The Effects of Vagus Nerve Stimulation Mediated Protection of Intestinal Epithelial Glycocalyx on Acute Lung Injury Associated With Traumatic Hemorrhagic Shock/Resuscitation

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Research

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Abstract

Background

Acute lung injury (ALI) is a common complication after THS/R, and vagus nerve stimulation (VNS) could alleviate lung injury by activating cholinergic anti-inflammatory pathway (CAP) during traumatic hemorrhagic shock/resuscitation (THS/R). The purpose of this study was to explore the effects of VNS on intestinal epithelial glycocalyx and acute lung injury associated with THS/R.

Methods

Sprague-Dawley rats were subjected to traumatic hemorrhagic shock/ resuscitation to induce ALI. The measurements of intestinal barrier permeability, intestinal epithelial glycocalyx, and the level of inflammation factors and histology of lung and gut tissue were made in each group.

Results

The level of TNF-α, IL-6 and MPO in lung and gut tissue were significantly decreased in the VNS-treated group. In addition, the shedding of intestinal epithelial glycocalyx and increased gut barrier permeability were alleviated in the VNS-treated group, and the gut tissue and lung tissue injury were mitigated in the VNS-treated group. However, pretreated with methyllycaconitine could reverse the protective effect of VNS.

Conclusions

VNS could relieve lung injury associated with THS/R by alleviating the damage of intestinal epithelial glycocalyx, which might be achieved by activating CAP.

Background

Traumatic Hemorrhagic shock (THS) is the most common type of shock. Although significant advances in treatment of THS, the one of most effective therapy is volume resuscitation. The process of traumatic hemorrhagic shock/resuscitation (THS/R) could lead to intestinal ischemia-reperfusion injury, disrupting the gut barrier and increasing intestinal permeability, then the cytokines generated by gut could across to sterile internal milieu and induce systematic inflammatory response(1, 2). Overwhelming gut-derived factors could cause pulmonary inflammatory reaction, increasing lung vascular permeability causing tissue edema and eventually leading to acute lung injury(ALI) (3) or acute respiratory distress syndrome (ARDS). ALI/ARDS after THS/R might trigger the process of multiple organ dysfunction failure and increase disability rate and mortality rate(4).

Cholinergic anti-inflammatory pathway (CAP) is a kind of neuroimmunomodulation mechanism to regulate inflammation reaction(5), which processed by the end of vagus nerve releasing acetylcholine (Ach) to combine with the alpha 7 nicotinic acetylcholine receptor(α7nAchR) on the surface of
inflammatory cells to regulate the level of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), nuclear transcription factor-κB (NF-κB) and interleukin-6 (IL-6) (6). Many studies show that vagus nerve stimulation (VNS) could alleviate the damage of gut barrier associated with THS/R by the activation of CAP but the specific mechanism is still unknown (7, 8).

Glycocalyx is a kind of complex compound located at the surface of endothelial cells and epithelial cells, consisting mostly of glycosaminoglycans (GAGs), including heparan sulfate (HS) (9), hyaluronic acid (HA), and chondroitin sulfate (CS). Hyaluronidase could diminish glycocalyx layers by cleavage HA. Although many studies showed that endothelial glycocalyx is the first barrier to maintain vascular endothelial permeability (10, 11), the function of glycocalyx on intestinal epithelial cells (IECs) is still unclear. Thus, the aim of this study is to explore the effects of VNS on glycocalyx located on IECs and acute lung injury associated with THS/R.

**Methods**

2.1 Animals

Male Sprague-Dawley rats (SD rats, weighing 250 ± 20 g) were purchased from Wuhan Center for Disease Control and Prevention, China. All rats were kept in air-conditioned room with 12 hours (h) light and 12 h dark cycle, free access to water, but 12-hour fast before experiment. All experiments were approved by the Ethics Investigation Board of the Central Theater Command General Hospital of the Chinese People’s Liberation Army, Wuhan, China.

2.2 Animal model constructing

This experiment consisted of two parts. In first part, twelve SD rats (n=12) were randomly divided into 2 groups (n=6), Control group and glycocalyx inhibitor group (GI group). Rats were anesthetized with 6% chloral hydrate (5 ml/kg) intraperitoneally. We incised the skin along the midline of the animals’ neck and separated the trachea and performed the tracheotomy, then imbedded 14G catheter into the trachea, retaining spontaneous respiration. Temperature measurement probe was inserted into anus to monitor temperature. A midline laparotomy length 5 cm was performed, and the cecum was identified and protected with moist gauze. A 10-cm segment of ileum was identified at 20-30 cm from cecum, ligated both ends of the ileum, and incised distally and flushed with 2.0 mL of 0.9% normal saline to remove feces. Once flushed, the incision was closed. 12 mg/kg Hyaluronidase and 20 mg/ml Fluorescein isothiocyanate dextran (FD4) were prepared. A total of 1 mL Hyaluronidase or 0.9% normal saline was injected into the 10-cm segment of ileum in GI or Control group respectively, then the abdominal incision is closed using 4-0 suture. 30 minutes later, midline laparotomy incision was released, and 1 mL 20 mg/ml FD4 was injected into the 10-cm segment of ileum carefully preventing any spillage onto the external bowel allowing for a circulation of 30 minutes. Then 1 mL of venous blood was taken from the right ventricle into a heparinized syringe, protected from light and placed on ice, then the rats were sacrificed. During the whole procedure, we used thermal equipment to maintain rats’ body temperature between 36.0°C and 38.0°C.
In second part, twenty-four (n=24) SD rats were randomly divided in 4 groups (n =6), Sham surgery group(SS group), traumatic hemorrhagic shock/resuscitation (THS/R group), Vagus nerve stimulation group(VNS group) and Vagus nerve stimulation-methyllycaconitine group(VSM group). SD rats were anesthetized, intubated and monitored temperature as above. Right carotid artery was dissected and cannulated with PE-50 tubing for blood withdraw, anticoagulated with heparin(5U/mL) infusion and attached to a BP-100 blood pressure monitor. Left internal jugular vein was dissected and cannulated with PE-50 tubing, and the right vagus nerve was identified and separated carefully along the right carotid artery. THS/R group, VNS group and VSM group received abdominal trauma and traumatic hemorrhagic shock/ resuscitation. A midline laparotomy length 5cm was performed to simulate trauma, then the incision was closed using 4-0 suture. Blood was withdrawn through the right carotid artery into an anticoagulation solution syringe until the mean arterial pressure (MAP) reached 30 to 35 mmHg at the rate of 1 mL per minute and maintained for 60 minutes. Before the resuscitation, VNS group and VSM group received VNS, VSM group also received methyllycaconitine(MLA,10mg/Kg) intraperitoneally before VNS, and THS/R group only separated the right vagus nerve. The process of VNS was as following: a platinum electrode was placed across the nerve, attached to the neurostimulator, and the VNS was applied for 15 minutes at 1.0mA·0.1ms·1Hz. Then the rats were resuscitated with shed blood and 0.9% normal saline at the rate of 1 mL per minute through internal jugular vein to make MAP reach the 90% of baseline, and maintained for 2.0 hours. 20 mg/kg Evans Blue dye (EBD) and 25 mg/mL FD4 were prepared. Then, midline laparotomy incision was released, and the cecum was identified and disposed as above. Then a total of 1mL FD4 was injected into the 10-cm segment, carefully preventing any spillage onto the external bowel. Meanwhile, EBD (20mg/kg) was administered via the internal jugular vein. After circulating for 30min, 2mL of venous blood was removed from the internal jugular catheter into a heparinized syringe, the lungs were perfused with 0.9% normal saline through ventriculus dexter, then the rats were sacrificed to obtain lung tissue and gut tissue. During the whole procedure, we used thermal equipment to maintain temperature between 36.0℃ and 38.0℃.

2.3 Lung vascular permeability assay

Lung vascular permeability was measured as Huang. et al described(12). We used EBD extravasation to assess the pulmonary vascular leakage. The lungs were excised and imaged, then the lungs were homogenized in formamide. Following overnight extraction, the tissue fluid was centrifuged at 12000g for 10 min. The EBD concentration of the supernatant was obtained by comparing with a standard curve measured on Microplate Reader at 620 nm absorption.

2.4 Gut Permeability Assay

Gut permeability was measured as Levy. et al described(13). 1 ml venous blood was protected from light and placed on ice. Plasma FD4 was obtained by centrifuging 3000 rpm for 10 minutes at 4℃ and the concentration was obtained by comparing with a standard curve measured on Microplate Fluorescence reader at excitation 485/20, emission 528/20, and a sensitivity of 40.

2.5 ELISA
ELISA assays were used to measure the levels of cytokines in lung tissues and Ach in plasma. The TNF-α, IL-6, NF-κB, and Ach ELISA kits were purchased from Coolaber (Shanghai, China). All procedures were performed according to the manufacturers’ protocols.

2.6 Immunofluorescence

After rats sacrificed, gut tissues were collected and fixed with 4% paraformaldehyde, paraffin embedded, and cut into 4 µm slices. Those slices were deparaffinized with xylene, and then dehydrated in gradient ethanol. Subsequently, antigen retrieval was performed, and slices were washed three times in PBS (5 min/wash). After blocking, the slices were incubated with primary antibodies to SDC-1 and FITC-conjugated secondary antibody. After washing, the slices were stained with DAPI and then sealed with an anti-fade fluorescence medium. The slices were observed with a fluorescence microscope and analyzed by ImageJ software.

2.7 H&E staining

The lung tissues and gut tissues were collected and fixed with 4% paraformaldehyde, paraffin embedded, and cut into 5 µm slices. Those slices were deparaffinized with xylene, and dehydrated in gradient ethanol, then stained with hematoxylin and eosin and observed under an optical microscope. Five random fields were identified in a blinded fashion at 100x magnification to quantify morphologic damage. The lung injury scores (14) were obtained by assessments of inflammatory cell infiltration in the airspace or vessel wall, alveolar congestion, hemorrhage, alveolar wall thickness, and hyaline membrane formation. The degree of gut injury was identified if any one of the following were present (15): a subepithelial space, moderate lifting of the epithelial layer from the lamina propria, massive epithelial lifting down the sides of the villi, denuded villi with lamina propria and dilated capillaries and/or digestion and disintegration of the lamina propria with hemorrhage, and ulceration.

2.8 Western blot analysis

The lung tissues and gut tissues were frozen at -80°C. The extraction of proteins was performed using protein extraction kit (Coolaber, China) in accordance with the manufacturer’s instructions. The lung tissues and gut tissues were homogenized respectively on ice with RIPA buffer, and the protein concentrations were determined by BCA protein assay kit (Coolaber, China). The protein samples were boiled in sample buffer and loaded into each lane, separated by 10% SDS-PAGE, and transferred to PVDF membranes. The membranes were washed three times in Tris-buffered saline with Tween 20 (TBST) and blocked with 5% nonfat milk for 2 h at room temperature. Subsequently, the samples were incubated with primary antibodies (MPO, TNF-α, IL-6, IL-10, NF-κB) overnight at 4 °C, followed by membrane washing three times for 10 min. Then, the samples were incubated with the second antibody at room temperature for 1 h. The protein bands were visualized using an enhanced chemiluminescence kit. The images were quantitatively analyzed by using the Image J analysis software.

2.9 Quantitative real time polymerase chain reaction (RT-qPCR)
Total RNA was isolated from lung tissues and gut tissues respectively using TRizol reagent (Simgen, China), according to the manufacturer's instructions. cDNA was synthesized using a ReverTra Ace qPCR RT kit (Simgen, China), according to the manufacturer's protocol. RT-PCR was performed using SYBR Premix Ex Taq (Simgen, China). The levels of mRNA expression of target gene were measured using primers purchased from TSangon Biotech (Shanghai, China); these primer sequences have been shown in Table 1. Target gene expression was quantified as the average of triplicate samples using the $\Delta \Delta CT$ equation and normalized to glyceraldehyde 3-phosphate dehydrogenase gene expression.

Table 1. Primer sequences

| Gene name | Forward (5’→3’) | Reversed (5’→3’) |
|-----------|-----------------|------------------|
| MPO       | TCGTATTTCAAGCAGGCGGT | GCATGTCCCTGTGACATTGAA |
| TNF-α     | AACTCGAGTGACAAGCCCGTG | GTACCACCAGTTGGTTGCTTTGA |
| IL-6      | ACTTTCCATCCAGTTGCTTCTTTT | TCATTGCCACGATTTCCCAGAG |
| IL-10     | AAGGTTACTTGGGTTGCCA | GTGTCACGTAGGCTTCTATGC |
| NF-κB     | TACCACGTCAACAGATGGCCC | ATATGCCGTCACAGT |
| GAPDH     | GGCACAGTCAGGCTGAGAATG | GTACCACCAGTTGGTTGCTTTGA |

2.10 Statistical Analysis

Data were presented as Mean ± SD and compared by independent Sample T test and one-way analysis of variance (ANOVA), and the Student–Newman–Keuls (SNK) post hoc test was used for statistical analysis to compare the data among all groups. A significant difference was presumed when $p<0.05$.

Results

3.1 Hyaluronidase damaged glycocalyx on IECs and increased intestinal barrier permeability

Syndecan-1 (SDC-1) is the main component of the core and side-chain structures of the glycocalyx, and is often used as an indicator of the integrity of the glycocalyx. In this study, compared with Control group, the results showed that pretreatment with hyaluronidase could damage the glycocalyx on IECs in GI group (Figure 1A-B, $P<0.0001$). The gut permeability was reflected by FD4 concentration in plasma, and higher FD4 concentration meant higher gut permeability. The results shown that, compared with Control group, pretreatment with hyaluronidase in GI group could increase FD4 concentration in plasma ($P=0.0043$ (Figure 1C).

3.2 Hyaluronidase induced intestinal and lung histology injury
In our study, the results showed that, compared with Control group, pretreatment hyaluronidase could induce slight intestinal and lung histology injury, such as the formation of some subepithelial spaces (Figure.2A) and inflammatory cells infiltrating into pulmonary interstitium (Figure.2B). In addition, the results showed that, in GI group, the concentration of TNF-α and NF-κB in lung tissues were significantly increased versus Control group, but the level of IL-6 didn’t show statistically significant difference (Figure.2C-2E). Figure.2. Hyaluronidase induced intestinal and lung histology injury. (A) Histopathological examination of intestine by HE staining. (B) Histopathological examination of lung by HE staining. (C–E) The levels of TNF-α, IL-6, and NF-κB in lung tissues were measured by ELISA. GI represent glycocalyx inhibitor group, ****p<0.0001, ***p<0.001, ns p>0.05.

3.3 VNS attenuated the increased pulmonary vascular permeability and alleviated lung histology damage induced by THS/R

ALI is characterized by increased pulmonary vascular permeability, inflammatory cell infiltration and pulmonary edema. The results showed that VNS could alleviate the increased pulmonary vascular permeability caused by THS/R, but the treatment of MLA, a kind of selective α7nAchR antagonist, could inhibit part of protective effect of VNS on pulmonary vascular permeability (Figure3A-3B, p<0.001). In addition, the results showed that, compared with SS group, lung tissues would be dramatically damaged by THS/R, specifically showing inflammatory cell infiltration in the airspace and increased alveolar wall thickness. However, VNS could alleviate lung injury, which could be partly inhibited by MLA (Figure3C).

3.4 VNS decreased the expression of TNF-α, IL-6 and MPO, and increased the expression of IL-10 in lung tissue

Neutrophil-derived inflammation plays an important role in the development of ALI, and the expression of IL-10, TNF-α and IL-6 could be the biomarkers of inflammation and the level of MPO could reflect the degree of neutrophil activation(16). As shown by the results (Figure 4A-E), compared with SS group, the mRNA and protein level of IL-10 was decreased, and the expression of TNF-α, IL-6 and MPO were increased in THS/R group; after treatment with VNS, the expression of IL-10 was increased, and the level of TNF-α, IL-6 and MPO were decreased, however, after administrating MLA, the effect of VNS was reversed remarkably.

3.5 VNS attenuated glycocalyx damage induced by THS/R and decreased intestinal barrier permeability.

Previous studies suggested that THS/R could damage gut barrier and increase intestinal barrier permeability(17). Our results showed that, compared with SS group, THS/R could damage the glycocalyx on IECs and increase gut permeability, but the treatment of VNS before resuscitation could mitigate the damage of glycocalyx and alleviate the elevation of gut permeability. However, the intraperitoneal injection of MLA before VNS could inhibit the effect of VNS on alleviating the glycocalyx damage and relieving the increase of gut permeability (Figure.5).

3.6 VNS reduced the gut histology injury induced by THS/R
During THS/R, gut tissue could be damaged because of ischemia-reperfusion (18). As the results shown, compared with SS group, the gut tissue was markedly damaged after THS/R, showing the information of subepithelial spaces, massive epithelial lifting down the sides of the villi, denuded villi with lamina propria as well as dilated capillaries, but treated with VNS before liquid resuscitation could relieve the gut damage. However, the intraperitoneal MLA could partly offset the effect of VNS (Figure.6).

3.7 VNS increased the level of Ach in plasma and IL-10 in gut, and decreased the expression of TNF-α, IL-6, NF-κB and MPO in gut

As the results shown that, compared with THS/R group, the level of acetylcholine in plasma increased in VNS group (Figure.7A). Compared with THS/R group, the mRNA and protein expression of TNF-α, IL-6, NF-κB and MPO decreased significantly in VNS group, and the expression of IL-10 mRNA and protein upregulated. However, intraperitoneal MLA before VNS could reverse the anti-inflammatory effect of VNS (Figure.7B-7G).

**Discussion**

To the best of our knowledge, this study firstly demonstrated that the effect of glycocalyx on IECs on intestinal barrier permeability, and VNS could alleviate the lung injury after THS/R by relieving the damage of glycocalyx on IECs.

ALI which is characterized by increased pulmonary vascular permeability, inflammatory cell infiltration and pulmonary edema is a common complication in patients after THS/R, and could develop into ARDS which presents as persistent hypoxemia, eventually resulting in respiratory failure (19, 20). Previous studies suggested the gut injury induced by THS/R plays a crucial role in the development of ALI, which means that THS/R could damage gut barrier and increase intestinal barrier permeability, so that lots of gut-derived cytokines could be carried in the mesenteric lymph where they reach the pulmonary system and cause lung injury (21-23). Our study also suggested that increased intestinal barrier permeability could cause inflammatory cell infiltration and increased cytokines in lung tissue, such as TNF-α and NF-κB. Many studies showed that kinds of treatments, including vagus nerve stimulation (VNS), could alleviate inflammation reaction and lung injury by protecting intestinal barrier, but the specific mechanisms were still unclear (13, 21, 24). In this study, we showed that THS/R could cause evident gut injury and markedly increased the intestinal barrier permeability, as well as cause inflammatory cell infiltration and interstitial edema and increase the level of IL-6, MPO and TNF-α in lung tissue, however, after administrating VNS, the intestinal barrier permeability was decreased, then lung injury and pulmonary vascular permeability were alleviated.

The intestinal barrier consists of mucous layer, gut microbiota, intestinal immune system and the integrity of intestinal epithelial cells. Glycocalyx is a kind of complex compound located at the surface of epithelial cells and endothelial cells, consisting mostly of glycosaminoglycans (GAGs) including heparan sulfate (25), hyaluronic acid (HA), and chondroitin sulfate (CS) (26), and the destruction of any element would lead to glycocalyx shedding. The function of endothelial glycocalyx had been studied in previous
researches, which is to constitute the first barrier to maintain vascular endothelial permeability (11, 27). However, the function of epithelial glycocalyx was still unclear, and some scholars proposed that mucosal epithelial glycocalyx could maintain physical barrier (28). In our study, we injected hyaluronidase, an enzyme for cleavage HA, into the ileum to find out the effect of intestinal epithelial glycocalyx on intestinal permeability for the first time, and used syndecan-1 as an indicator to detect the content of glycocalyx (12, 29). Our results showed that intestinal barrier permeability increased after intestinal epithelial glycocalyx was damaged by hyaluronidase.

Glycocalyx could be damaged by inflammation factors and ischemia-reperfusion injury, the change of wall shear stress, the increase of cholesterol and decrease of high-density lipoprotein (27, 30-32). Previous studies showed that inflammation factors, such as matrix metalloproteinase-1 (MMP-1), MMP-7, MMP-15 and MMP-9, are capable of cleaving GCX components (9, 30, 33-36). Recently, many clinical trials demonstrated that the increase of other inflammatory factors, like IL-6, TNF-α, and the decrease of anti-inflammatory, such as IL-10, also could damage glycocalyx (37, 38).

In this study, we found that there were extensive damage of glycocalyx on IECs after THS/R, and the level of IL-6, TNF-α and NF-κB in gut were increased. After VNS treatment, the level of IL-6, TNF-α and NF-κB were decreased and the level of IL-10 was increased, then the damage of glycocalyx on IECs was alleviated, which meant that VNS could alleviate the shedding of glycocalyx by suppressing inflammatory reaction in gut. Vagus nerve is an important part of CAP, which is a kind of neuroimmunomodulation mechanism to regulate inflammation reaction, and VNS could activate CAP by releasing Ach to combine with α7nAchR which located on the surface of inflammatory cells or nerve cells (5). Many research have revealed that VNS is an effective way to reduce the level of inflammatory factors, like IL-1, IL-6, TNF-α and NF-κB as well as alleviate tissue damage following THS/R by activating CAP (7, 21, 25).

In this study, when we pretreated with MLA, a selective α7nAchR antagonist, the above protective effect of VNS were reversed, which suggest that the glycocalyx protective effect of VNS might be mediated by activating CAP.

**Conclusion**

Our study firstly demonstrated that the shedding of glycocalyx on IECs could increase intestinal barrier permeability, and that the intestinal epithelial glycocalyx was damaged after THS/R, and VNS could relieve lung injury via alleviating the damage of glycocalyx on IECs by activating CAP. The results could provide a potential mechanism for the protective effect of VNS on lung injury after THS/R.

**Abbreviations**

Our study firstly demonstrated that the shedding of glycocalyx on IECs could increase intestinal barrier permeability, and that the intestinal epithelial glycocalyx was damaged after THS/R, and VNS could relieve lung injury via alleviating the damage of glycocalyx on IECs by activating CAP. The results could provide a potential mechanism for the protective effect of VNS on lung injury after THS/R.

**Declarations**
Ethics approval and consent to participate: All animals studies were performed in accordance with the guide for the Care and Use of laboratory Animals established by the US National Institutes of Health and permitted by the Central Theater Command General Hospital of the Chinese People's Liberation Army Institutional Review Board.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analysed during this study are included in this published article.

Conflicts of interest statement: All authors declare that there are not conflicts of interest.

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Figures

Figure 1

Hyaluronidase damage glycocalyx and increase gut permeability. (A) The content of glycocalyx on IECs was detected by Immunofluorescence (magnification 200x). GI represent glycocalyx inhibitor group.
(B) The mean fluorescence intensity of SDC-1 of glycocalyx on IECs. (C) FD4 concentration in plasma was measured by fluorescence microplate. ****P<0.0001, ** P<0.01.

**Figure 2**

Hyaluronidase induced intestinal and lung histology injury. (A) Histopathological examination of intestine by HE staining. (B) Histopathological examination of lung by HE staining. (C–E) The levels of TNF-α, IL-6, and NF-κB in lung tissues were measured by ELISA. GI represent glycocalyx inhibitor group, ****p<0.0001, ***p<0.001, ns p>0.05.

**Figure 3**

VNS decrease lung pulmonary vascular permeability and mitigate lung injury. (A-B) Lung permeability was measured as the amount (μg) of EBD in 100mg lung tissue that permeated through the lung parenchyma, ***p<0.001. (C) Histopathological examination of lung by HE staining.
Figure 4

VNS decreased the expression of TNF-α, IL-6 and MPO, and increased the expression of IL-10 in lung tissue. (A) The level of IL-10 mRNA in lung was measured by RT-qPCR. SS vs. THS/R, p=0.0015; THS/R vs. VNS, p=0.0015; VNS vs. VSM, p=0.0014. (B) The level of TNF-α mRNA in lung was measured by RT-qPCR. SS vs. THS/R, p< 0.0001; THS/R vs. VNS, p=0.0031; VNS vs. VSM, p=0.0453. (C) The level of IL-6 mRNA in lung was measured by RT-qPCR. SS vs. THS/R, p<0.0001; THS/R vs. VNS, p=0.0074; VNS vs. VSM, p=0.1735. (D) The level of MPO mRNA in lung was measured by RT-qPCR. SS vs. THS/R, p=0.0003;
THS/R vs. VNS, p=0.0441; VNS vs. VSM, p=0.1002. (E) The expression of IL-10, TNF-α, IL-6, NF-κB and MPO in lung tissue were measured by western-blot.

Figure 5

VNS attenuated the glycocalyx damage and decreased the intestinal barrier permeability. (A) The content of glycocalyx of IECs was detected by immunofluorescence (magnification 200x). (B) The mean fluorescence intensity of SDC-1 of glycocalyx on IECs. SS vs. THS/R, p<0.0001; THS/R vs. VNS, p=0.016; VNS vs. VSM, p=0.009. (C) The intestinal barrier permeability was showed by the concentration (μg/ml) of FD4 in plasma, ***p<0.001.

Figure 6

Histopathological examination of intestine by H&E staining.
VNS up-regulated the expression of IL-10, and reduced the expression of inflammatory factors in gut. (A) The level of acetylcholine in plasma was measured by ELISA. SS vs. THS/R, p=0.0035; THS/R vs. VNS, p=0.0031; VNS vs. VSM, p=0.0446. (B) The level of IL-10 mRNA in gut was measured by RT-qPCR. SS vs. THS/R, p=0.0007; THS/R vs. VNS, p=0.0018; VNS vs. VSM, p=0.0105. (C) The level of TNF-α mRNA in gut was measured by RT-qPCR. SS vs. THS/R, p<0.0001; THS/R vs. VNS, p=0.0070; VNS vs.
(D) The level of IL-6 mRNA in gut was measured by RT-qPCR. SS vs. THS/R, p<0.0001; THS/R vs. VNS, p=0.0001; VNS vs. VSM, p=0.0045. (E) The level of NF-κB mRNA in gut was measured by RT-qPCR. SS vs. THS/R, p<0.0001; THS/R vs. VNS, p=0.0033; VNS vs. VSM, p=0.0305. (F) The level of MPO mRNA in gut was measured by RT-qPCR. SS vs. THS/R, p<0.0001; THS/R vs. VNS, p=0.0010; VNS vs. VSM, p=0.0326. (G) The expression of IL-10, TNF-α, IL-6, NF-κB and MPO in gut tissue were measured by western-blot.