Cigarette Smoke Extract and Lipopolysaccharides Induce Pyroptosis in Rats Pulmonary Microvascular Endothelial Cells

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Research Article

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Abstract

Objective: To investigate the effects of cigarette smoke extract (CSE) and lipopolysaccharide (LPS) on the activity and pyroptosis of pulmonary microvascular endothelial cells (PMVECs).

Methods: PMVECs were cultured without treatment or with CSE (1%-25%), LPS, or CSE+LPS. Cell viability was detected using the CCK8 method. Apoptosis was evaluated by flow cytometry. Cell morphology was evaluated using optical microscopy. The content of IL-1β and IL-18 was measured by ELISA.

Results: CSE decreased cell viability in a dose-dependent manner. The cells in the CSE+LPS group showed the most obvious cytomorphological changes and the highest pyroptosis rate under the microscope. Flow cytometry showed that the CSE and LPS groups showed higher apoptosis rates than the blank group; the apoptotic rate in the CSE+LPS group was even higher ($P<0.01$). Compared with the bkank group, the levels of IL-18 and IL-1β in the cell supernatant of the CSE, LPS, and CSE+LPS groups increased significantly, with significant differences ($P<0.01$). There were no differences between the CSE and LPS groups ($P>0.05$). Compared with the CSE and LPS groups, the CSE+LPS group had higher IL-18 and IL-1β ($P<0.01$).

Conclusion: The effect of CSE on cell viability is dose-dependent. CSE+LPS can induce cell pyroptosis and increase the levels of inflammatory cytokines in PMVECs. These observations demonstrated that pyroptosis caused by CSE and LPS might play an important role in pulmonary vascular remodeling.

Introduction

Pulmonary arterial hypertension (PAH) is a rare type of pulmonary hypertension characterized by hemodynamic and pathophysiologic states of increased mean pulmonary arterial pressure (MPAP) (>20 mmHg at rest, previously ≥25 mmHg) in the absence of accompanying lung or left-sided cardiac abnormalities as a cause for the increased pressure [1–3]. The reported prevalence of PAH is 15-60 per million individuals [1]. PAH can be idiopathic, heritable, drug- or toxin-induced, or caused by conditions such as HIV infection, congenital heart diseases, portal hypertension, schistosomiasis, and connective tissue diseases [1, 4]. Complications of PAH may include right ventricular dysfunction/right heart failure, peripheral edema, ascites, and tachyarrhythmias [1].

PAH is a lung disease characterized by incompletely reversible airflow limitation and recurrent and progressive development [5]. Its pathogenesis is complex and not yet clear. In addition to the theory of hypoxia pathogenesis, the disease might be related to the infiltration of inflammatory cells and the accumulation of inflammatory factors caused by cigarette smoke or harmful gases, which can cause oxidative stress and damage to vascular endothelial cells and cause lung or systemic inflammation [6]. Pulmonary microvascular endothelial cells (PMVECs) are mainly composed of flat epithelial cells on the inner surface of blood vessels and lymphatic vessels of the lungs. Their main function is to form the blood-air barrier and provide endocrine function. They can synthesize and release a variety of endothelial-derived vasoactive factors, with physiological functions such as reducing vascular permeability, inhibiting

vascular endothelial cell proliferation, mediating inflammation and immune cell chemotaxis, inhibiting cell migration, regulating vasoconstriction and vasodilatation, inhibition of platelet aggregation and anti-adhesion. They are closely related to various lung diseases such as pulmonary arterial hypertension, acute lung injury and systemic inflammatory response syndrome [7].

Pyroptosis is a newly discovered inflammatory programmed cell death pattern accompanied by varying degrees of an inflammatory response [8]. The morphological characteristics of pyroptosis include a concentrated nucleus, enlarged mitochondria and endoplasmic reticulum, many pores on the cell membrane, the loss of ability to regulate the entry and exit of substances, and increased cell permeability that leads to membrane rupture, the release of cellular contents and other active substances, recruitment of immune response, induction of additional inflammatory cells, and expansion of the inflammatory response [9]. Studies have shown that cell pyroptosis is intimately involved in respiratory diseases [10, 11]. Cigarette smoke extract (CSE) [12, 13] and lipopolysaccharide (LPS) [14, 15] are well-known to be involved in the death of various pulmonary cell types. However, whether they can jointly induce PMVEC pyroptosis to play a role in pulmonary vascular remodeling remains unknown.

Therefore, this study aimed to investigate the effects of CSE and LPS on the activity and pyroptosis of PMVECs, which will provide a novel strategy for studying the pathogenesis of PAH.

Materials And Methods

Cell line, drugs, and reagents

The rat PMVECs used in this experiment were purchased from Beijing Beina Biotechnology Company (Beijing, China). DMEM medium (d5796, Sigma, St. Louis, MO, USA), trypsin digestion solution (c0201, Biyuntian, Shanghai, China), fetal bovine serum (FBS) (10099141, GIBCO, Invitrogen Inc., Carlsbad, CA, USA), phosphate-buffered saline (PBS) (sh30256.01, Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), penicillin/streptomycin (sv30010, Biyuntian), T25 cell culture bottle (n707003, Nest, Shanghai, China), CCK8 Kit (nu679, Japan Tongren, Tokyo, Japan), FAM-YVAD-FMK pyrolysis kit (ab219935, Abcam, Cambridge, United Kingdom), IL-18 ELISA kit (ab0213909, Abcam), and IL-1 β ELISA kit (ab100767, Abcam) were purchased. The main equipment included an ultra-clean worktable (YT-CJ-2NB, Beijing Yatailong, Beijing, China), direct heating CO_2 incubator (DH-160I, Santo Instrument Product, Beijing, China), inverted biological microscope (DSZ2000X, Beijing Zhongxian Hengye Instruments), low-speed centrifuge (SL02, Zhixin Instruments, Shanghai, China), flow cytometry (FACSCanto□, BD Biosciences, Franklin Lake, NJ, USA), ultraviolet-visible photometer (Eppendorf, Hamburg, Germany), and Imark Microplate Reader (Bio-Rad, Hercules, CA, USA).

Cell culture

PMVECs were cultured in a DMEM medium containing 10% Gibico fetal bovine serum (FBS), 1% double antibiotics, and high glucose in a 5% CO_2 incubator at 37°C. When confluence reached 70%-80%, the old

medium was discarded for trypsinization and passage. The second- to third-generation cells with good growth were used for the experiments.

Preparation of CSE and LPS

CSE was prepared according to a published method [16]. A filter-removed cigarette (Huangguoshu cigarette, tar 10 mg, nicotine 0.9 mg) was connected to a 50 ml syringe through a rubber tube. After it was ignited, negative pressure suction was performed. Each cigarette was smoked at a 50 ml/min rate six times. The smoke (300 ml) was introduced into 25 ml of serum-free DMEM medium. The pH was adjusted to 7.4 with 1 mM NaOH. The container was gently shaken to dissolve the smoke in the medium. The bacteria and particles were removed by filtering through a filter membrane of 0.22 μm. This solution was defined as the 100% CSE stock solution, stored at -20℃ for later use. The concentration of CSE was detected by ultraviolet spectrophotometry at a wavelength of 320 mm. The absorbance difference of different batches of CSE solutions was about ±0.2. The stock solution was diluted to different concentrations according to the experimental needs.

The LPS stock solution (5 mg/ml) was diluted 1000 times to prepare a solution with an intermediate concentration of 5 μg/ml. The solution with a 5 μg/ml concentration was diluted 250 times to get the final concentration of 20 ng/ml.

Screening of drug intervention concentration with the cell counting kit-8 (CCK8) method

The cells in the logarithmic growth phase were digested with 0.25% trypsin, and the cell suspension at a density of 5×10^4/ml was inoculated on 96-well plates. After the cells adhered to the wall, the culture medium was changed. Then, CSE was added at 0%, 1%, 2.5%, 5%, 10%, 20% and 25% for 24 h (six groups) in 100 μl of complete medium. Then, 10 μl of CCK8 solution was added to each well, and the plates were incubated at 37℃ for 2 h. The optical density (OD) value was detected at 450 nm using a microplate reader. The formula for cell viability was cell viability (%) = [A (dosing) - A (blank)] / [a (0 dosing) - A (blank)] ×100. The experiments were repeated three times.

Cell grouping and treatment

The cells were divided into four groups, with six wells in each group. In the blank control group, PMVEC cells were cultured without treatment. In the CSE group, the concentration of CSE was set as 20% for 12 h, according to the results of the cell viability experiment. In the LPS group, 20 ng/ml LPS was added for 12 h. In the CSE+LPS group, the cells were treated with 20% CSE and 20 ng/ml LPS for 12 h. The cell supernatant was collected for measurements.

Cell morphology observation

Cell growth and morphological changes were observed under a microscope.

Double staining with propidium iodide (PI) and carboxyl fluorescein (FAM-YVAD-FMK)

The cells were digested with trypsin (without EDTA) and collected, and then inoculated at 1×10^5/well in 96-well plates. The culture medium was discarded after centrifugation. The cells were rinsed twice with PBS, centrifuged at 2000 rpm for 5 min, and about 3×10^5 cells were collected. The PI/ FAM-YVAD-FMK staining solution was prepared with the cell staining buffer solution. Then, 5 μl of PI staining solution and 5 μl of FAM-YVAD-FMK staining solution were added to each well. The cells were resuspended with the prepared staining solution and incubated at room temperature for 1 h in the dark. Flow cytometry was used for observation within 1 h.

IL-1β and IL-18 by ELISA

PMVECs in the logarithmic phase were selected, cultured for 24 h, and centrifuged at 3000 rpm for 5 min. The supernatant was taken. ELISA was performed according to the manufacturer's instructions. The OD value of each well was measured at a wavelength of 450 nm within 5 min after stopping the reaction.

Statistical analysis

SPSS 21.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The continuous data were expressed as mean ± standard deviation. Since the data were consistent with a normal distribution and homogeneity of variance, ANOVA with the Dunnett post hoc test for pairwise comparison. The independent sample t-test was used for comparison between two groups. P-values <0.05 were considered statistically significant.

Results

Determination of the best CSE concentration

Different concentrations of CSE (1%-25%) had a significant effect on the viability of PMVECs ($P<0.001$). Among them, the 20% CSE had the best inhibitory effect on the viability of PMVECs (Figure 1). Therefore, the experimental intervention concentration was determined as 20% CSE.

Morphological comparison of PMVECs treated with CSE and LPS

In the blank control group, PMVECs grew into monolayers, grew well, were arranged like paving stones, adhered to the wall, were distributed evenly, had a clear boundary, and were mostly long fusiform and polygonal. In the CSE and LPS groups, the number of cells was decreased, some cells floated, and some adherent cells were oval. The CSE+LPS group showed the most obvious inhibitory effect. The number of PMVECs decreased significantly, the cells became round, swollen, and smaller, the cell membrane shrunk, and the number of non-adherent cells increased significantly (Figure 2).

CSE and LPS increased PMVEC apoptosis rates

The results of the double-staining with PI and FAM-YVAD-FMK showed that compared with the blank control group, the number of apoptotic cells in the LPS, CSE, and LPS+CSE group was significantly different ($P<0.05$), but there were no significant differences between the CSE and LPS groups ($P>0.05$). The number of apoptotic cells in the LPS+CSE group was increased, and the difference was statistically significant compared with the CSE and LPS groups ($P<0.05$) (Figure 3).

CSE and LPS increased the IL-18 and IL-1β content in PMVECs

Compared with the normal group, the levels of IL-18 and IL-1β in the cell supernatant of the CSE, LPS, and CSE+LPS groups increased significantly, with significant differences ($P<0.05$). There were no differences between the CSE and LPS groups ($P>0.05$). Compared with the CSE and LPS groups, the CSE+LPS group had higher IL-18 and IL-1β ($P<0.05$) (Table 1).

Table 1
Effects of drug interventions on IL-18, IL-1β, Caspase1, and GSDMD contents in PMVEC cells in each group.

Group	IL-18	IL-1β	Caspase1	GSDMD
Blank	0.1±0.01#	0.3±0.01#	0.08±0.01#	0.06±0.02#0
CSE	0.2±0.02*#	0.4±0.02*#	0.2±0.01*#	0.08±0.03*#
LPS	0.2±0.01*#	0.4±0.01*#	0.3±0.04*#	0.07±0.01*#
CSE +LPS	0.3±0.01*	0.5±0.01*	0.6±0.02*	0.3±0.01*
Notes: *P<0.05 vs. the blank group. #P<0.05 vs. the CSE + LPS group.				

Discussion

Pulmonary vascular remodeling is an important pathological mechanism of PAH [17, 18]. It refers to the changes in vascular structure in the pulmonary vascular wall caused by hypoxia, inflammation, or other reasons. Bacterial-triggered inflammation and smoke stimulation are important factors for pulmonary vascular remodeling [17]. Long-term chronic inflammation could lead to pulmonary vasoconstriction, changes in endothelial cells' structure, function, and metabolism, and then cause pulmonary vascular remodeling [17–20].

PMVECs form a barrier in the pulmonary microvascular vessels, with characteristics of active metabolism and being non-fenestrated. They are continuously distributed in the endovascular wall of the alveoli microvessel [21, 22]. PMVECs can be injured by inflammation, which is closely related to the development

of many respiratory diseases such as pulmonary arterial hypertension, acute lung injury, and systemic inflammatory diseases [12, 14, 15, 21, 22]. Under pathological conditions, PMVECs become the main target cells of the inflammatory response and participate in regulating intravascular coagulation, maintaining vascular tension, regulating lung local and systemic inflammatory response functions, and maintaining normal lung function. Therefore, the protection of PMVECs might be an important way to inhibit the remodeling of the pulmonary blood microvessels. Some studies showed that inflammation and smoke stimulation could induce the onset of PAH, cause pulmonary vascular damage, and lead to tracheal and vascular remodeling [23, 24]. The components and metabolites in cigarettes interact with the pulmonary blood vessels, activate inflammatory cells, stimulate the proliferation of pulmonary artery cells, thicken elastic tissues, and change the structure of pulmonary blood vessels [25]. Inflammation stimulates pulmonary microvascular endothelial cells to produce and release many cytokines directly or induce the expression of cytokines by secreting inflammatory cytokines such as IL-1β and IL-18 [26, 27].

LPS is the main component of bacterial endotoxin and is a powerful inflammatory stimulus. The increased permeability of PMVECs is related to LPS [28] and involves increased oxidative stress and inflammatory reaction [29]. LPS can also activate the pyrolysis reaction in cells and promote the production of pyrolysis cells [30, 31]. LPS induces endothelial cells in blood vessels to secrete inflammatory factors, promotes the production of inflammatory reactions, and induces pyroptosis [32]. Vascular endothelial cells secrete inflammatory factors such as IL-1β and IL-18 to induce inflammatory damage [32]. As an inflammatory programmed cell death process, the activation of pyroptosis is related to inflammation [32]. Pyroptosis is divided into the caspase-1-mediated classical pyroptosis pathway and the caspase-4/5/11-mediated non-classical pyroptosis pathway [8, 9, 33, 34]. Still, there were no clear studies on whether CSE and LPS could induce pyroptosis in PMVECs. It is also not clear whether apoptosis is related to pulmonary vascular remodeling.

In the present study, the PMVECs treated with CSE+LPS showed changes in cytomorphology. Without CSE and LPS, PMVECs grew into monolayers, were arranged like paving stones, adhered to the wall, were distributed evenly, had a clear boundary, and were mostly long fusiform and polygonal. In the CSE+LPS group, the number of PMVEC cells was decreased significantly, and cytomorphology suggested pyroptosis in many cells. The supernatant IL-1β and IL-18 levels were also sharply increased, supporting the inflammatory process. The comparison among groups shows that there was no significant difference in content between CSE and LPS groups (all $P>0.05$). Therefore, these results suggest that CSE and LPS have a detrimental synergetic effect on PMVECs, suggesting that CSE and LPS might be involved in the pathogenesis of PAH.

Conclusion

This study showed that CSE and LPS can induce the pyroptosis of rat PMVECs, and may play an important role in pulmonary vascular remodeling in PAH. Because of the complexity of the pulmonary vascular remodeling factor in PAH, the exact mechanism needs to be further explored in future studies.

Abbreviations

CSE: cigarette smoke extract

LPS: lipopolysaccharide

PMVECs: pulmonary microvascular endothelial cells

PAH: Pulmonary arterial hypertension PI: propidium iodide

FAM-YVAD-FMK: carboxyl fluorescein

Declarations

Ethics approval and consent to participate

All animal experiments followed stated protocols from the The Affiliated Hospital of Hunan Academy of Chinese Medicine and Hunan Shrek Jingda [permit to syxk (Xiang) 2019-0017.].This experiment was approved by the animal ethics committee of Hunan University of traditional Chinese medicine (No 2020-0092) and conforms to the principles of experimental animal welfare and ethics. All experiments were performed in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Qizhi Wang and Yu Liu designed the study and were responsible for data acquisition. All authors contributed to the analysis and interpretation of data, writing, critical reviewing, and approval of the final version.

Acknowledgments

Not applicable

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Figures

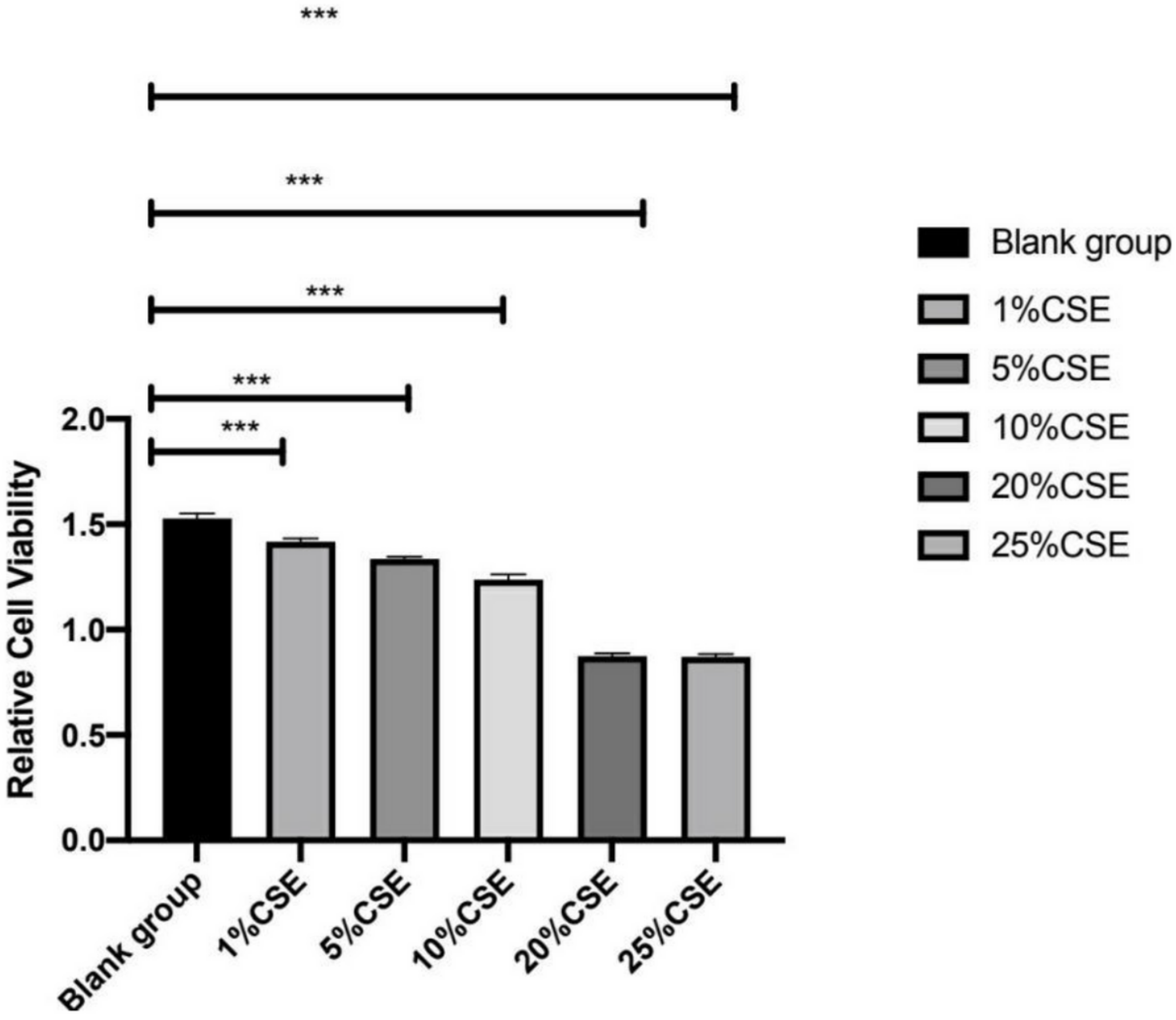

Figure 1

Effects of different concentrations of cigarette smoke extract (CSE) on the viability of pulmonary microvascular endothelial cells (PMVECs). The CCK8 assay confirmed that CSE had an inhibitory effect on PMVEC viability. A CSE concentration of 20% resulted in the lowest cell viability. ***P<0.001 vs. the blank group.

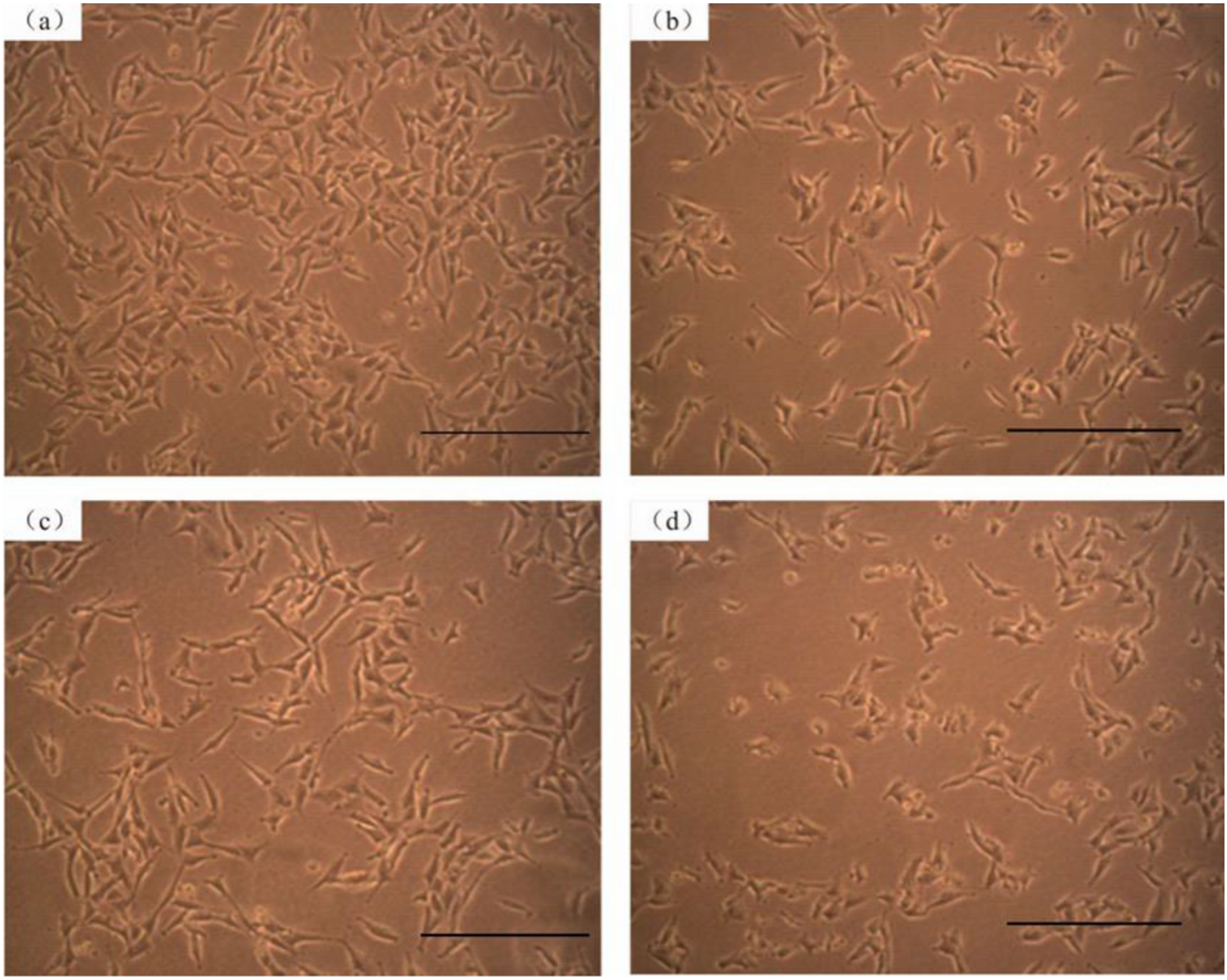

Figure 2

Morphological comparison of pulmonary microvascular endothelial cells (PMVECs) treated with different drugs. a: blank control group; b: cigarette smoke extract (CSE) group; c: LPS group; d: CSE+LPS group. Scale bars = 200 μm. In the blank control group (a), PMVECs grew into monolayers, grew well, were arranged like paving stones, adhered to the wall, were distributed evenly, had a clear boundary, and were mostly long fusiform and polygonal. In the CSE (b) and LPS groups (c), after treatement, the number of cells decreased, some cells floated, and some adherent cells were oval. The CSE + LPS group (d) shows the most obvious inhibitory effect. The number of PMVECs was decreased significantly, the cells became round, swollen, and smaller, the cell membrane shrunk, and the number of suspended cells increased significantly.

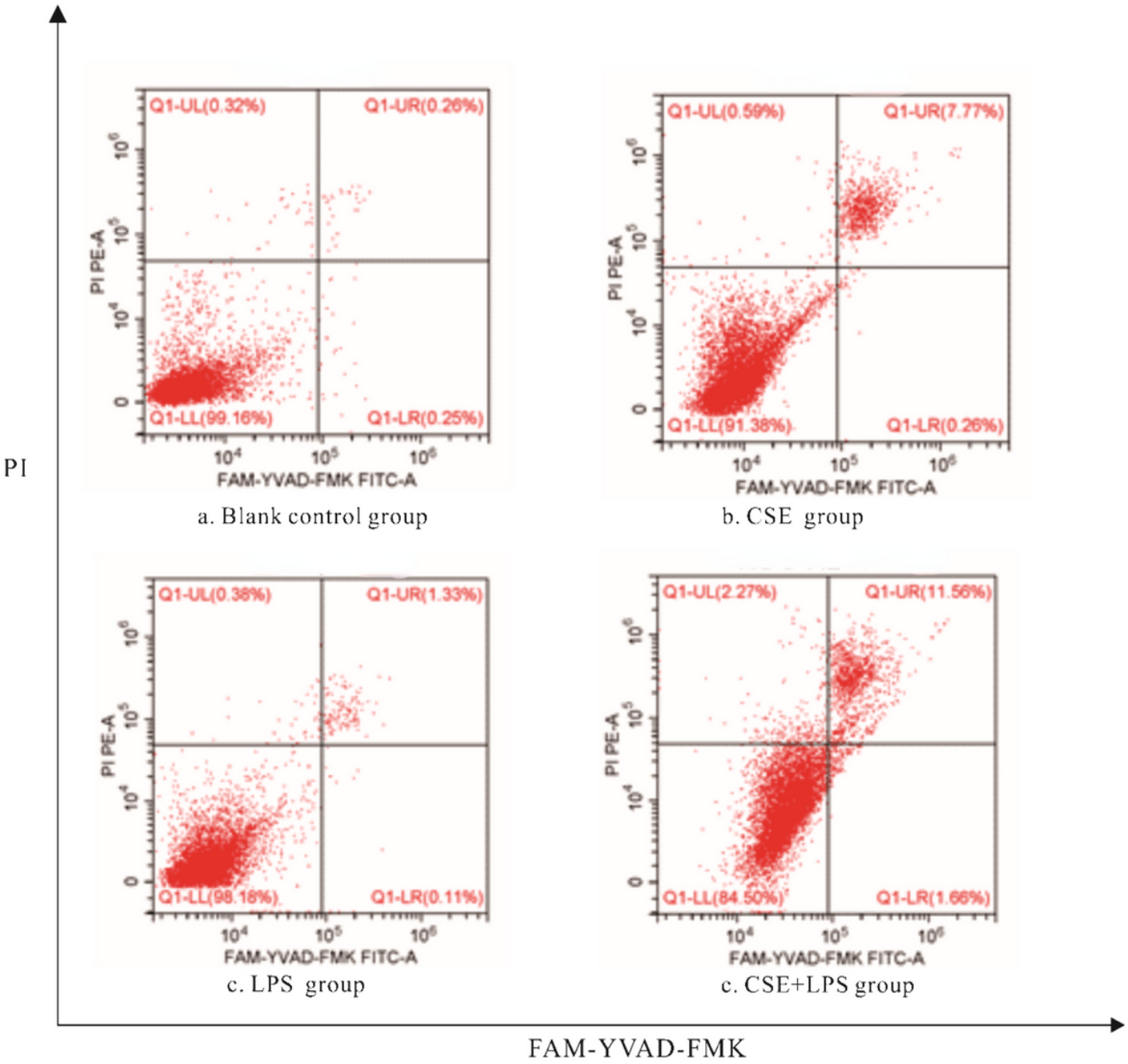

Figure 3

Comparison of staining and cell death rate in each group by flow cytometry. The CME and CME+LPS groups showed the highest cell death rates. Notes: *P<0.05 vs. the blank group.