinfected after anesthetization by intraperitoneal injection of a lethal dose of pentobarbital. The abdominal cavity was opened under general anesthesia and analgesia to expose the cecum, ligation was performed under the ileocecal valve, and a needle puncture was performed. A small amount of stool is squeezed out by puncture and the cecum is repositioned to the abdomen. The peritoneal wall was closed with continuous sutures and the skin with interrupted sutures using 3.0 silk. At the end of the operation, normal saline 3 mL/100 g was injected subcutaneously, and penicillin 20,000 U was injected intramuscularly to prevent incision infection. Animals subjected to sham laparotomy underwent the same procedure but without ligation and puncture of the cecum. (31, 32). For best consistency and reproducibility, the procedure was done at the same time of the day and performed by the same operator.

Collection of plasma and tissue samples

Fundus vein blood was collected and stood at room temperature for 2 h. After centrifugation at 3000 RPM for 15 min, precipitation was discarded, and the supernatant was subloaded and stored at -80°C for subsequent experiments. The rats in each group were anesthetized with 1% pentobarbital sodium at a dose of 40 mg/kg through intraperitoneal injection. The tissues were taken and fixed in 4% neutral formaldehyde for 24 h for histopathological observation.

Hematoxylin and Eosin staining and TUNEL assay

The tissue was fixed in 4% paraformaldehyde (Solarbio, China) for 24 h, then embedded in paraffin, and the tissue was cut into 4 μm sections. After depar-affinization and hydration, the sections were stained with hematoxylin and eosin (C0105, Beyotime, China) and TUNEL kit (11684817910, Roche, Germany), the sections were sealed with neutral resin and examined by a light microscope.

Measurement of inflammatory cytokines

IL-6 and TNF-α levels in p were detected using an IL-6 ELISA kit (ERC003.96T, Neobioscience, China), TNF-α ELISA kit (ERC102A.96T, Neobioscience, China), respectively. The experiments were performed strictly according to the requirements of the kit.

Determination of oxidative stress levels

The classical NBT chromogenic method was used to measure the levels of SOD. the SOD in injured organs was detected by a SOD kit (A001-1-1, Nanjing Jiancheng, China) and MDA kit (A003-1-1, Nanjing Jiancheng, China).

Statistical analysis

Data for the plasma cytokine levels measured by ELISA were subjected to one-way analysis of variance (ANOVA) using SPSS 26.0 software (IBM, USA). Differences among the groups were obtained using the least significant difference option. A statistical analysis of the oxidation product was carried out using one-way ANOVA. It was considered as significant at $P < 0.05$. All the results were expressed as the mean ± SD.

Results

Ani HBr reduces injury of organs in CLP-induced septic rats

CLP caused degenerative alterations in the brains of rats in comparison to the sham group, which were then treated with Ani HBr (Fig. 1).

Myocardial cells in the septic shock group were disordered compared to the sham group, with atrophy of myocardial fibers, obvious enlargement of nuclei, and abundant dark blue chromatin. The arrangement of rat cardiomyocytes recovered regularly after Ani HBr treatment, and the nuclei remained enlarged compared to the sham

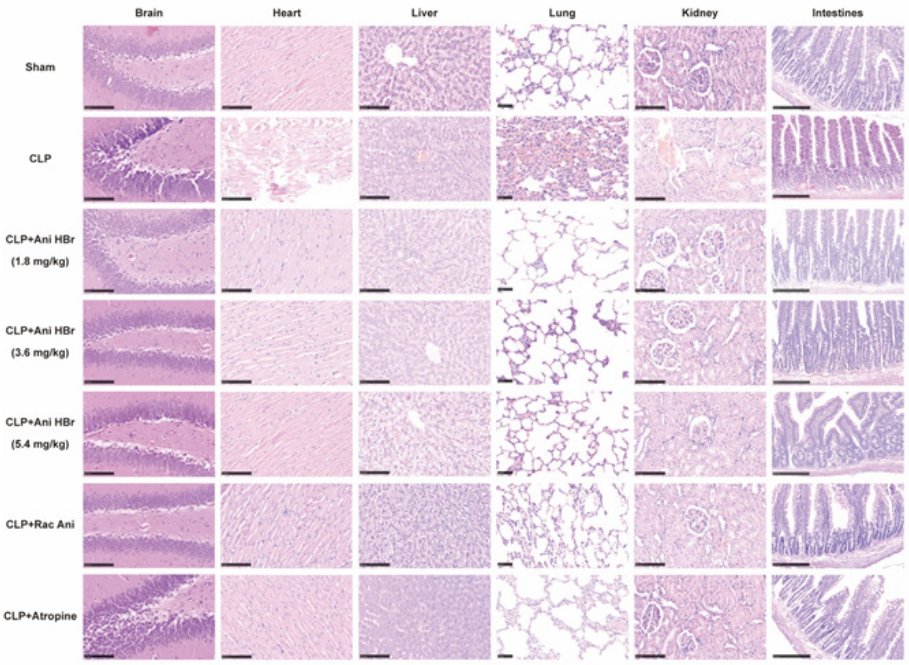

Figure 1. The effects of anisodamine hydrobromide on injury of various organs in CLP rats. The bar is 100 μm for brain, heart, liver, and kidney, 50 μm for lung, and 250 μm for small intestine.

group, but the number of cells with dense dark blue chromatin was significantly reduced.

CLP causes fractures, bleeding, and increased chromatin density in the liver tissues of septic shock rats. The edema and bleeding of liver tissues were greatly improved after Ani HBr therapy, as were the chromatin-packed cells and the damage.

There was hemorrhage and alveolar thickening in the lung tissues in the septic shock group, with inflammatory cell infiltration of the alveolar wall. Ani HBr treatment can decrease CLP-induced bleeding, alveolar thickness, and inflammatory cell infiltration of the alveolar wall.

In the septic shock group, renal tubule cells exhibited renal tubule dilatation, cell death, and bleeding. Ani HBr treatment significantly attenuated the injuries in the kidney.

The intestinal villi grew thick and bled, and their intestinal glands swelled in the septic shock rats. Ani HBr therapy can dramatically improve intestinal villi thickening and bleeding, as well as intestinal gland thickening.

Ani HBr inhibits apoptosis in organs induced by CLP

Apoptotic cells were stained using the TUNEL assay (Fig. 2A). TUNEL-staining-positive cells were apoptotic cells. The quantitative results are shown in Figure 2B-G. The results showed that the TUNEL-positive cells in the brain tissues were more concentrated in the hippocampus in the septic shock group. After treatment with Ani HBr injection, fewer TUNEL-positive cells were found in rats after CLP.

Cardiomyocytes were found to be normal in the sham group's cardiac tissue sections. TUNEL-positive cardiomyocytes were seen in slices of cardiac tissues from the septic shock group. We found a reduction in TUNEL-positive cardiomyocytes and the widespread occurrence of typical cardiomyocytes in cardiac tissue slices from the Ani HBr treated group.

Hepatocyte morphology was normal in the sham group. However, the number of TUNEL-positive hepatocytes was considerably high in the liver tissue samples of the septic shock group. We saw a reduction in TUNEL-positive hepatocytes in the liver tissue sections of the Ani HBr therapy group, and most hepatocytes displayed normal morphology.

The TUNEL-positive cells in the lung tissues of the septic shock group were concentrated in the alveoli. Compared with the CLP group, the number of TUNEL-positive cells in the lung tissues of rats treated with Ani HBr was significantly lower.

Compared with the sham group, the TUNEL-positive cells were substantially more prevalent in renal tissue sections in the septic shock rats, and most positive cells were located in the renal tubules. TUNEL-positive cells in rats with sepsis caused by CLP are reduced after Ani HBr therapy.

Most TUNEL-positive cells were concentrated in the intestinal villi epithelium in the septic shock group. Rats treated with Ani HBr had considerably fewer TUNEL-positive cells in their small intestines than those in the CLP group.

Therefore, Ani HBr can inhibit CLP-induced apoptosis in the brain, heart, liver, lung, kidney, and small intestine.

Ani HBr inhibited plasma TNF-α and IL-6 levels in CLP-induced septic shock rats

In the present study, the plasma levels of inflammatory cytokines TNF-α and IL-6 in the rats were detected (Fig. 3). The levels of both cytokines were found to be significantly increased in the septic shock group compared with the sham group (30.57 ± 5.19 pg/mL vs. 8.94 ± 2.17 pg/mL, TNF-α, $P<0.001$; 1480.37 ± 180.08 pg/mL vs. 404.61 ± 51.09 pg/mL, IL-6, $P<0.001$; CLP vs. Sham) (Fig. 3). With Ani HBr administration, the plasma levels of TNF-α were significantly reduced to 14.76 ± 1.52 pg/mL at 1.8 mg/kg Ani HBr, 12.51 ± 1.28 pg/mL at 3.6 mg/kg Ani HBr, and 11.44 ± 1.23 pg/mL at 5.4 mg/kg Ani HBr, respectively ($P< 0.001$). Also, administration of 1.8, 3.6 and 5.4 mg/kg Ani HBr decreased the plasma levels

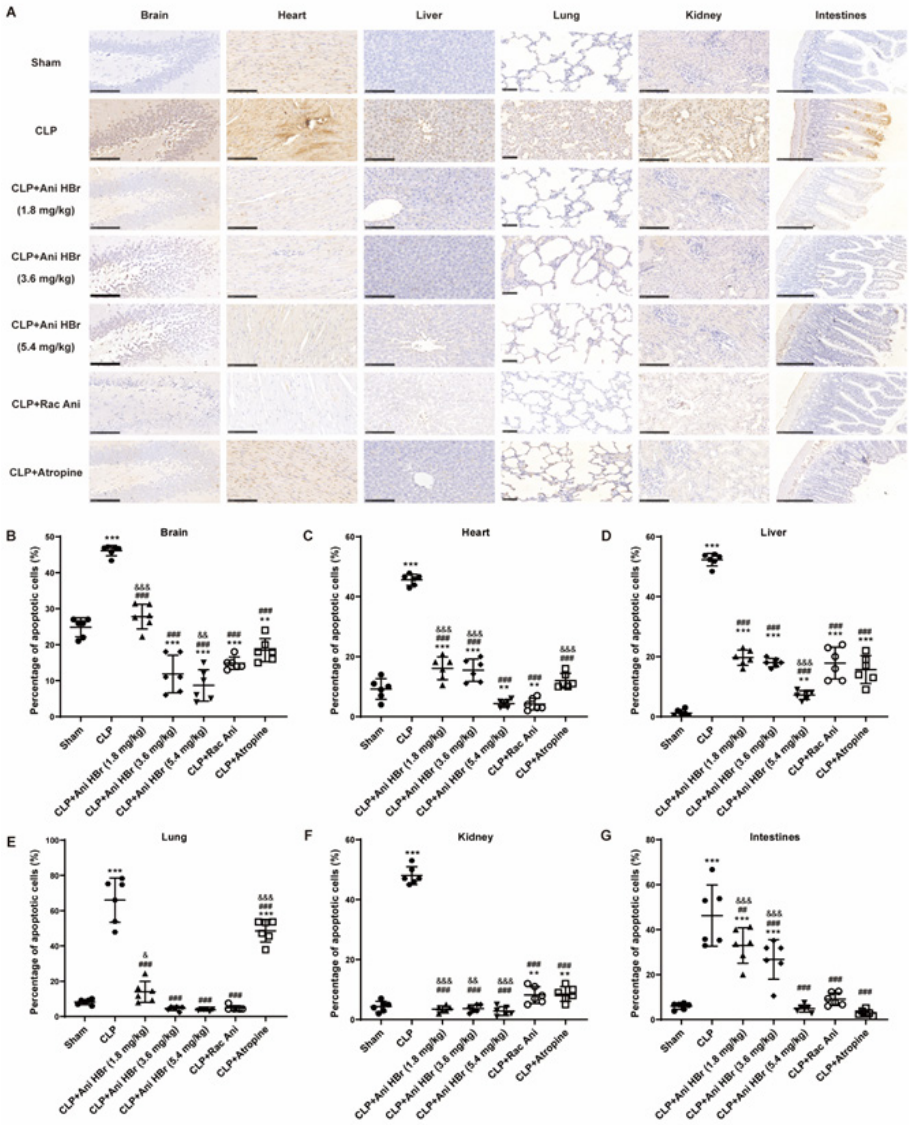

Figure 2. The protective effect of anisodamine hydrobromide on apoptosis in organs in CLP rats. A. TUNEL staining. The bar is 100 μm for brain, heart, liver, and kidney, 50 μm for lung, and 250 μm for small intestine. B-G, TUNEL-positive cells in organs (n=6). ***P*<0.01, ****P*<0.001 vs. Sham; ##*P*<0.01, ###*P*<0.001 vs. CLP; &*P*<0.05, &&*P*<0.01, &&&*P*<0.001 vs. CLP+ Rac Ani.

of IL-6 to 1099.43 ± 133.08 (*P*<0.01), 961.53 ± 66.24 (*P*<0.001) and 800.16 ± 106.31 pg/mL (*P*<0.001), respectively. In contrast to Ani HBr, the levels of both cytokines were found to be increased in the Rac Ani group (TNF-α: 20.37 ± 2.69 pg/mL; IL-6: 1131.01 ± 73.94 pg/mL) when compared with the septic shock group (*P*<0.01). These results suggested that Ani HBr could significantly inhibit the plasma levels of inflammatory cytokines TNF-α and IL-6 in septic shock rats in a dose-dependent manner, and the anti-inflammatory effect of Ani HBr was better than that of Rac Ani at 5.4 mg/kg each.

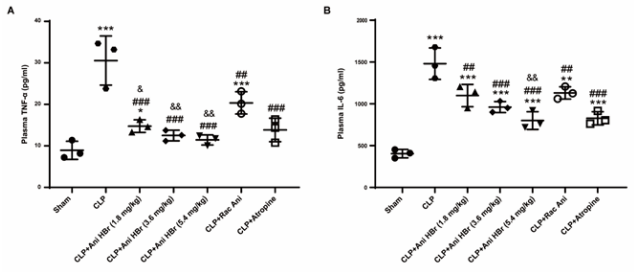

Figure 3. The effects of Ani HBr on plasma levels of TNF-α (A) and IL-6 (B) in CLP-induced septic rats. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. Sham; ##*P*<0.01, ###*P*<0.001 vs. CLP; &*P*<0.05, &&*P*<0.01 vs. CLP+ Rac Ani.

Ani HBr inhibits plasma SOD activity and increases MDA content in CLP-induced septic rats

To explore the effects of oxidant and antioxidant defenses on the sepsis process, plasma antioxidant levels (SOD) and oxidant levels (MDA) were evaluated in septic shock rats. As shown in Figure 4, compared with the Sham group, the SOD activity in CLP rats was significantly decreased (*P*<0.01) while the MDA levels were increased significantly (*P*<0.001). The SOD and MDA levels were determined as 62.08 ± 14.16 U/mL and 15.73 ± 1.80 nmol/mL in the CLP group, respectively, and significantly higher than 100.70 ± 17.12 U/mL and 2.03 ± 1.01 nmol/mL in the Sham group. Compared with the Rac Ani group, administration of 5.4 mg/kg (*P*< 0.001) of Ani HBr significantly increased the SOD activity to 141.21 ± 1.29 U/mL(*P*<0.001), while, administration of 1.8, 3.6, and 5.4 mg/kg Ani HBr could significantly down-regulate MDA levels to 12.64 ± 0.58 (*P*<0.01), 8.34 ± 0.110 (*P*<0.001) and 6.32 ± 0.69 nmol/mL (*P*<0.001), respectively. Atropine could significantly up-regulate SOD activity to 41.56 ± 2.27 U/mL, but Rac Ani had no obvious effect on SOD compared with the CLP group. Rac Ani and atropine also

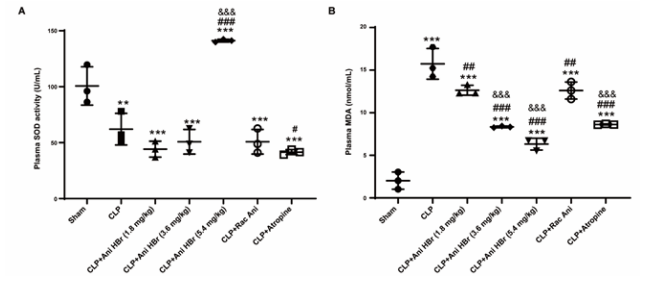

Figure 4. The effects of Ani HBr on plasma SOD activities (A) and MDA contents (B) in CLP-induced septic rats. ***P*<0.01, ****P*<0.001 vs. sham; #*P*<0.05, ##*P*<0.01; ###*P*<0.001 vs. CLP; &&&*P*<0.001 vs. CLP+ Rac Ani.

could significantly down-regulate MDA levels to 12.61 ± 1.01 (*P*<0.01) and 8.63 ± 0.05 nmol/mL (*P*<0.001), respectively.

These results suggested that Ani HBr treatment could ameliorate the plasma levels of SOD and MDA in rats with septic shock, and at a dose of 5.4 mg/kg, Ani HBr was better than Rac Ani in enhancing the activity of SOD and inhibiting the level of MDA in rats with septic shock. Thus, there is extensive oxidative stress in CLP rats, which could be suppressed by Ani HBr.

Discussion

It was demonstrated that in mice with septic shock caused by CLP, maackiain, a naturally occurring compound derived from Sophora flavescent, was found to lessen organ damage, systemic inflammation, and oxidative stress (33). Therefore, we questioned if Ani HBr may similarly protect shock animals by reducing the levels of oxidative stress and inflammation in CLP rats. In the present study, we have established a septic shock rat model via the CLP procedure to explore the potential protective effects of Ani HBr. We observed that the Ani HBr treatment exerted a substantial protective effect on the septic rats. We found that the infiltration of inflammatory cells and apoptosis cells was reduced, and the tissue injury was also attenuated after the Ani HBr treatment. Furthermore, we discovered that Ani HBr reduced inflammatory cytokines including TNF-α and IL-6, as well as ameliorated the change in plasma MDA and SOD levels under these conditions. Basing on these observations, we can suggest that the therapeutic administration of Ani HBr prevented oxidative stress changes and cytokine changes, protecting vital organs.

Studies have shown that all the length of cecal ligation, the type of needle used in puncture and the puncture numbers are important factors affecting the mortality of rats. The increase of proinflammatory factors in serum is closely related to the length of cecal ligation. By adjusting the position of the cecal ligation, different severities of septic shock can be caused, which is an advantage of the CLP model. At the same time, because different experimental factors will seriously affect the severity of septic shock of rats and thus affect the experimental results, this is also a major disadvantage of the CLP model (34, 35). However, we conducted the experiment with the same person under uniform conditions, which minimize the influence of human factors on the experimental results.

Ani HBr is commonly applied in clinical practice. It has recently been used in combination with various medications for anesthesia and endoscopy, in addition to treating septic shock, smooth muscle spasm, organophosphorus pesticide poisoning, renal colic, and bronchial asthma. A future large-scale clinical randomized controlled study of Ani HBr will support its responsible usage. Rac Ani, often known as "654-2," was synthesized chemically in 1975. The structures of Ani HBr and Rac Ani differ somewhat. Despite the fact that both can improve microcirculation and reduce the injury of organs in CLP-induced septic rats, there is no proof that they are operative by the same molecular mechanism.

It was found that the excessive oxidative stress induced by pro-inflammatory substances will damage the cells, leading to an increase in the permeability of mitochondrial membrane that results in the release of proapoptotic factors such as cytochrome C (36). When a pathogen enters the body, the innate immune system is triggered, and the neutrophils were rapidly accumulated in order to capture and destroy the pathogen at the site of pathogen invasion. However, following the inflammatory response, neutrophils can produce the inflammatory chemicals, causing tissue damage. TNF-α is the most important proinflammatory cytokine, controlling the expression of several inflammatory genes, oxidative stress, and antiapoptotic signaling pathways (37). Thus, therapeutic targeting of TNF-α signaling has been widely employed to treat a variety of inflammatory disorders (38, 39). IL-6, IL-10, and IL-18 are inflammatory markers that can be utilized to detect the onset of sepsis and are induced by TNF-α (40). Previous research has revealed that blood levels of TNF-α and IL-6 in the CLP-induced septic shock rats were much higher than sham rats, while inhibiting their increase had a protective impact on septic shock rats (41-43). Following the administration of CLP, Ani HBr therapy led to substantial reductions in pro-inflammatory cytokines TNF-α and IL-6. The suppression of inflammatory cytokines might responsible for attenuating the organ damage by Ani HBr. The lowering of a variety of proinflammatory mediators generated by leukocytes and macrophages is likely to contribute to Ani HBr's anti-inflammatory actions in CLP-induced septic shock.

ROS are intermediary metabolites of oxygen or derivatives of oxygen (44, 45). MDA is the end-product of lipid peroxidation generated by the interaction of free radicals with polyunsaturated fatty acids. The ROS and MDA levels are crucial markers of the body's oxidative stress levels (46). ROS and MDA harm tissue by attacking polyunsaturated fatty acids in the cell membrane, rupturing the mitochondrial membrane, and increasing the permeability of the cell membrane. This results in cell death and edema (47). Antioxidants like SOD help the body rid itself of free radicals. SOD protects cells by catalyzing the conversion of superoxide to hydrogen peroxide and scavenging superoxide free radicals, and its activity reflects the body's capacity to neutralize free radicals (48). In septic shock, as neutrophils will generate a large amount of ROS to kill microorganisms, the level of oxidative stress in the body increases, while SOD, as a free radical scavenger, may be reduced in consumption. As a result, monitoring the levels of MDA and SOD can reveal the extent of oxidative stress in the body (49). Our study found that the MDA level in the plasma of rats was significantly higher than that of the sham group, while the activity of SOD was significantly lower than that of the sham group. In the 1.8, 3.6, and 5.4

mg/kg Ani HBr and Rac Ani treatment groups, the level of MDA in rat plasma was significantly decreased, while the plasma SOD activity of 5.4 mg/kg Ani HBr group in rat plasma was significantly increased. Previous studies have shown that MDA levels in serum (50) were significantly increased during septic shock and were closely related to the prognosis of septic shock. An oxidative stress disorder in rats with septic shock is improved by both Ani HBr and Rac Ani, but the improvement effect of Ani HBr is better than that of Rac Ani. Even though both can improve microcirculation, further research must be done to determine the difference in exact mechanism between Ani HBr and Rac Ani in the future.

Conclusions

The present study suggests that a huge number of inflammatory cells are activated during septic shock, increasing oxidative stress and causing cell damage and organ malfunction. Ani HBr can reduce inflammatory cytokines including TNF-α and IL-6 and alleviate the detrimental change in the plasma levels of SOD and MDA. It is noteworthy that Ani HBr may be a better alternative for the clinical treatment of septic shock since it has a greater therapeutic impact than atropine and Rac Ani. Taken together, the therapeutic administration of Ani HBr shielded vital organs from injuries induced by cytokines and oxidative stress during septic shock.

Interest conflict

The authors have none conflicts of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

Authors' Contribution

Y.Z. designed the studies, all authors contributed to the data analysis and manuscript writing.

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Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee

of Sichuan University (K202102).

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