Modulation of Oxidative and Nitrosative Stress Attenuates Microvascular Hyperpermeability in Ovine Model of Pseudomonas Aeruginosa Sepsis

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Research

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Abstract

Background: Sepsis is one of the most frequent causes of death in the ICU, and microvascular hyperpermeability caused by oxidative/nitrosative stress plays an important role in tissue edema leading to multi-organ dysfunctions and increased mortality. This study tested the efficacy of a novel compound R-107, a modulator of oxidative/nitrosative stress, in an ovine model of sepsis. We hypothesized that R-107 effectively ameliorates the severity of microvascular hyperpermeability and preserves multi-organ function.

Methods: Sepsis was induced in twenty-two adult female Merino sheep by intravenous infusion of *Pseudomonas aeruginosa* ($1x10^{10}$ CFUs) for 60 minutes. After injury, animals were allocated into the following groups: 1) Control: intramuscular injection (IM) of saline, n=13; and 2) Treatment: IM of 50 mg/kg R-107, n=9. The IM treatment was given after the completion of the *Pseudomonas aeruginosa* injection. Animals were placed on a mechanical ventilator, fluid resuscitated, and monitored for 24 hours in a conscious state.

Results: R-107 treatment attenuated 24-hour mortality (11 vs. 30%). R-107 significantly reduced fluid requirement (15 – 24 hours, $p<0.05$), net fluid balance (9 – 24 hours, $p<0.05$), and water content in lung, heart, and kidney ($p=0.02$, 0.04, and 0.01, respectively) compared to control. R-107 treatment significantly delayed the onset of positive qSOFA (3.3 vs. 6.8 hours, $p=0.04$), and significantly decreased lung injury score and modified sheep SOFA score at 24 hours ($p=0.01$ and 0.04). The R-107 treatment group had significantly lower arterial lactate (21 – 24 hours, $p<0.05$), shed syndecan-1 (3 – 6 hours, $p<0.05$), and interleukin-6 (6 – 12 hours, $p<0.05$) levels in plasma, and significantly attenuated lung tissue 3-nitrotyrosine and vascular endothelial growth factor-A expression ($p=0.03$ and 0.002) compared to control. There was no adverse effect observed during R-107 treatment.

Conclusions: Modulation of oxidative/nitrosative stress by R-107 reduced lung tissue vascular endothelial growth factor-A, plasma shed syndecan-1, and interleukin-6 and attenuated severe microvascular hyperpermeability resulting in improved multi-organ function and survival in *Pseudomonas aeruginosa* sepsis.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection, and the most frequent cause of death in intensive care units (ICUs) due to multiple organ failure [1, 2]. The overall rate of hospital mortality of sepsis is reported at 25 – 35 % [3 – 5]. Recent work by Luhr et al. reported that mortality rate of septic patients has remained unchanged over the last two decades [6]. Endothelial cell damage and increased microvascular hyperpermeability caused by excessive oxidative/nitrosative stress (O&NS) produce interstitial tissue and multi-organ edema, leading to multiple organ dysfunctions and increased mortality [7 – 11].

Various therapies targeting O&NS (i.e., superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite) have been proposed for treatment of sepsis, however none of them has advanced to clinical practice as a standard therapy [12 – 14]. The exact reasons for these failed translational studies are unknown, however, it may be related to the lack of approaches that consider the complexity and multifactorial mechanism of O&NS-induced tissue injury, specifically the imbalance of nitric oxide (NO) and superoxide anion (O_2^-) and peroxynitrite in the septic condition [15 – 20]. The imbalance of these free radical species during sepsis impacts the distribution of extracellular water, disrupts epithelial and endothelial tight junctions, impairs endothelial function and vascular smooth muscle tone, chokes off microcirculatory blood flow, triggers pulmonary arterial hypertension, and raises endothelial hyperpermeability [16 – 20].

Previously, we tested the effects of the novel anti-O&NS agent R-100, which has triple actions– O_2^- catalytic degradation, NO donation, and peroxynitrite decomposition catalysis in a clinically-relevant ovine model of pneumonia/sepsis [21].

In the present study, we further tested the hypothesis that the novel drug R-107, a prodrug of R-100, attenuates microvascular hyperpermeability and improves multi-organ function and survival in an ovine model of PA sepsis.

Methods

Animal Model and Experimental Design

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch and conducted in compliance with the guidelines of the Animal Research: Reporting of In Vivo Experiments [22], the National Institutes of Health, and the American Physiological Society for the care and use of laboratory animals, as previously described [23, 24].

Twenty-two adult female Merino sheep (body weight [BW] 36.8±1.0 kg) were used. Briefly, animals were anesthetized with an intravenous injection of ketamine and isoflurane inhalation, and multiple vascular catheters were surgically inserted (Swan-Ganz, femoral arterial, and left atrial catheters). Pre- and post-surgical analgesia was provided with long-acting (for 72 hours) Buprenorphine SR^{TM} (0.05 mg/kg, SR Veterinary Technologies, Windsor, CO). Merino sheep were chosen because of their close resemblance of the pathophysiologic and immune responses to infection that are seen in humans [25 – 27].

After 5 – 7 days following instrumentation, baseline (BL) cardiopulmonary hemodynamic variables were collected (**Table 1**), as previously described [23, 24]. After the BL data were collected, a tracheostomy tube and urine catheter were inserted under ketamine and inhaled isoflurane anesthesia, and animals were placed on a mechanical ventilator (AVEA; Carefusion, Yorba Linda, CA) with the initial settings of a pressure-regulated volume control assist-control mode, tidal volume (TV) of 12 mL/kg, positive end-expiratory pressure of 5 cmH_2O, respiratory rate (RR) of 20 breaths/minute, and inspired oxygen concentration (FiO_2) of 0.21. Then, 1.0 x 10^{10} CFUs of *Pseudomonas aeruginosa* (PA; strain; PD-05144 [12-4-4, BRK-1244, NCIB-10780, NRRL-B-3224], catalog #: ATCC® 27317™, ATCC, Manassas, VA) suspended in 50 mL of warm 0.9% sodium chloride were intravenously injected (IV) via the jugular vein over 60 minutes in a conscious state. The variables of systemic hemodynamics were continuously monitored during and until 180 minutes after the initiation of the bacterial IV infusion. (**Table 2**). Arterial lactate levels were also determined during this time period.

Table 1: Cardiovascular Hemodynamics, Pulmonary Mechanics, Hematocrit, Biochemical Variables, and Systemic Neutrophil Counts during Baseline and at 24-hours.

Parameter	Group / Time	BL	6 hr	12 hr	15 hr	18 hr	21 hr	24 hr
Temperature (°C)	Control	39.0±0.1	40.8±0.2	40.5±0.2	40.3±0.3	40.1±0.2	40.2±0.3	40.2±0.2
	R-107	39.1±0.1	40.3±0.1	40.6±0.1	40.7±0.2	40.8±0.2 *	40.8±0.2 *	40.8±0.2
Heart Rate (beats/min)	Control	92±3	151±8	177±7	181±8	172±5	159±9	164±10
	R-107	97±4	169±8	181±9	184±10	168±11	174±7	163±10
Mean Arterial Pressure (mmHg)	Control	99±2	98±5	86±4	88±4	86±3	87±5	83±6
	R-107	96±2	104±4	87±3	91±4	93±3	91±4	92±2
Mean Pulmonary Artery Pressure (mmHg)	Control	18±1	31±1	35±2	39±2	38±2	35±2	32±2
	R-107	17±1	31±2	33±2	34±2	35±2	30±2	27±2
Left Atrial Pressure (mmHg)	Control	6±1	10±1	12±1	15±1	16±1	17±1	17±1
	R-107	6±1	9±1	11±1	11±1	12±1	12±1 **	11±1 ***
Central Venous Pressure (mmHg)	Control	4±1	8±1	11±1	13±1	13±1	15±1	15±1
	R-107	4±1	8±1	10±1	11±1	10±1	10±1 **	10±1 **
Cardiac Index ($mL/min/m^2$)	Control	6.4±0.3	4.8±0.4	8.1±0.6	9.5±0.6	9.7±0.8	8.6±0.9	9.3±1.1
	R-107	6.3±0.4	6.2±0.5	8.7±0.5	8.2±0.7	8.2±0.5	8.6±0.7	8.2±0.7
Stroke Volume Index ($mL/m^2/beat$)	Control	71±5	33±4	45±3	53±3	57±5	55±5	57±6
	R-107	66±5	37±3	49±4	46±5	50±3	49±3	51±4
Left Ventricular Stroke Work Index ($gm-m/m^2/beat$)	Control	86±6	38±6	44±3	50±4	54±5	52±6	54±6
	R-107	78±6	44±4	48±5	48±6	52±3	52±3	54±3
Right Ventricular Stroke Work Index ($gm-m/m^2/beat$)	Control	13±1	10±1	15±1	19±2	19±2	15±2	13±1
	R-107	11±1	12±1	15±1	13±1 *	17±2	14±1	11±1
Systemic Vascular Resistance Index ($dyne-sec/cm^5/m^2$)	Control	1214±65	1710±262	794±86	672±78	631±48	755±125	700±113
	R-107	1212±85	1303±129	728±54	822±71	834±65	796±73	845±86
Pulmonary Vascular Resistance Index ($dyne-sec/cm^5/m^2$)	Control	111±10	273±30	223±26	188±20	181±32	192±33	155±40
	R-107	98±9	220±30	176±30	202±32	189±14	160±12	131±16
PaO_2/FiO_2 Ratio (mmHg)	Control	513±6	467±29	402±33	362±35	320±45	274±45	229±45
	R-107	520±9	507±27	433±50	383±63	380±80	363±84	356±85
Shunt Fraction (%)	Control	6±1	7±1	15±3	22±4	27±6	31±8	32±5
	R-107	5±1	5±1	15±3	20±5	20±7	19±6	22±8
Peak Airway Pressure (cmH_2O)	Control	20±1	26±1	32±2	33±2	33±2	34±2	32±2
	R-107	17±1	26±2	31±3	33±3	29±3	28±3	27±3
Plateau Airway	Control	18±1	22±1	30±2	31±2	31±2	32±2	29±2

Pressure (cmH_2O)	R-107	16±1	23±2	28±3	29±3	27±3	25±3	25±3
Lung Static Compliance (mL/cmH_2O)	Control	35±2	26±1	17±2	15±2	13±2	14±2	17±2
	R-107	40±3	28±3	22±4	20±4	24±5	26±4 *	24±3
Hematocrit	Control	26±1	36±1	29±1	27±1	26±1	25±1	26±1
(%)	R-107	26±1	32±2	27±2	27±2	26±2	25±1	25±1
Glucose	Control	62±2	47±2	51±10	46±4	47±4	46±4	49±5
(mg/dL)	R-107	69±2	47±4	48±5	47±2	46±2	44±1	45±2
Neutrophil	Control	2.0±0.2	0.6±0.1	2.1±0.4	-	3.6±0.7	-	3.2±1.5
(x 10^3 cell/µL)	R-107	2.3±0.3	1.4±0.7	4.0±1.2	-	5.4±1.7	-	5.7±2.0

Data are expressed as mean±SEM. Two-way analysis of variance with a mixed-effects model with post hoc Bonferroni multiple comparison tests was performed. * p<0.05, ** p<0.01, and *** p< 0.001.

Animal Grouping, Drug Treatment, and Post-injury Care

After the injury, animals were randomly allocated into two groups: 1) control: treated with an intramuscular injection (IM) of saline, n=13; and 2) R-107: administered with IM 50 mg/kg R-107, n=9. The R-107 was injected into the animal's right quadriceps immediately after completing the infusion of bacteria.

Briefly, R-107 has a multi-functional prodrug technology to target redox imbalance of O&NS. R-107 is a prodrug ester that hydrolyzes to form R-100 [21], a molecule serving as: a NO donor via its organic nitrate, and a broad-spectrum catalyst of O&NS degradation via its nitroxide moiety (hydroxymethylproxyl). The spectrum of O&NS degradation of R-100 includes: O_2^- dismutation, catalase-like activity (detoxifying H_2O_2), and peroxynitrite decomposition. There are at present no approved agents with this multi-functional action. R-107 was provided from the Salzman Group Inc. (Beverly, MA).

Cardiopulmonary hemodynamic variables were continuously monitored (IntelliVue MP50; Philips Medical Systems, Andover, MA) (**Table 1**), and recorded hourly for a 24-hour study period in mechanically-ventilated conscious sheep. RR and FiO_2 were adjusted to maintain $PaCO_2$ between 30 - 40 mmHg and PaO_2 ~ 100 mmHg, respectively. Arterial and venous blood gas (i.e., arterial and venous PO_2, PCO_2, saturation, lactate, hematocrit) were determined using a blood gas analyzer (RAPIDPoint 500; Siemens Healthcare, Erlangen, Germany). Lactate clearance [28] was calculated using the following formula: lactate clearance = (lactate $_{\text{3-hours value}}$ – lactate $_{\text{delayed time-point value}}$) / lactate $_{\text{3-hours value}}$ × 100 (expressed as percentage). Plasma protein concentration was measured using a handheld refractometer (National Instrument Company Inc., Baltimore, MD).

Animals were fluid resuscitated with lactated Ringer's solution (LR; Baxter Healthcare Corporation, Deerfield, IL), starting with an initial infusion rate of 2 mL/kg/hr for 3 hours. Thereafter, the LR rate was adjusted every 3 hours to maintain hematocrit close to the BL levels ± 3%. Fluid input and urine output were monitored hourly and cumulative fluid balance was calculated, as previously described [23, 24].

Quick SOFA and Modified Sheep SOFA Score

In order to assess the onset of sepsis and the severity of multi-organ dysfunction during sepsis, we used quick SOFA (qSOFA) and modified sheep SOFA (mSOFA) scores, as previously described [1, 24, 29]. We measured the time to meet the qSOFA criteria from the initiation of the PA injury. Also, the mSOFA score included the values of animal neurological status, mean arterial pressure (MAP), PaO_2/FiO_2 ratio, total platelet counts measured by ADVIA-120 (Siemens Healthcare Diagnostics, Deerfield, IL), and plasma total bilirubin and creatinine concentrations measured by the hospital clinical chemistry laboratory. The neurological status of animal was assessed by the Simplified Sheep Neurological/Alertness

Assessment score [29]. The qSOFA score was measured hourly until the animals met the criteria, and the mSOFA score was measured at BL and at the 24-hour timepoint.

Euthanasia, Tissue Collection, and Tissue Extravascular Water Content Analysis

After completion of the 24-hour study period, animals were euthanized with injection of ketamine (40 mg/kg), buprenorphine (0.01 mg/kg), and xylazine (3.0 mg/kg), following the IACUC approved protocol and American Veterinary Medical Association Guidelines for Euthanasia [30]. Immediately after euthanasia, organs and tissues were collected, and lung, heart, and kidney tissue water content were measured by wet-to-dry weight ratio (W/D), as previously described [23, 24, 31].

Bacterial Clearance in Lung and Kidney

To assess bacterial clearance in the lung and kidney, a 1.0-gram section of the dorsal edge of right lung middle lobe and the kidney cortex were taken at necropsy and homogenized in 2 mL of 1x phosphate-buffered saline. Then, 200 µL of the tissue homogenates were transferred onto soy agar plates. The plates were incubated for 24 hours at 37°C for bacterial CFUs counts, as previously described [29].

Western Blotting and Enzyme-linked Immunosorbent Assay

Levels of lung tissue 3-nitrotyrosine [24] and vascular endothelial growth factor-A (VEGF-A), which is a major mediator of microvascular hyperpermeability [32 – 34], were determined by Western blot analysis (Automated capillary Western analysis, Wes^{TM} (ProteinSimple, San Jose, CA), as previously described [24].

The plasma shed syndecan-1 (Sdc1) and interleukin-6 (IL-6) levels were measured by enzyme-linked immunosorbent assay (ELISA) kits following the instructions (Sdc-1; Cat #: MBS745791, MyBiosource Inc., San Diego, CA, IL-6; Cat #: SEA079Ov, Cloud-Clone Corp., Katy, TX), and as previously described [29].

Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 8.3.1 (GraphPad Software, Inc., La Jolla, CA). Results were compared between the groups at each timepoint by a two-way analysis of variance with a mixed-effects model with *post hoc* Bonferroni multiple comparison tests. The values measured at a single timepoint were compared by unpaired t-test or Mann-Whitney U test, based on the normality of the data distribution (Shapiro-Wilk test). All values are expressed as Mean ± standard error of mean (Mean±SEM). Statistical significance was considered for p value < 0.05.

Results

Changes in Systemic Cardiopulmonary Hemodynamics and Biochemical Variables During and After Intravenous Bacterial Infusion

During the 60-minute intravenous infusion of PA, body temperature (BT), heart rate (HR), MAP, mean pulmonary artery pressure (mPAP), central venous pressure (CVP), systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI), and RR were increased, and cardiac index (CI) was decreased in all animals with the peak at 20 – 30 minutes after the start of the PA infusion. These changes returned to the BL values by 90 minutes after the start of the bacterial infusion, with the exception of the BT, HR, mPAP, PVRI, and plasma lactate level, which gradually increased until 180 minutes after the initiation of the bacterial infusion. The greatest increases of end-tidal CO_2 ($EtCO_2$) and CO_2 production (VCO_2) were observed at 30 minutes; these values then started decreasing 180 minutes after the start of the bacterial infusion. There was no significant difference in all variables between the two groups, indicating comparable injury in the two groups (**Table 2**).

Table 2: Changes in Systemic Hemodynamics and Biochemical Variable During and After (Baseline – 180 mins) Bacterial Intravenously Administration.

Parameter	Group /Time	BL	30 min	60 min	90 min	120 min	150 min	180 min
Temperature (°C)	Control	39.0±0.1	40.1±0.1	40.2±0.1	40.4±0.1	40.2±0.1	40.5±0.2	40.6±0.2
	R-107	39.1±0.1	40.2±0.2	40.3±0.2	40.1±0.1	39.9±0.2	40.0±0.2	40.2±0.2
Heart Rate (beats/min)	Control	92±3	110±8	126±10	163±6	164±6	174±8	161±6
	R-107	97±4	116±9	127±8	171±11	174±13	176±13	162±11
Mean Arterial Pressure (mmHg)	Control	99±2	116±5	104±3	90±4	93±3	94±5	98±4
	R-107	96±2	110±3	108±7	89±5	90±3	93±3	96±2
Mean Pulmonary Artery Pressure (mmHg)	Control	18±1	43±2	35±1	31±2	31±2	34±2	31±1
	R-107	17±1	43±2	34±2	27±2	27±1	29±1	29±2
Central Venous Pressure (mmHg)	Control	4±1	8±1	6±1	6±1	7±1	7±1	6±1
	R-107	4±1	7±1	5±1	5±1	6±1	5±1	5±1
Cardiac Index ($mL/min/m^2$)	Control	6.4±0.3	4.1±0.5	4.6±0.4	6.7±0.4	6.9±0.5	5.8±0.4	5.7±0.5
	R-107	6.3±0.4	3.7±0.4	5.7±0.4	7.1±0.4	6.5±0.4	6.5±0.4	5.4±0.4
Systemic Vascular Resistance Index ($dyne-sec/cm^5/m^2$)	Control	1214±65	2472±2729	1901±199	1037±72	1063±87	1256±100	1449±155
	R-107	1212±85	2427±280	1520±157	973±86	1085±100	1131±102	1418±101
Pulmonary Vascular Resistance Index ($dyne-sec/cm^5/m^2$)	Control	111±10	474±50	320±31	194±24	193±14	255±15	253±24
	R-107	98±9	525±66	269±42	167±20	166±16	190±15	223±29
Respiratory Rate (rate/min)	Control	20	25±2	25±2	23±2	23±2	23±2	23±1
	R-107	20	24±2	22±1	23±2	22±2	22±2	22±1
End-tidal CO_2 (mmHg)	Control	-	32.0±2.9	30.1±2.1	30.7±1.5	32.4±1.1	34.4±2.2	33.1±1.7
	R-107	-	36.3±2.4	31.1±1.6	29.5±1.5	30.2±2.2	33.7±1.9	34.6±2.5
CO_2 Production (VCO_2) (mL/min)	Control	-	352±33	252±28	264±34	276±22	294±23	290±21
	R-107	-	333±28	249±15	253±13	246±21	284±22	285±23
Lactate (mmol/L)	Control	0.51±0.04	-	2.65±0.29	2.90±0.37	3.00±0.40	-	3.37±0.52
	R-107	0.52±0.05	-	2.20±0.49	2.89±0.60	2.96±0.62	-	2.85±0.61

Data are expressed as mean±SEM. Two-way analysis of variance with a mixed-effects model with post hoc Bonferroni multiple comparison tests was performed.

Survival, qSOFA and mSOFA Scores, Plasma Lactate Clearance, and Lung Injury Score

During the 24-hour study period, in both groups, MAP was decreased and BT, HR, mPAP, and CI were increased from their BL values, reflecting a state of hyperdynamic sepsis (**Table 1**). The BT was significantly higher in R-107 treated sheep at 18 – 21 hours ($p<0.05$) compared to control. R-107 significantly attenuated the increases in LAP and CVP at 21 – 24 hours ($p< 0.01$) compared to control. R-107 treatment also significantly decreased right ventricular stroke work index at 15 hours ($p<0.05$), and significantly attenuated the decreases in static lung compliance at 21 hours ($p<0.05$) as compared to control (**Table 1**).

The 24-hour survival rate was 89% (8 out of 9 sheep) for the R-107 treatment group, and 69% (9 out of 13 sheep) for control (no statistical significance).

R-107 treatment significantly delayed the onset of sepsis compared to control, which was confirmed by the time that sheep met qSOFA criteria. Six of 9 sheep in the R-107 group met the qSOFA score criteria within 6.8±2.0 hours, whereas all 13 sheep in the control group met these criteria within 3.3±0.7 hours from the start of the PA infusion ($p=0.04$) (**Figure 1A**).

R-107 treatment significantly attenuated the increases in the mSOFA score at 24-hours in the survived animals (n=8 in the R-107 treatment group and n=9 in control, 2.9±1.0 vs. 5.9±1.0, $p=0.04$) as compared to control (**Figure 1B**). The measured variables of mSOFA score in both groups are shown in **Table 3**.

Table 3: Lung Injury Score and mSOFA Score Variables at 24-hours.

Variable / Group	Respiration PaO_2/FiO_2 (mmHg) / mSOFA Score	Coagulation Platelets (x $10^3/\mu L$) / mSOFA Score	Liver Bilirubin (mg/dL) / mSOFA Score	Cardiovascular MAP Decrement from BL (mmHg) / mSOFA Score	Central Nervous System SSNAA (2 – 11) / mSOFA Score	Renal Creatinine (mg/dL) / mSOFA Score	Total mSOFA Score (at 24-hours)
Control	229±44 / 2.1±0.5	274±39 / (0)	0.5±0.1 / (0)	11.3±5.2 / 1.2±0.4	6.2±0.9 / 2.2±0.4	1.0±0.2 / 0.3±0.2	**5.9±1.0**
R-107	356±85 / 1.6±0.7	341±72 / 0.4±0.4	0.2±0.1 / (0)	4.5±2.6 / 0.3±0.2	9.6±0.7 / 0.6±0.3	0.7±0.1 / (0)	**2.9±1.0 ***
Data are expressed as mean±SEM. Mann-Whitney U test was performed in the Total mSOFA Score, * $p<0.05$.							

Also, the lung injury score (LIS) at 24-hours in the survived animals was significantly lower in the R-107 treatment group as compared to control (n=8 in the R-107 treatment group and n=9 in control, 1.5±0.2 vs. 2.2±0.2, $p=0.01$) (**Figure 1C**).

R-107 treatment significantly attenuated the increase in plasma lactate levels at 21 – 24 hours ($p<0.01$) (**Figure 1D**), and significantly improved lactate clearance during sepsis compared to control at 21 – 24 hours ($p<0.05$) (**Figure 1E**).

Fluid Balance, Hematocrit Changes, Plasma Protein Concentration, and Total Amount of Pleural and Ascitic Fluid at Necropsy

The fluid requirement was significantly lower in the R-107 treated group at 15 – 24 hours ($p<0.05$) (**Figure 2A**), and cumulative urine output at 15 – 24 hours ($p<0.05$) (**Figure 2B**) was significantly higher in the R-107 treated group compared to control. The net fluid balance was significantly attenuated in the R-107 treated group as compared to control at 9 – 24 hours ($p<0.05$) (**Figure 2C**). The hematocrit changes during the study period in both groups were comparable (**Table 1**).

The plasma protein concentration was significantly higher in the R-107 treated group compared to control at 21 – 24 hours ($p<0.05$) (**Figure 2D**).

The combined volume of pleural and ascitic fluid at euthanasia was significantly lower in the R-107 treated group as compared to control (21.1±6.2 vs. 57.7±4.7 mL/kg BW, $p<0.001$) (**Figure 2E**).

Organ Extravascular Water Content and Bacterial Clearance

The organ extravascular water content was determined by measuring organ W/D at euthanasia. The R-107 treated group displayed significantly lower water content in the lung, heart, and kidney compared to control (lung: 7.5±0.3 vs. 8.5±0.4, heart: 4.1±0.1 vs. 4.4±0.1, and kidney: 4.1±0.02 vs. 4.2±0.04, $p=0.02$, 0.04, and 0.01, respectively) (**Figure 3A – 3C**).

The number of bacteria in lung and kidney tissue homogenate cultures was significantly lower in the R-107 treated group compared to control (0.4±0.1 vs. 1.3±0.3 and 0.4±0.3 vs. 1.1±0.3 $\times 10^3$ CFUs/gram tissue in control, $p=0.02$ and 0.02, respectively) (**Figure 3D, 3E**).

Oxidative and Nitrosative Stress, Glycocalyx, and Inflammatory Mediators

The modulation of O&NS by the R-107 treatment was measured by the levels of 3-nitrotyrosine in the lung tissue. The 3-nitrotyrosine levels in lung tissue homogenate collected at the euthanasia was significantly lower in the R-107 treated group compared to control (0.07±0.01 vs. 0.13±0.02, $p=0.02$) (**Figure 4A**).

R-107 significantly decreased plasma levels of shed Sdc1 at 3 and 6 hours (37.0±6.6 vs. 88.9±13.4 and 26.7±7.7 vs. 68.8±10.9, $p=0.005$ and 0.03, at 3 and 6 hours, respectively) compared to control (**Figure 4B**).

The plasma IL-6 concentration was significantly lower in the R-107 treated group at 6 and 12 hours (2178±467 vs. 8732±918 and 3817±1115 vs. 7585±1287, $p<0.001$ and 0.03, respectively) compared to control (**Figure 4C**). The lung tissue VEGF-A levels were significantly lower in the R-107 treated group compared to control (0.89±0.11 vs. 1.59±0.12, $p<0.001$) (**Figure 4D**).

Discussion

In this study, we demonstrated that modulation of O&NS by R-107 significantly reduced microvascular hyperpermeability and body fluid retention, resulting in improved multiorgan function and survival in septic sheep. Previously, we demonstrated that the modulation of O&NS by R-100 significantly reduces systemic fluid retention and improves pulmonary gas exchange in the model of ovine pneumonia/sepsis [21]. The present study results were in an agreement with previous work in terms of reduced fluid retention, organ edema, and microvascular hyperpermeability. In the present study, we further showed that R-107 treatment improved bacterial clearance in the lung and kidney, inhibited increases in inflammatory mediators (plasma shed Sdc1, IL-6, and lung VEGF-A levels), delayed the onset of sepsis (measured by the qSOFA score), attenuated multi-organ dysfunction (measured by the mSOFA score), and improved multi-organ function.

In ICU patients with sepsis and septic shock, positive fluid balance, at least in part, due to this microvascular hyperpermeability is not only the independent predictor of mortality, but it also is linked to the increased ICU-days, ventilator-days, and total hospital care costs [35 – 39]. Therefore, therapies targeting microvascular hyperpermeability and edema formation during sepsis are of particular interest.

As mentioned, sepsis increases microvascular hyperpermeability and excessive fluid retention and causes multiple organ dysfunction by excessive O&NS [7 – 9, 40, 41]. Excessive O&NS causes endothelial cell damage and endothelial glycocalyx layer disruption [40 – 43]. The endothelial glycocalyx layer controls endothelial permeability to water and serves as a barrier to neutrophil/bacteria migration into organ interstitial space from the bloodstream [7 – 9, 41, 42, 44, 45]. The Sdc1 is one of the major components of the glycocalyx, and the levels of shed Sdc1 in plasma are considered as a marker of glycocalyx layer disruption during sepsis [45]. Moreover, the plasma shed Sdc1 levels correlate with the increments of plasma IL-6 level during sepsis [46, 47].

In addition, VEGF-A, a well-known inflammatory mediator and potent vascular hyperpermeability factor, is increased in septic conditions [32, 34, 48]. In our previous studies, we have shown increases in lung tissue VEGF and myeloperoxidase activity associated with sever microvascular hyperpermeability [34].

The results of the present study demonstrated that R-107 treatment significantly reduced the degree of O&NS as evidenced by increases in lung tissue 3-nitrotyrosine levels. We also showed that R-107 significantly reduced the plasma levels of shed Sdc1 and IL-6 and significantly attenuated VEGF-A levels in lung tissue compared to control. Although, these findings do not represent causation, we speculate that R-107 attenuated microvascular permeability in this model by inhibiting the potent permeability factor VEGF and preserving endothelial glycocalyx through modulation of O&NS stress.

In the present study, we reported that the number of bacteria in lung and kidney tissue were significantly lower in the R-107 treatment group compared to control. The underlying mechanisms for this finding are not fully understood. Lubkin et al. reported that PA secretes toxins that disrupt the endothelial cell monolayer barrier mechanism via the induction of oxidative stress [49, 50]. We speculate that protection of the endothelial barrier by R-107 may be attributed to the reduced number of bacteria in organ tissues. Of note, circulating numbers of neutrophils were not affected by R-107, indicating that differences in bacterial numbers between the two groups were not related to variations in the number of neutrophils.

Further, increases in plasma shed Sdc1 in control were unlikely to have been impacted by the volume of resuscitation fluid because the hematocrit, plasma protein concentration, and hemodynamic variables were comparable in both groups at 3 – 6 hours, when the plasma shed Sdc1 levels were significantly lower in the R-107 treated group compared to control.

Our present study has several limitations. First, we did not show causative factors by which R-107 reduced microvascular hyperpermeability, rather we showed co-association of the numbers of potent inflammatory mediators with increased vascular permeability. Second, we did not use the supportive therapies, such as antibiotics and vasopressors, which are the standard therapies for sepsis. However, we aimed to explore a “pure” effects of testing compound without any drug interactions. Finally, the study period was relatively short (24-hour), which does not consider the concomitant diseases or factors that are associated with human sepsis. However, the model allowed us to most closely mimic the human hyperdynamic sepsis, continuously monitoring cardiopulmonary hemodynamics in a conscious state without the effects of anesthetics.

Conclusions

In conclusion, modulating excessive O&NS stress by R-107 may be considered as an effective and safe therapeutic option for management of sepsis-induced microvascular hyperpermeability. Future studies are warranted to further investigate the mechanisms of how moderation of O&NS by this potential therapeutic compound "R-107" attenuates the pathophysiology of sepsis-induced microvascular hyperpermeability.

Abbreviations

ICU; intensive care unit, O&NS; oxidative/nitrosative stress, NO; nitric oxide, O_2^-; superoxide anion, IACUC; institutional animal care and use committee, TV; tidal volume, RR; respiratory rate, FiO_2; inspired oxygen concentration, PA; *Pseudomonas*

aeruginosa, IV; intravenous injection, IM; intramuscular injection, BW; body weight, LR; lactated Ringer's solution, qSOFA; quick Sequential Organ Failure Assessment, mSOFA; modified sheep Sequential Organ Failure Assessment, MAP; mean arterial pressure, W/D; wet-to-dry weight ratio, VEGF-A; vascular endothelial growth factor-A, Sdc-1; Syndecan-1, IL-6; interleukin-6, ELISA; enzyme-linked immunosorbent assay, BT; body temperature, HR; heart rate, mPAP; mean pulmonary artery pressure, CVP; central venous pressure, SVRI; systemic vascular resistance index, PVRI; pulmonary vascular resistance index, CI; cardiac index, $EtCO_2$; end-tidal CO_2, VCO_2; CO2 production, LIS; lung injury score.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch and conducted in compliance with the guidelines of the Animal Research: Reporting of In Vivo Experiments, the National Institutes of Health, and the American Physiological Society for the care and use of laboratory animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

EG, GS, and AS are employed in Salzman group.

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Authors' contributions

SF, AS, and PE: Design of the work. SF, YN, YH, and EM: Data acquisition and analysis. SF, YN, YH, EG, GS, AS, and PE: Interpretation of data. SF, YH, DP, and PE: Drafted the work or substantively revised it.

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Figures

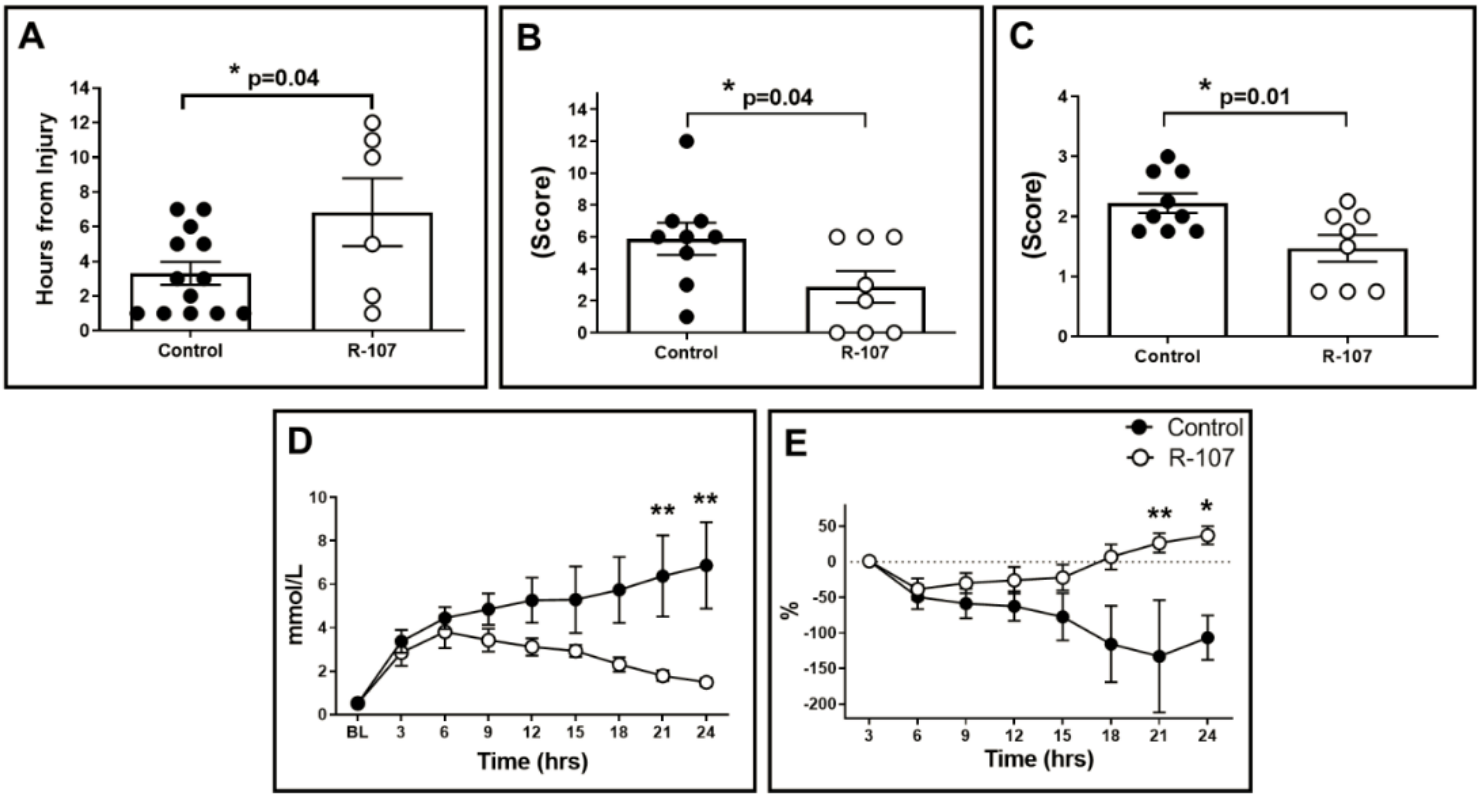

Figure 1

Quick SOFA and Modified Sheep SOFA Scores, Plasma Lactate Clearance, and Lung Injury Score (A) Time met the quick SOFA criteria from the injection of Pseudomonas aeruginosa in two groups (n=13 in control and n=6 in the R-107 treatment group). (B) Modified sheep SOFA scores at baseline and at 24-hours in two groups (n=9 in control and n=8 in the R-107 treatment group at 24-hours). (C) Lung injury score at 24-hours in two groups (n=9 in control and n=8 in the R-107 treatment group). (D) Plasma lactate concentration during the baseline and at 24-hours in two groups. (E) Lactate clearance from the 3-hours value during the baseline and at 24-hours in two groups. Closed circles represent control group and open circles

represent the R-107 treatment group. Data are expressed as mean ± SEM. (* p<0.05, ** p<0.01, and *** p<0.001, Control vs. R-107 treatment group.)

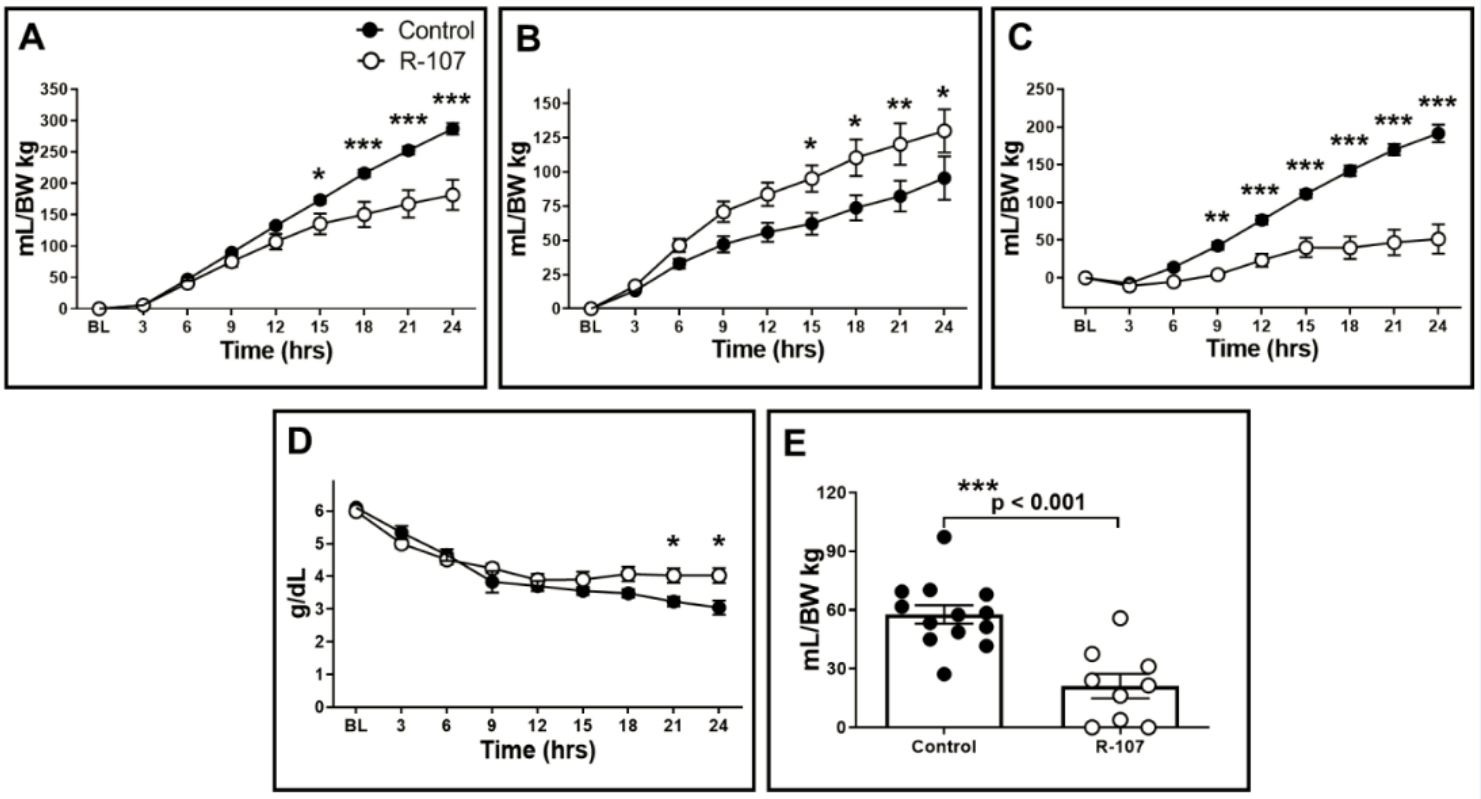

Figure 2

Fluid Balance, Plasma Protein Concentration, and Total Amount of Pleural and Ascitic Fluid at Necropsy (A) Fluid requirement during the baseline and at 24-hours in two groups. (B) Cumulative urine output during the baseline and at 24-hours in two groups. (C) Net fluid balance during the baseline and at 24-hours in two groups. (D) Plasma protein concentration during the baseline and at 24-hours in two groups. (E) Combined volume of pleural and ascitic fluid measured at euthanasia in two groups. Closed circles represent control group and open circles represent the R-107 treatment group. Data are expressed as mean ± SEM. (* p<0.05, ** p<0.01, and *** p<0.001, Control vs. R-107 treatment group.)

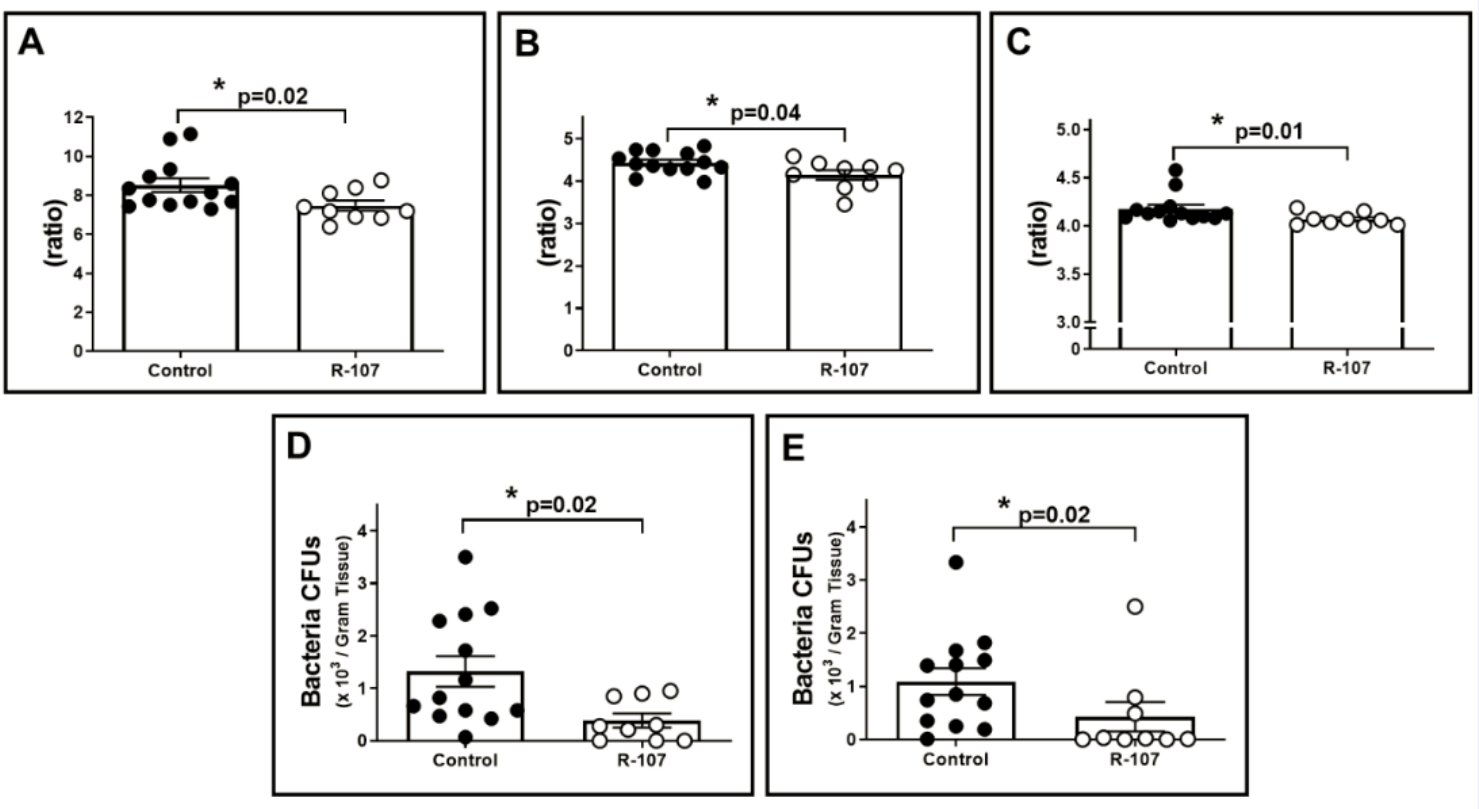

Figure 3

Organ Extravascular Water Content and Bacterial Clearance (A) Lung extravascular water content at euthanasia in two groups. (B) Heart extravascular water content at euthanasia in two groups. (C) Kidney extravascular water content at euthanasia in two groups. (D) Numbers of bacteria in lung tissue homogenate cultures collected at euthanasia in two groups. (E) Numbers of bacteria in kidney tissue homogenate cultures collected at euthanasia in two groups. Closed circles represent control group and open circles represent the R-107 treatment group. Data are expressed as mean ± SEM. (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$, Control vs. R-107 treatment group.)

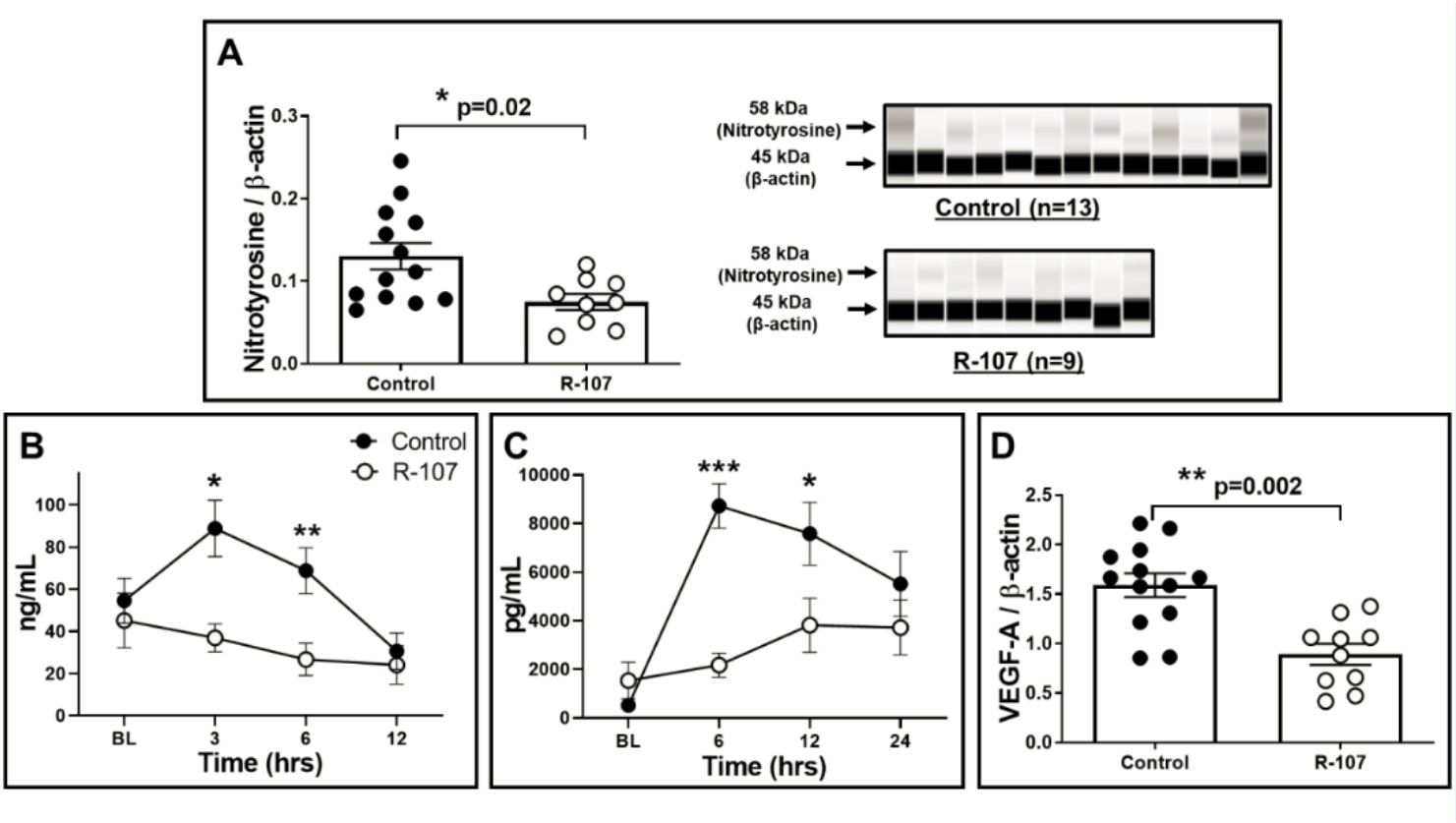

Figure 4

Oxidative and Nitrosative Stress, Glycocalyx, and Inflammatory Mediators (A) 3-nitrotyrosine level in lung tissue homogenate collected at euthanasia in two groups. (B) Plasma shed syndecan-1 concentration during the baseline and at 12-hours in two groups. (C) Plasma interleukin-6 concentration during the baseline and at 24-hours in two groups. (D) Vascular endothelial growth factor-A level in lung tissue homogenate collected at euthanasia in two groups. Closed circles represent control group and open circles represent the R-107 treatment group. Data are expressed as mean ± SEM. (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$, Control vs. R-107 treatment group.)