

Effects of Combined Levosimendan and Vasopressin on Pulmonary Function in Porcine Septic Shock

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Abstract—This study aims to determine whether levosimendan combined with arginine vasopressin infusion supplemented with norepinephrine can improve hemodynamics and pulmonary dysfunction. The study was tested in a fecal peritonitis-induced septic shock model, we observed that levosimendan combined with arginine vasopressin supplemented with norepinephrine therapy resulted in lower mean pulmonary artery pressure, lactate concentrations, arterial total nitrate/nitrite, and high-mobility group box 1 levels; decreased lung wet/dry ratio, and pulmonary levels of interleukin-6, total histological scores, and improved pulmonary gas exchange when compared with norepinephrine group. Levosimendan combined with arginine vasopressin supplemented with norepinephrine infusion shows potential benefit in sepsis-induced acute lung injury by decreasing mean pulmonary artery pressure and attenuating inflammatory responses in the lung compared to norepinephrine infusion alone.

KEY WORDS: sepsis; vasoactive agents; inflammatory responses; oxygenation.

INTRODUCTION

Despite a significant improvement in diagnosis and treatment, mortality rates associated with sepsis and septic shock remain unacceptably high [1]. Refractory arterial hypotension plays a key role in the development of multiple organ failure, and the lung has been identified as one of the most important targets of injury

during septic shock [2]. Thus, hemodynamic support with vasoactive agents is necessary when fluid administration fails to maintain hemodynamic stability [3], norepinephrine is the most widely used first-line vasopressor in the treatment of volume-refractory septic shock [4]. However, high doses of norepinephrine are required due to diminished responsiveness of adrenergic receptors in septic shock. This in turn may be associated with significant side effects, including arrhythmias, increased myocardial oxygen consumption, and pulmonary vascular resistance [5].

Arginine vasopressin (AVP) is a unique vasoactive hormone with the characteristics of pulmonary vasodilation and systemic vasoconstriction [6]. A growing number of studies have demonstrated that AVP is inappropriately low in patients with septic shock and replacement of physiologic levels of AVP can restore vascular tone, suggesting the usefulness of exogenous replacement treatment [7]. Furthermore, recent studies suggest low-dose AVP may decrease sepsis-induced pulmonary inflammation [8, 9]. However, considering AVP may potentially reduce cardiac output and tissue perfusion, the addition of a drug with positive inotropic and vasodilatory properties, with the purpose of improv-

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ing cardiac performance and organ blood flow, would be reasonable. It has been proposed that the calcium sensitizer, levosimendan, increases contractility with decreased side effects on oxygen consumption and simultaneously produce pulmonary vasodilation [10, 11]. More importantly, both clinical and experimental studies demonstrate that levosimendan also has anti-inflammatory and anti-apoptotic properties [12, 13]. Because hyperactive inflammation is a well-recognized feature of sepsis-induced acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), it is reasonable to speculate that the combination therapy holds a promising strategy for the treatment of septic shock-induced ALI/ARDS.

The primary aim of this study was to test whether a combined infusion of levosimendan and AVP supplemented with norepinephrine would attenuate inflammatory response, improve pulmonary hemodynamics, and gas exchange compared to either AVP supplemented with norepinephrine, levosimendan supplemented with norepinephrine, or norepinephrine alone. The study hypothesis was tested in a porcine model of septic shock induced by peritonitis.

MATERIALS AND METHODS

Animal Care

All procedures were approved by the ethics committee of Nanjing University Medical School and were performed in accordance with the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publication No. 85–23, revised 1996). Thirty-two domestic female swine were fasted for one night with free access to water.

Instrumentation and Surgical Procedures

After anesthesia induction with intramuscular ketamine 20 mg kg⁻¹ (HenRui Co., Jiangsu, China), the pigs were placed in the supine position and the cephalic vein was cannulated with a peripheral venous catheter. The animals were then orally intubated (5.5–6.5; TuoRen Co., Henan, China) and mechanically ventilated in controlled volume mode (Servo ventilator 900 C; Siemens-Elima, Solna, Sweden) with a positive end-expiratory pressure of 5 cm H₂O, a tidal volume of 7–10 ml kg⁻¹ min⁻¹, an inspired oxygen fraction of 0.21, and an inspiratory time/expiratory time of 1:2. Anesthesia was maintained with continuous intravenous infusions of fentanyl (10 µg kg⁻¹ h⁻¹;

Renfu Co., Hubei, China) and propofol (2 mg kg⁻¹ h⁻¹; AstraZeneca, WuXi, China); vecuronium (0.3 mg kg⁻¹ h⁻¹; Renfu Co., Hubei, China) was used for muscle relaxation. Tidal volume and respiratory rates were adjusted to maintain end-tidal carbon dioxide tension between 35 and 45 mmHg. The right femoral artery was catheterized for monitoring of arterial blood pressure and withdrawal of arterial blood samples. Through the right jugular vein, an introducer was inserted, and a 7.0 F Swan–Ganz catheter (Edwards Life Sciences, Irvine, CA, USA) was floated into the pulmonary artery with pressure waveform monitoring.

Peritonitis was induced as Rehberg *et al.* [14] described. Briefly, the cecal and ileocecal was identified through a midline laparotomy. After a 1 cm perforation in the cecal tip, spillage of fecal material (1 g kg⁻¹ of body weight) was collected in a 100 mL syringe. The cecum and the abdominal cavity were then closed by fascial and cutaneous sutures. Finally, peritonitis was induced by inoculating the autologous feces (1 g kg⁻¹) into the peritoneal cavity via a suction catheter that remained *in situ*.

Experimental Protocol

In each animal, lactated Ringer's solution (10 ml kg⁻¹ h⁻¹; WanTong, Co., Jilin, China) and hydroxyethyl starch (5 ml kg⁻¹ h⁻¹; 6% hydroxyethyl starch 130/0.6; Fresenius, Beijing, China) were infused as maintenance fluid. Additional fluids (crystalloid/colloid ratio 2:1) were infused if the hematocrit increased. After the onset of septic shock (defined as mean artery pressure [MAP] < 60 mmHg), animals were randomly assigned to the following four groups (each, *n* = 8). (1) Norepinephrine group: an open norepinephrine (2 mg ml⁻¹; WanTong, Co., Jilin, China) infusion was titrated to maintain MAP between 65 and 75 mmHg. (2) Levosimendan + norepinephrine group: levosimendan (Simdax, Abbott Pharma, North Chicago, IL, USA; 0.4 µg kg⁻¹ min⁻¹) was added at the same time as norepinephrine to maintain MAP between 65 and 75 mmHg. (3) AVP + norepinephrine group: an AVP (American Regent Inc, Shirley, NY, USA) infusion was started at a constant infusion rate of 0.57 mU kg⁻¹ min⁻¹. (4) Levosimendan + AVP + norepinephrine group: levosimendan (0.4 µg kg⁻¹ min⁻¹) and AVP (0.57 mU kg⁻¹ min⁻¹) were infused at the same time. If necessary, an open norepinephrine infusion was titrated to maintain MAP between 65 and 75 mmHg in the AVP + norepinephrine and levosimendan + AVP + norepinephrine groups.

Measurements and Calculations

All intravascular pressures were referenced to the mid-axillary and determined at end-expiration. MAP, mean pulmonary arterial pressure (MPAP), pulmonary capillary wedge pressure (PCWP), infusion volume, and blood gas analysis were evaluated at the following time points: baseline, shock time, 4, 8, and 12 h after septic shock. Norepinephrine requirements were recorded every 30 min after the onset of septic shock. Venous admixture (Qs/Qt), systematic vascular resistance (SVR), and pulmonary vascular resistance (PVR) were calculated by standard formula. Heart rate was determined by calculating the mean frequency of arterial

pressure curve peaks. Cardiac output (CO) was measured in triplicate by the thermodilution technique, using 10 ml iced saline solution (0°C). Arterial and mixed venous blood samples were simultaneously obtained for immediate determination of arterial and mixed venous oxygen saturations, partial pressure of oxygen, arterial hematocrit, arterial pH, arterial base excess (BE), and arterial lactate concentrations (GEM Premier 3000, Guangzhou, China).

Histological Analyses and Apoptosis Assessment

Animals surviving the 12-h study period after the onset of septic shock were killed under deep anesthesia

Table 1. Changes in Cardiopulmonary Hemodynamics

Variable	Time/GROUP	Baseline	ST	4 h	8 h	12 h
HR (beats·min ⁻¹)	NE	100±9	121±13	135±9	142±9	150±11
	LEVO+NE	109±16	123±16	127±21	134±20	147±13
	AVP+NE	108±16	126±13	118±6*	124±7*	139±9
	LEVO+AVP+NE	103±16	119±16	129±10	131±7	146±10
CO (ml kg ⁻¹ min ⁻¹)	NE	104±13	108±6	165±17	164±18	147±16
	LEVO+NE	102±11	109±7	174±15	174±9	160±9
	AVP+NE	106±12	111±6	133±10*,**	149±16**	142±9**
	LEVO+AVP+NE	106±11	109±5	138±14*,**	156±9	147±20
MAP (mmHg)	NE	97±12	59±0	70±3	71±2	70±3
	LEVO+NE	102±14	59±1	72±2	70±3	69±4
	AVP+NE	96±16	59±0	70±2	71±4	70±2
	LEVO+AVP+NE	97±15	59±1	69±3	70±3	69±3
CVP (mmHg)	NE	8±2	11±2	12±2	13±2	14±2
	LEVO+NE	9±2	11±1	11±2	13±2	13±1
	AVP+NE	8±2	12±2	12±2	12±1	13±2
	LEVO+AVP+NE	7±2	12±1	11±3	12±2	14±2
SVR (dynes scm ⁻⁵)	NE	2,297±441	1,190±170	955±165	976±183	1,005±180
	LEVO+NE	2,369±252	1,159±148	914±88	868±180	910±118
	AVP+NE	2,212±452	1,117±106	1,163±147*,**	1,064±202**	1,092±70
	LEVO+AVP+NE	2,254±525	1,153±137	1,120±180**	969±101	1,042±231
MPAP (mmHg)	NE	21±3	27±3	30±3	36±3	42±4
	LEVO+NE	21±3	26±4	29±3	33±3	38±3
	AVP+NE	21±3	28±4	30±3	31±4*	36±2*
	LEVO+AVP+NE	21±3	28±2	29±2	32±2	36±2*
PCWP (mmHg)	NE	10±2	14±2	15±2	16±2	18±1
	LEVO+NE	10±2	14±1	15±2	16±1	17±1
	AVP+NE	10±1	15±2	16±2	17±1	17±1
	LEVO+AVP+NE	10±2	15±2	16±1	16±1	17±1
PVR (dynes scm ⁻⁵)	NE	281±114	335±52	249±67	304±68	400±100
	LEVO+NE	289±84	297±95	202±68	257±81	351±53
	AVP+NE	294±72	318±119	277±54	259±87	366±42
	LEVO+AVP+NE	306±116	313±91	272±76	264±38	375±102

Values are represented as mean±SD, each group n=8

LEVO levosimendan, AVP arginine vasopressin, NE norepinephrine, BL baseline, ST shock time, HR heart rate, CO cardiac output, MPAP mean pulmonary artery pressure, CVP central venous pressure, PCWP pulmonary capillary wedge pressure, SVR systemic vascular resistance, PVR pulmonary vascular resistance

*P<0.05 versus NE group

**P<0.05 versus LEVO+NE group

with a lethal dose of 10% potassium chloride. An isolated central lobe in the right lung was excised and immediately immersed into 4% formalin. The samples were sectioned and stained with hematoxylin and eosin for light microscopy. The degree of microscopic injury was scored based on the scoring system previously described by Gloor *et al.* [15].

The pulmonary terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to monitor the extent of DNA fragmentation as a measure of apoptosis in paraffin-embedded sections. The assay was performed according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Fluorescein-conjugated dUTP incorporated in nucleotide polymers were detected and quantified using fluorescence microscopy (Zeiss LSM 410, Wetzlar, Germany). Only nuclear staining was considered positive.

Lung Wet-to-Dry Weight Ratio

Lung wet-to-dry weight ratio (W/D) was determined immediately after the experiment. Briefly, the lungs were removed, weighted, and then dried in an oven at 80°C for 48 h to obtain pulmonary W/D ratios.

Measurement of Secreted Cytokines, NO_x, and High-Mobility Group Box 1

Arterial blood was withdrawn and immediately centrifuged for 10 min at 3,000 rpm at the corresponding time points, and the isolated plasma was stored at -70°C for the determination of inflammatory mediators later. Tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-10 in the lung and serum were quantified using specific ELISA kits for swine according to the manufacturers' instructions (Quantikine, R&D systems, Abingdon, UK). Pulmonary tissue levels of TNF- α , IL-6, and IL-10 were normalized to the protein concentration in the sample. Arterial total nitrate/nitrite concentrations (NO_xa, a surrogate of NO) were measured using the Griess reagent (Cayman Chemical Nitrite/Nitrite Assay Kit; Cayman Chemical Co., Ann Arbor, MI, USA). Serum levels of high mobility group box 1 (HMGB1) secretion were assayed by sandwich method according to the manufacturers' instructions (Shino-Test Corporation, Tokyo, Japan).

Statistical Analyses

Kolmogorov-Smirnov test was applied to determine if the collected data form normal distribution. Data

collected from experiments forming normal distribution were expressed as mean \pm standard deviation unless otherwise stated. Variables from baseline to septic shock time, W/D ratio, total histological changes, TNF- α , IL-6, IL-10, and apoptotic cells in the lung tissue were analyzed using analysis of variance (ANOVA) with Bonferroni post hoc test. ANOVA was also employed for group comparison of norepinephrine requirements at the given time points. Missing values of norepinephrine consumptions were estimated by the norepinephrine consumption of the last 30 min multiplied the corresponding missing time period. Intergroup comparisons during the intervention period from shock time to 12 h after septic shock were tested using analysis of covariance (ANCOVA) followed by Bonferroni test. The data collected at baseline were used as a covariate in the analysis to account for potential intergroup differences before intervention except for HMGB1, in which shock time (ST) was used as the covariate because plasma HMGB1 was not detected at baseline. Statistical analysis was performed using the SPSS 16.0 software for Windows (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

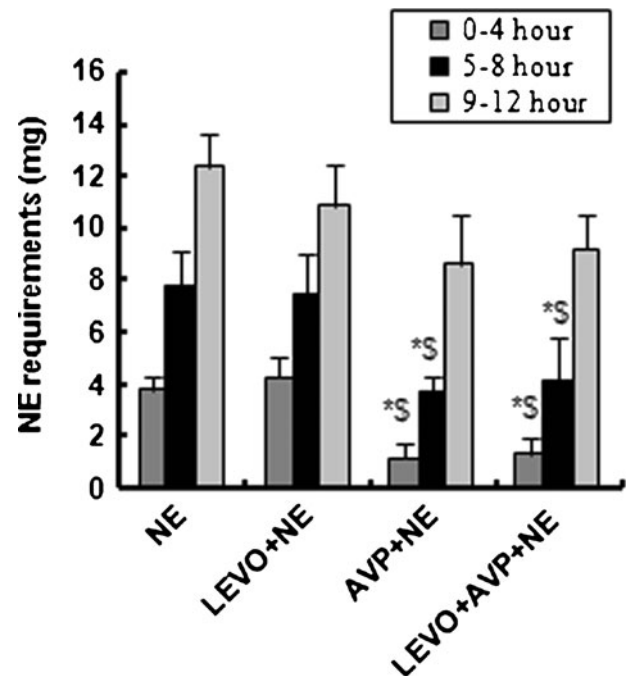


Fig. 1. Cumulative norepinephrine requirements at given time. AVP infusion significantly reduced norepinephrine requirements as compared to NE group and LEVO+NE group in the first 8 h ($n=8$ each group). Data are mean \pm SD; * $P < 0.05$ versus NE group, § $P < 0.05$ versus LEVO+NE group. NE norepinephrine, LEVO levosimendan, AVP arginine vasopressin.

RESULTS

Three animals in the norepinephrine group and six in the other three groups (two animals in each group) died before the end of the study due to refractory arterial hypotension. There was no significant difference in mean body weight, time to the onset of septic shock, and any of the investigated variables at baseline and septic shock time points among the four groups (Supplemental Digital Content). Hematocrit, PCWP, central venous pressure, BE, pHa, and fluid requirements were similar among the four groups ($P>0.05$).

Cardiopulmonary Hemodynamics and Metabolic Changes

All the groups presented with comparable MAP. CO in the AVP+norepinephrine group decreased significantly 4, 8, and 12 h after septic shock as compared with levosimendan+norepinephrine group ($P<0.05$; Table 1).

When compared with norepinephrine group, levosimendan+AVP+norepinephrine group significantly decreased MPAP 12 h after ST while AVP+norepinephrine group decreased this parameter 8 and 12 h after ST (Table 1). SVR was higher in the AVP+norepinephrine group when compared with norepinephrine group 4 and 8 h after septic shock, whereas there was no significant difference in PVR among groups (Table 1). Animals receiving AVP required less norepinephrine requirements comparing to norepinephrine or levosimendan+norepinephrine group in the first 8 h ($P<0.05$; Fig. 1). Lactate concentrations were significantly lower in the levosimendan+AVP+norepinephrine group 12 h after septic shock as compared with norepinephrine group (Table 2).

Inflammatory Changes

There was no statistical difference in pulmonary TNF- α and IL-10 among groups ($P>0.05$). Furthermore, we did not detect any difference in serum levels of

Table 2. Changes in Metabolic Parameters, Lactate Concentrations, PaO₂/FiO₂, Venous Admixture, Cumulative Fluid Infusion and Hematocrit

Variable	Time/Group	BL	ST	4 h	8 h	12 h
PHa	NE	7.49±0.07	7.41±0.08	7.38±0.05	7.33±0.05	7.30±0.08
	LEVO+NE	7.48±0.08	7.41±0.07	7.38±0.05	7.34±0.06	7.30±0.05
	AVP+NE	7.47±0.07	7.39±0.08	7.37±0.07	7.32±0.03	7.28±0.06
	LEVO+AVP+NE	7.50±0.08	7.41±0.07	7.36±0.07	7.33±0.04	7.27±0.03
BE (mmol L ⁻¹)	NE	6±4	0±4	-1±3	-4±3	-6±6
	LEVO+NE	6±5	1±4	-1±4	-3±4	-7±3
	AVP+NE	5±4	1±4	0±4	-5±2	-7±4
	LEVO+AVP+NE	6±3	0±4	-1±4	-5±3	-5±3
Lactate concentration (mmol L ⁻¹)	NE	1.0±0.4	2.0±0.4	2.3±0.5	2.9±0.4	3.8±0.4
	LEVO+NE	1.2±0.5	1.8±0.5	2.2±0.5	2.7±0.3	3.3±0.6
	AVP+NE	1.0±0.3	1.8±0.5	2.3±0.4	2.6±0.4	3.0±0.3
	LEVO+AVP+NE	1.1±0.5	1.9±0.6	2.2±0.4	2.4±0.4	2.7±0.5*
PaO ₂ /FiO ₂	NE	447±23	369±57	300±18	258±36	192±23
	LEVO+NE	430±54	353±44	308±43	282±26	233±35
	AVP+NE	425±42	339±28	304±20	288±10	239±27
	LEVO+AVP+NE	415±52	349±37	317±24	300±18*	267±52*
Venous admixture (%)	NE	12±4	19±3	25±4	37±4	46±4
	LEVO+NE	14±5	19±3	26±4	33±5	43±7
	AVP+NE	16±5	19±3	24±3	32±4	38±5
	LEVO+AVP+NE	15±6	20±2	24±4	31±4*	35±9*
Cumulative fluid requirements (ml)	NE	880±162	10,960±783	13,100±718	15,480±575	17,950±310
	LEVO+NE	905±110	10,600±580	12,540±554	14,960±580	17,520±461
	AVP+NE	920±176	10,720±505	12,780±545	15,350±345	17,840±276
	LEVO+AVP+NE	910±175	10,900±370	12,765±154	15,380±320	17,878±265
Hematocrit (%)	NE	28±4	30±3	31±3	32±3	34±2
	LEVO+NE	30±2	30±3	31±3	31±3	33±4
	AVP+NE	28±3	29±3	30±3	31±4	33±4
	LEVO+AVP+NE	29±2	30±2	30±4	31±3	32±4

Values are represented as mean±SD

PaO₂ partial pressure of oxygen, LEVO levosimendan, AVP arginine vasopressin, NE norepinephrine, BL baseline, ST shock time

* $P<0.05$ versus NE group, each group $n=8$

Table 3. Changes in Serum Cytokines, NO_xa, and HMGB1

Variable	Time/group	BL	ST	4 h	12 h
T N F - α (pg ml ⁻¹)	NE	42 (0–78)	3,675 (2,765–5016)	64 (382–1,440)	38 (15–92)
	LEVO+NE	36 (0–102)	2,987 (1,745–4,620)	646 (286–1,540)	31 (16–68)
	AVP+NE	24 (0–90)	3,820 (2,806–4,428)	492 (321–1,024)	43 (0–132)
	LEVO+AVP+NE	28 (0–65)	3,218 (1,892–5,087)	527 (275–1,347)	49 (17–87)
IL-6 (pg ml ⁻¹)	NE	142 (56–206)	2,045 (1,218–3,645)	1,580 (960–2,692)	586 (186–853)
	LEVO+NE	96 (37–203)	2,716 (1,084–4,123)	1,232 (782–2,131)	422 (136–672)
	AVP+NE	130 (28–283)	1,982 (1,228–3,765)	1,025 (568–1,923)	387 (115–721)
	LEVO+AVP+NE	126 (22–261)	2,386 (1,326–4,120)	687 (454–1,325)*	262 (87–482)*
IL-10 (pg ml ⁻¹)	NE	12 (0–32)	108 (65–295)	124 (45–283)	62 (38–111)
	LEVO+NE	16 (8–46)	130 (86–213)	106 (57–236)	78 (27–123)
	AVP+NE	8 (0–36)	98 (56–234)	121 (65–195)	72 (42–134)
	LEVO+AVP+NE	15 (0–48)	161 (78–312)	94 (52–186)	82 (32–150)
N O _x a (μ mol L ⁻¹)	NE	26 \pm 7	36 \pm 8	51 \pm 14	56 \pm 6
	LEVO+NE	28 \pm 6	38 \pm 10	46 \pm 8	37 \pm 7*
	AVP+NE	29 \pm 9	38 \pm 7	43 \pm 11	44 \pm 11
	LEVO+AVP+NE	28 \pm 8	34 \pm 8	39 \pm 9	35 \pm 7*
H M G B 1 (μ g L ⁻¹)	NE	ND	8 \pm 1	16 \pm 5	26 \pm 6
	LEVO+NE	ND	9 \pm 3	15 \pm 3	23 \pm 5
	AVP+NE	ND	8 \pm 4	14 \pm 3	21 \pm 4
	LEVO+AVP+NE	ND	8 \pm 4	12 \pm 3	16 \pm 4*

Data are presented as mean \pm SD or median (interquartile range)

LEVO levosimendan, AVP arginine vasopressin, NE norepinephrine, BL baseline, ST shock time, ND not detected. * P <0.05 versus NE group, each group n =8

* P <0.05 versus NE group, each group n =8

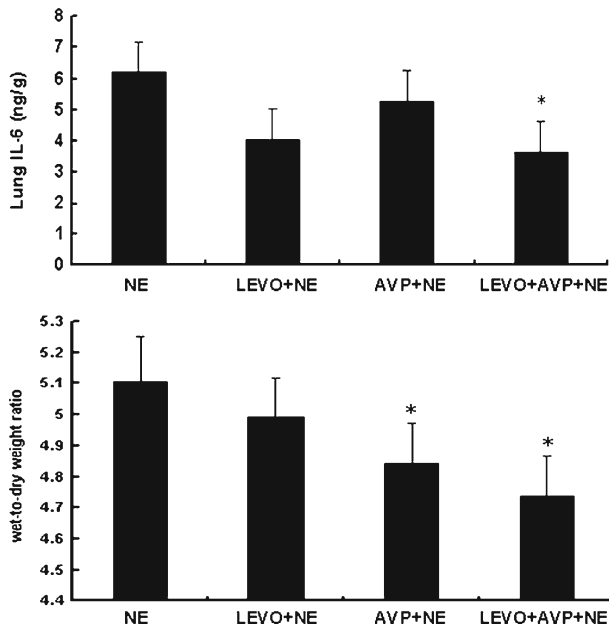


Fig. 2. Pulmonary levels of IL-6, wet-to-dry (W/D) ratio. LEVO combined with AVP supplemented with NE reduced pulmonary levels of IL-6, and W/D ratio (n =8 each group). Data are presented as mean \pm SD; * P <0.05 versus NE group. NE norepinephrine, LEVO levosimendan, AVP arginine vasopressin.

TNF- α and IL-10 at any time point among the groups (P >0.05). Arterial total nitrate/nitrite and HMGB1 concentrations were lower in the levosimendan+AVP+norepinephrine group comparing to norepinephrine group 12 h after septic shock (p <0.05; Table 3). Pulmonary IL-6 levels were lower in the levosimendan+AVP+norepinephrine group than the norepinephrine group 4 and 12 h after septic shock (p <0.05; Fig. 2).

Other Laboratory Analyses

The lung W/D ratio, histological scores, and apoptotic cells in levosimendan+AVP+norepinephrine group were significantly attenuated when comparing to norepinephrine group (Figs. 2, 3, and 4). Gas exchange as evidenced by Qs/Qt was more severely compromised in the norepinephrine group than the levosimendan+AVP+norepinephrine group 12 h after septic shock (Table 2).

DISCUSSION

In the present study, P demonstrated that a combination of levosimendan and AVP, supplemented with norepinephrine improved sepsis-induced ALI as

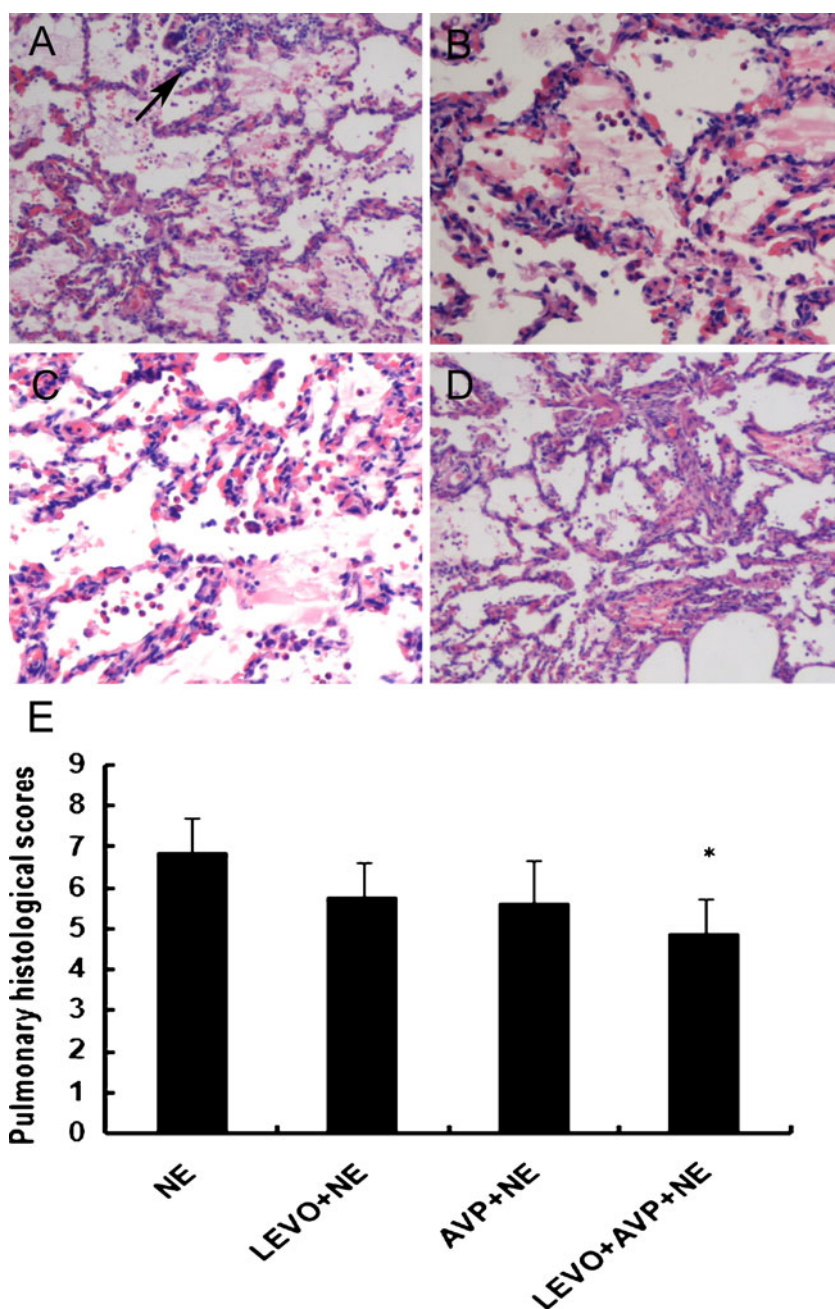


Fig. 3. Pulmonary histological scores. Hematoxylin-eosin $\times 400$, LEVO combined with AVP supplemented with NE infusion (d) decreased histological scores significantly when compared with NE group. The arrow indicates the infiltrating inflammatory cells ($n=8$ each group). Data are presented as mean \pm SD; * $P < 0.05$ versus NE group. NE norepinephrine, AVP arginine vasopressin, LEVO levosimendan. a NE group, b LEVO+NE group, c AVP+NE group, d LEVO+AVP+NE group.

reflected by decreased MPAP, reduced inflammatory mediators and apoptosis, and improved gas exchange as compared with norepinephrine infusion alone.

In the present study, we observed that AVP infusion significantly decreased norepinephrine requirements,

which has been well demonstrated in the literature [5, 6, 9]. However, the addition of levosimendan to AVP failed to improve CO when compared with AVP+norepinephrine group, we cannot rule out that higher doses may produce more pronounced effects. In addi-

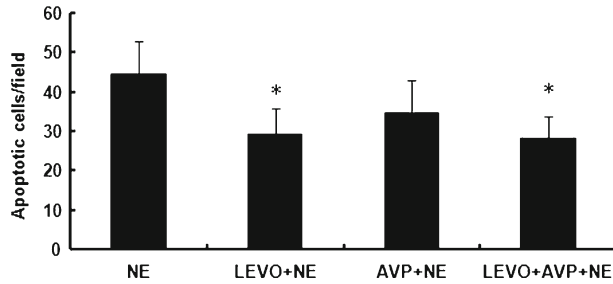


Fig. 4. Pulmonary apoptotic cells. LEVO or LEVO combined with AVP supplemented with NE infusion decreased apoptotic cells significantly when compared with NE infusion alone ($n=8$ each group). Data are presented as mean \pm SD; * $P<0.05$ versus NE group. NE norepinephrine, AVP arginine vasopressin, LEVO levosimendan.

tion, the norepinephrine sparing effect of AVP was not sustained and was limited to the first 8 h after septic shock. Possible explanations for this phenomenon included the decreased AVP responsiveness over time in septic shock [16] and inadequate of AVP was administered. Although increasing amounts of AVP appear to provide the intended effect on MAP or organ's "perfusion pressure", it is possible that the higher doses may induce more adverse effects, actually reduces organ's "perfusion flow", and therefore counterbalances the beneficial effects of low-dose AVP [17]. On the other hand, pulmonary arterial hypertension is a common clinical feature of ALI/ARDS and increased pulmonary arterial hypertension is associated with poor prognosis [11]. Previous studies have demonstrated that both levosimendan and AVP infusion result in a significant decrease of pulmonary artery pressure in septic shock [6, 11, 18]. In this regard, a lower MPAP might be expected in levosimendan+AVP+norepinephrine group. However, we noted that the decrease in MPAP was even more pronounced in AVP+norepinephrine group. One reason might be that lower norepinephrine was infused in AVP+norepinephrine group, therefore reduced the detrimental effects of norepinephrine [5]. In addition, the attenuated lactate concentrations observed in the levosimendan+AVP group suggested an enhanced tissue perfusion and improved oxygenation.

Previous study has suggested that the lung is the major organ expressing a large amount of inducible nitric oxide synthase (iNOS) in response to bacterial endotoxin [19]. It is believed that overproduction of NO may exert pro-inflammatory, cytotoxic effects by reacting with super-oxide radicals to form toxic products such as peroxynitrite. These changes can cause endothelial damage and thus pulmonary edema, ultimately resulting in impairments in pulmonary gas exchange

[20]. The decreased arterial total nitrate/nitrite observed in the present study probably suggested the beneficial effects of the combination therapy of levosimendan and AVP infusion. In accordance with our observation, Sareila *et al.* [21] have shown that levosimendan reduces iNOS promoter activity, iNOS expression, and NO production by downregulating NF- κ B-dependent transcription *in vitro*. Studies have also demonstrated AVP decreases the synthesis of NO in response to endotoxemia stimuli, thus attenuates the arterial vasodilatation and organ damage in sepsis [22]. In this context, it seems that levosimendan acts synergistically with AVP to inhibit the increase of NO under this circumstance.

It has been demonstrated that the degree of acute inflammation is highly associated with the outcome of human ALI/ARDS [23]. IL-6 is a marker of inflammation and contributes to morbidity and mortality in patients with septic shock [24]. It has been reported that AVP infusion significantly decreases pulmonary IL-6 levels in a rat model of sepsis [7]. Similarly, IL-6 production is inhibited by about 30% by levosimendan in cells exposed to inflammatory stimuli in an *in vitro* study [21]. The greater reduction in IL-6 concentrations suggests that the group treated with the combination of levosimendan and AVP presented a less lung inflammation. Besides, TNF- α is an important pro-inflammatory cytokines and plays an important role in the pathogenesis of sepsis [25]. However, we did not notice any difference among groups with respect to this parameter at any given time point. One of the main reasons could be that early cytokines like TNF- α peak shortly after sepsis insult and return to the baseline levels within few hours [26], thus we might fail to detect the difference of TNF- α in the present study. On the other hand, HMGB1 protein is a late-phase mediator which is critically involved in the development of sepsis and sepsis-induced ALI [27]. More recently, Wolfson *et al.* [28] have demonstrated that HMGB1 induces human lung endothelial cell cytoskeletal rearrangement and barrier disruption. In the present study, we showed that combined infusion of levosimendan and AVP dramatically reduced serum levels of HMGB1 in a porcine model of septic shock. These observations and the results of the present study suggest that HMGB1 is a key mediator of cell injury and that its inhibition may be crucial in improving clinical outcomes. Besides, levosimendan combined with AVP supplemented with norepinephrine infusion markedly reduced severity of histological changes, and W/D ratio activity in the lung, suggesting the beneficial effects of the therapy.

Interestingly, a recent study has demonstrated that the activation of apoptotic and non-apoptotic/inflammatory Fas signaling is an important early pathophysiological event in the development of ALI after sepsis [29]. Notably, the anti-apoptotic property of levosimendan has been demonstrated in both experimental and clinical studies [11, 12]. In accordance with these, we observed the less pulmonary TUNEL-positive cells in the levosimendan+AVP+norepinephrine group. On the other hand, catecholamines may induce lymphocyte apoptosis in a murine model of polymicrobial sepsis [30] and/or enhance the inflammatory effects in response to endotoxin [31]. This may provide additional evidence of the value of levosimendan and/or AVP in the treatment of septic shock when the adrenergic vasopressors are no longer efficient.

Intravenous infusion of vasodilators such as levosimendan might be expected to worsen Qs/Qt due to their indiscriminate action on pulmonary vessels in ventilated as well as in nonventilated lung fields. However, the opposite result was observed in the present study suggesting the following factors may contribute to the improved oxygenation. First, the attenuated lung inflammation with subsequent less pulmonary edema in response to levosimendan+AVP+norepinephrine is one of most important explanation. Second, it has been reported that norepinephrine infusion further increased while AVP does not alter endotoxin-induced lung permeability [32]. Therefore, the beneficial effects of AVP may be that it reduced the norepinephrine requirements and thus reduced the detrimental effects of high dose of norepinephrine. Third, the reduction in pulmonary artery pressure may reduce the lung water.

Despite of these promising observations, there are some obvious limitations in the present study. First, neither causal therapy nor antibiotics was used in the present study in order to avoid the influence of these additional variables and to obtain a lethal model, however, this obviously did not reflect the actual clinical situation. Second, the present study was an exploratory study designed to find trends, owing to the complex actions of each drug as well as too many confounding factors, the results should be interpreted with cautions. Third, this short observational period precludes any conclusion whether the combination therapy is beneficial later or even may lead to subsequent deterioration. Finally, the dosage of AVP was similar to that used in clinical practice, whereas levosimendan was higher than previous clinical study, our pilot study suggested that levosimendan at the dosage of $0.4 \mu\text{g kg}^{-1} \text{min}^{-1}$

improved CO with minimal effects on MAP. Therefore, we chose $0.4 \mu\text{g kg}^{-1} \text{min}^{-1}$ as the final dose studied.

In conclusion, we demonstrated that continuous intravenous infusion of levosimendan combined with AVP, supplemented with norepinephrine, may decrease MPAP, attenuate pulmonary inflammatory responses and apoptosis, and ameliorate gas exchange when compared with norepinephrine administration alone.

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