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Role of mechanical stretching and lipopolysaccharide in early apoptosis and IL-8 of alveolar epithelial type Ⅱ cells A549

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ABSTRACT

Objective: To investigate the effects of mechanical stretching and lipopolysaccharide (LPS) on the early apoptosis and IL-8 production of alveolar epithelial type Ⅱ cells A549. **Methods:** The experimental matrix consisted of three integrated studies. In the first study, A549 cells were subjected to different stretching strain frequency and duration time to see the effects on the early apoptosis. In the second study, A549 cells were subjected to mechanical stretch (15% 4 h, 0.5 Hz) and LPS (1 or 100 ng/mL) to see whether mechanical strain and LPS also have an addictive effect on the early apoptosis. In the third study to investigate whether this addictive effect could be induced by LPS and mechanical stretch on IL-8 production, A549 cells were subjected to LPS (100 ng/mL) and mechanical strain (15%, 0.5 Hz, 4 h). Real time PCR and enzyme linked immunosorbent assay were used to measure mRNA and protein level of IL-8. The early apoptosis was detected by flow cytometry. **Results:** Mechanical stretch induced the early apoptosis in a force and frequency and time-dependent manner. In the presence of LPS, mechanical stretch enhanced LPS-induced early apoptosis, especially in 100 ng/mL LPS group compared with 1 ng/mL LPS and the control group. Mechanical stretch increased IL-8 production and enhanced LPS-induced IL-8 screation both in mRNA and protein levels. **Conclusions:** Mechanical stretch can induce the early apoptosis and IL-8 secretion. Mechanical stretch and LPS have an addictive effect on the early apoptosis and IL-8 production in alveolar type 2 cells, which is one of the mechanisms of ventilator-induced lung injury.

1. Introduction

Acute respiratory distress syndrome (ARDS), the severe form of alveolar lung injury (ALI), is characterized by flooded alveolar air spaces and increased microvascular and epithelial permeability due to disruption of the alveolar epithelium, damage to the alveolar capillary endothelium, and neutrophilic inflammation[1]. Mechanical ventilation has been an indispensable therapeutic modality for the respiratory failure. Nonetheless, it may cause or worsen lung injury[2,3]. Since acute lung injury is non-homogenous[4], overdistention of the lung tissue during mechanical ventilation may initiate ventilator-induced lung injury (VILI).

Apoptosis is a highly orchestrated form of cell death, being proposed to play an important role in tissue damage during the development of acute lung injury[5,6]. Alveolar type 2 cells reportedly undergo apoptosis as part of normal lung development and maturation[7,8] and as a consequence of acute lung injury[9]. Given that apoptosis play an important role in immune system and tissue repair after injury, understanding the response of cell apoptosis under the inflammatory conditions is important for the development of therapies for these diseases.

Lipopolysaccharide (LPS) is a major pathogenic factor potentially provoking local acute lung inflammation[10], commonly used as a molecular tool to study the cellular and molecular mechanism of acute inflammatory response. The

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human respiratory epithelium is an important environmental interface. LPS-induced epithelial responses have been reported[11].

Alveolar type Ⅱ cells were important in lung inflammation[12,13] and stretch-induced cytokine production[14]. IL-8 is a chemoattractant and activator of neutrophils and can contribute to the inflammatory responses associated with lung diseases[15]. But the effect on apoptosis and IL-8 production of alveolar type Ⅱ cells induced by mechanical stretch and LPS has never been studied.

We applied LPS to stimulate human alveolar epithelium A549, which analogue pathogen-associated molecular pattern of actue lung injury[16]. We assume that apoptosis and IL-8 secreation of human alveolar epithelium depend on mechanical stretch and LPS which may lead to different clinic outcomes.

2. Materials and methods

2.1. Experimental matrix

The experimental matrix consisted of three integrated studies. In the first study, A549 cells were subjected to different stretching strain frequency and duration time to see the effects on the early apoptosis. In the second study, A549 cells were subjected to mechanical stretch (15% 4 h, 0.5 Hz) and LPS (1 or 100 ng/mL) to see whether mechanical strain and LPS also have an addictive effect on the early apoptosis. In the Third study to investigare whether this addictive effect could be induced by LPS and mechanical stretch on IL-8 production, A549 cells were subjected to LPS (100 ng/mL) and mechanical strain (15%, 0.5 Hz, 4 h). Real time PCR and enzyme linked immunosorbent assay were used to measure mRNA and protein level of IL-8. The early apoptosis was detected by flow cytometry.

2.2. Cell culture

Human type Ⅱ-like alveolar epithelial cells (A549 cells) (Figure 1) were purchased from American Type Culture Collection. Ham′s F-12K medium containing L-glutamine (2 mM), FCS (10%), and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively, Sigma) was used as growth medium at 37 ℃ in 5% CO_2 incubation. The cells were passaged and seeded at a density of 5×10^5 cells/well on six-well culture plates (Flexcell International) before each experiment. The base of the plates consists of a flexible, collagen Ⅰ-impregnated silicoelastic membrane with 25 mm in diameter. After 22 h of adherence, the complete medium was removed and replaced with fresh medium containing 2% FCS. Cells were stimulated with *Escherichia coli* LPS serotype O55:B5 (Sigma) and/or mechanical stretch.

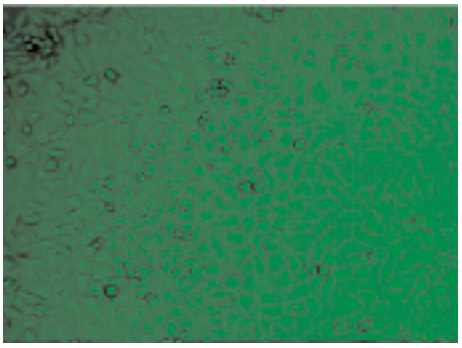

Figure 1. Human lung adenocarcinoma cell line A549 cultured in vitro 60×.
The A549 cells have grown to confluent monolayer.

2.3. Application of cyclical mechanical stretch

Confluent A549 cells were stretched using the Flexercell® Tension PlusTM FX-4000T system (Flexcell International Corp,USA) (Figure 2), which is a computer-regulated bioreactor applying strain to the cultured cells. Through vacuum pressure, cell cultures are regulated and deformed on a collagen Ⅰ coated culture wells when positioned on a vacuum base station. Moreover, we use BioFlex® Loading Stations to provide uniform radial and circumferential strain to a membrane surface in 5% and 15%, but not in 30% strain. Control plates, are not positioned on the base station but instead are placed adjacent to it in the same incubator. All cells were maintained in 5% CO_2 at 37 ℃ for the duration of the experiment. The cells were randomly subjected to different cyclic stretching patterns.

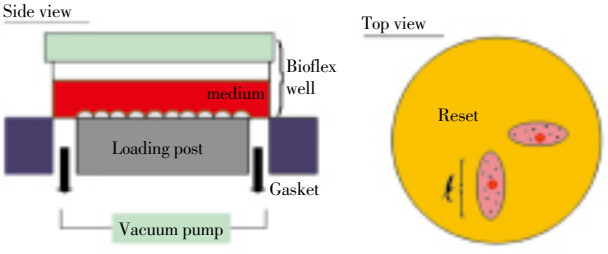

Figure 2. Principles of operation of Flexercell® Tension PlusTM FX-4000T system (Flexcell International Corp).

2.4. Annexin Ⅴ binding and propidium iodide staining

The cells were harvested, stained with FITC-conjugated annexin Ⅴ and propidium iodide (PI) using the Apoptosis Detection Kit (Jingmei Biotech Co. Ltd, China), and analyzed by flow cytometry (Beckman Coulter Co, USA). Both adherent and nonadherent cells were included in the analysis. Flow cytometry was performed at different mechanical stretching protocol. Each data point represents n=3 cell isolations with triplicate measurements.

2.5. RNA isolation and real time PCR

RNA was prepared using TRIzol® (Invitrogen, CA, USA). One microgram of total RNA was heated at 70 ℃ for 5 min. and placed on ice for 5 min. A mix of 5 μL M-MLV RT 5× reaction buffer, 10 mM of each of the 4 dNTPs, 200 U of M-MLV transcriptase enzyme(H-), 25 pg/μL of oligo (dT) primer and 20 U of RNase inhibitor (all from Promega, WI, USA) was added to each sample, followed by incubation at 40 ℃ for 60 min and 70 ℃ for 15 min. Real-time PCR was performed using double stranded DNA dye SYBR Green PCR Master Mix (PE Biosystems, Warrington, UK) on the ABI PRISM 7700 system (Perkin-Elmer, CA,USA). PCRs were performed in triplicate and GAPDH was co-amplified to normalize the amount of RNA added to the reaction. All data were analyzed using the ABI PRISM SDS 2.0 software. Primer sequences were as followed, IL-8: Sense 5′ACTCCAAACCTTTCCACCC3′ and antisense 5′AAACTTCTCCACAACCCTCTG3′, GAPDH: Sense 5′CGGATTTGGTCGTATTGGG3′ and antisense 5′TCTCGCTCCTGGAAGATGG3′. To compare the expression of mRNA levels among different samples, the relative expression of mRNA levels was calculated using the comparative delta C_T (threshold cycle number) method as described previously[17]. Briefly, the following formula was used: $2^{-\triangle\triangle CT}$ where $\triangle CT$ is the difference in C_T between the gene of interest and GAPDH, and $\triangle\triangle CT$ for the sample = $\triangle CT$ for the actual sample- $\triangle CT$ of the lowest expression sample.

2.6. Enzyme linked immunosorbent assay (ELISA)

According to the manufacturers′ instructions, IL-8 concentrations in the culture supernatants were determined by commercially available ELISA kits (R&D systems, Minneapolis, MN).

2.7. Statistical analysis

Students′ *t* test was used for comparisons between experimental groups. One way analysis of variance (one ANOVA) were performed, and followed by Student-Newman-Keuls test as a *post-hoc* analysis for multiple testing. Results are expressed as mean±SEM. Statistical significance was assumed if $P<0.05$.

3. Results

3.1. Annexin Ⅴ binding and PI saining

A representative plot of a flow cytometry analysis of stretched cells for annexin Ⅴ binding and PI staining is shown in Figure 3-8. Cells negative for PI and annexin Ⅴ were regarded as viable cells (lower left quadrant, B3). PI-negative cells, which bind annexin Ⅴ, were defined as cells early in the apoptotic process (lower right quadrant, B4).

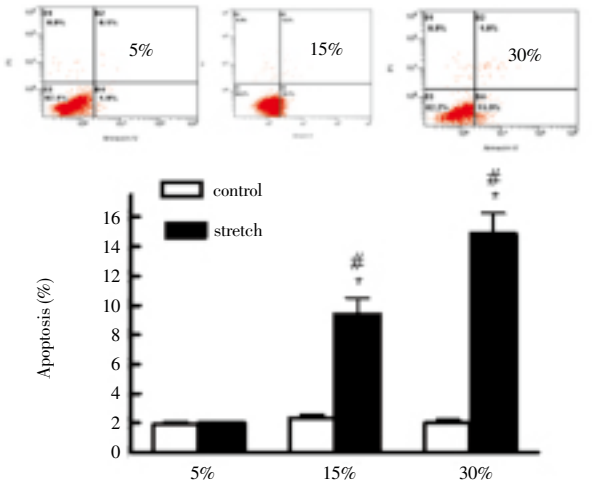

Figure 3. Effects of Stretch on apoptosis of A549 cells
Annexin V(+) and PI (-) straining. Flow cytometry data of cells subjected cyclic stretch (close bars) and cells from staticcultures (open bars). *$P<0.05$ versus control. #$P<0.05$ versus the other stretch groups.

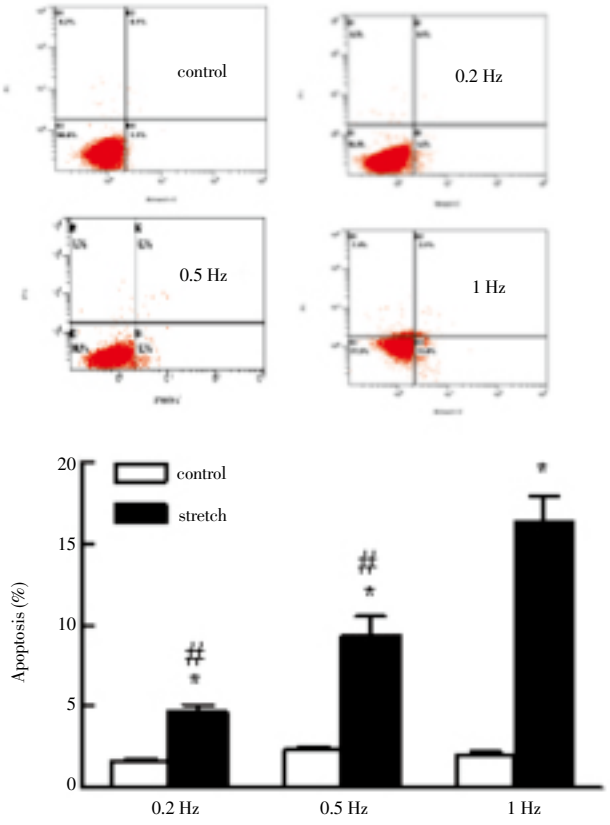

Figure 4. Frequency-dependent effect of mechanical stretch on apoptosis by Annexin V(+) and PI (-) straining.
Data are means±SEM of triplicate determination of 3 separate experiments. *$P<0.05$ versus control. #$P<0.05$ versus the other stretch groups.

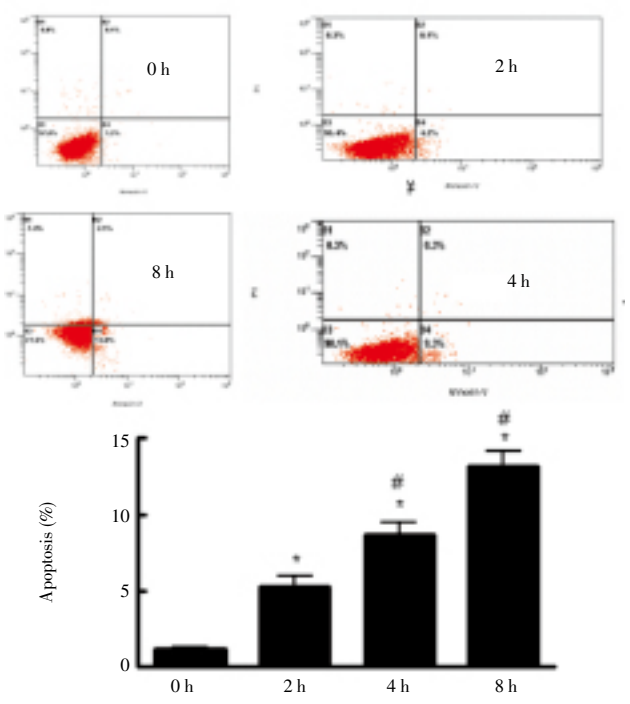

Figure 5. Time-dependent effect of mechanical stretch on apoptosis by Annexin V(+) and PI (-) straining.

Data are means±SEM of triplicate determination of 3 separate experiments. * $P<0.05$ versus control. # $P<0.05$ versus the other stretch groups.

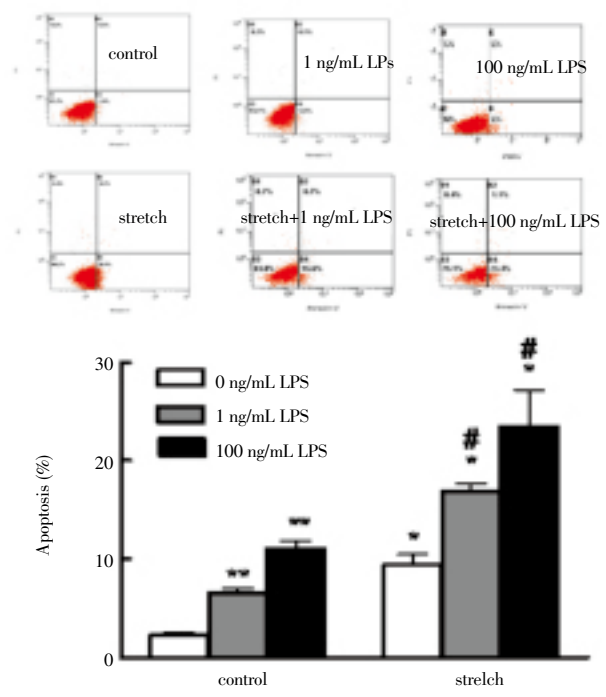

Figure 6. Effects of LPS on apoptosis of Stretch A549 cells.

*$P<0.05$ *vs.* the control group, ** $P<0.05$ *vs.* the other groups only in control group, # $P<0.05$ *vs.* the other groups only in stretch group.

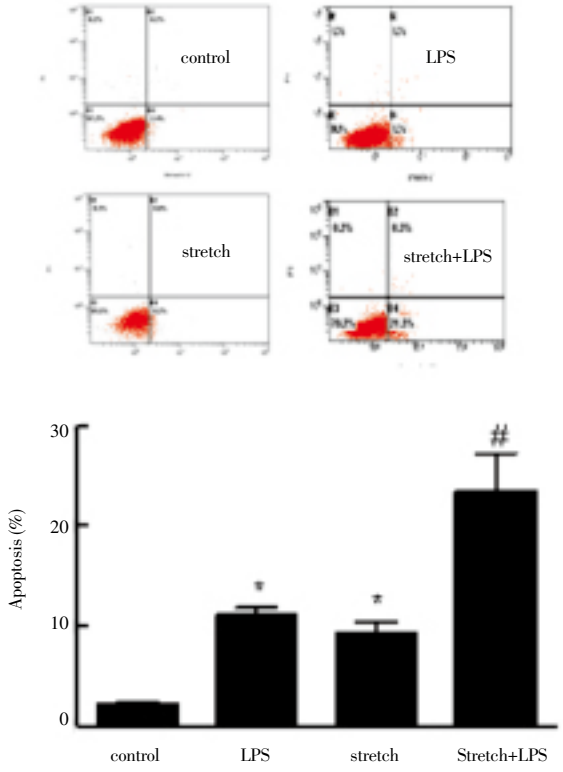

Figure 7. Effects of 100 ng/mL LPS and 15% stretch on apoptosis of A549 cells.

*$P<0.05$ versus control. #$P<0.05$ versus the other groups.

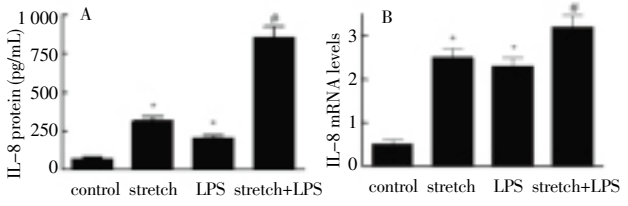

Figure 8. Effects of LPS and Stretch on Protein and mRNA levels of IL-8.

A: Addictive effect between LPS and mechanical stretch on IL-8 production. B: LPS and mechanical stretch elevated IL-8 mRNA levels respectively. *$P<0.05$ versus control. #$P<0.05$ versus the other groups.

Mechanical stretch induces apoptosis of AT II cells, annexin- Ⅴ and PI staining were assessed by flow cytometry (10 000 counted cells per sample). 15% and 30% not 5% strain enhanced apoptosis [(9.40±1.10)% and (14.90±1.40)% *vs.* (2.33±0.22)% and (2.07±0.18)%] (Figure 3). To investigate the effect of stretching frequency on the apoptotic rate of ATⅡ cells, we exposed A549 cells to different stretching frequency (0.2, 0.5, 1 Hz) at 15% stain for 4 h. Our results demonstrate a clear frequency dependence of stretching-induced apoptosis, increasing significantly upon stretching at 1 Hz, 0.5 Hz and 0.2 Hz up to (16.30±1.66)%, (9.40±1.10)% and (4.73±0.35)%, respectively (Figure 4). To investigate the

effect of stretching time on the apoptotic rate of AT Ⅱ cells, we exposed A549 cells to different periods (0 h, 2 h, 4 h and 8 h) at strain 15%, 0.5 Hz. We found a clearly time dependence of stretching-induced apoptosis (Figure 5).

A549 cells were subjected to cyclic mechanical strain (15% strain, 0.5 Hz, 4 h) and/or LPS (1 or 100 ng/mL). Mechanical strain significantly increased the early apoptotic rate of A549 cells especially in the presence of LPS 100 ng/mL compared with LPS 1 ng/mL and without LPS [(23.50±1.41)% *vs.* (16.90±0.81)% and (9.40±1.10)%]. With the increasing concentration of LPS, the addictive effect on the early apoptosis enhanced greatly (Figure 6).

In the presence of 100 ng/mL LPS, 15% mechanical strain enhanced LPS-induced early apoptosis significantly compared with stretch and LPS alone (Figure 7).

3.2. Protein and mRNA levels of IL-8

A549 cells were subjected to cyclic mechanical strain (15% strain, 0.5 Hz, 4 h) and/or LPS (100 ng/mL). IL-8 protein increased significantly induced by mechanical strain and LPS compared with LPS and stretch alone [(856.30±69.32) pg/mL *vs.* (203.00±19.66) pg/mL and (313.30±29.45) pg/mL] (Figure 8A). mRNA of IL-8 also enhanced significantly by cyclic stretch and LPS [(3.20±0.26) *vs.* (2.51±0.19) and (2.30 ±0.20) (Figure 8B).

4. Discussion

Although many therapeutic have been developed clinically[18-20], the mortality and morbidity associated with ARDS remains very high[21]. Investigators have focus on the normal alveolar type II cells by mechanical stretch. But Little attention is paid in the effect on apoptosis and IL-8 production of alveolar type II cells under pathologic state.

Apoptosis is an important mechanism of cell death in alveolar type 2 cells in ARDS. It has been reported that alveolar type 2 cells apoptosis were important in acute lung injury[22]. Tesfaigzi and colleagues found in the ealy stage of endotoxin induced acute lung injury, apoptosis of type II cells increased significantly[23]. In sublethal hyperoxic lung injury apoptosis of type II cells also increased significantly[24]. It has also been reported that high volume ventilator produced significant pathological changes of ALI in rats, which includes acute inflammatory reaction and extensive lung cell apoptosis[25]. Some investigators also found that cell apoptosis play an important role in the pathogenesis of VILI[26,27]. All of above indicate that apoptosis is an additional mechanism for acute lung injury.

Apoptotic epithelial cells have been found in the damaged alveolar epithelium of patients with ARDS[28]. One of the possible consequences of epithelial (type II) cell apoptosis in ARDS or ALI is the reduction of lung surfactant pools.

The continuous human alveolar epithelial cell line (A549) was studied as a surrogate for human alveolar epithelium. A549 cells have many features consistent with alveolar type II epithelial cells. Morphologically, when grown at subconfluence, they retain a cuboidal shape, are able to synthesize lecithin and phosphatidylcholine[29].

Annexin Ⅴ and PI double staining has been used to define the stage of cell death. Double negative cells are considered as non-apoptotic, annexin Ⅴ positive cells as early apoptotic with normal plasma membrane permeability, and double positive cells as late apoptotic or secondary necrotic with increased permeability of cytoplasm membrane[30-32]. In this study we use flow cytometry to detect the early apoptosis cells.

4.1. Relationship between strain and total lung capacity

There still have not definite conceptions about what are the strain levels that alveolar epithelial cells (AECs) encounter during a normal breathing pattern. The normal range of strain an AEC encounters varies with the changes in tidal volume associated with normal breathing.

Strain is a measure of the degree of stretch and is expressed as the percentage of the change in cell length to resting cell length. Increases in epithelial basement membrane surface area have been reported to occur predominantly with lung inflations (residual together with tidal volume) exceeding 40% of total lung capacity (TLC) in the rat[33]. Free alveolar surface area as much as doubles when the lungs are inflated from 40% TLC to 100% TLC, some of the area change reflects unfolding of alveolar septal ''pleats'' as opposed to extension of elastic cell and tissue elements[34]. By mathematical modeling, a change in lung volumes from 42% of the TLC to 64% of the TLC was associated with 32% of the alveolar surface area, which corresponded to 15% linear strain of alveolar cells. It is generally estimated that AECs are exposed to 1%-5% strain during normal breathing, and levels of 30% strain might represent a very high tidal volume mechanical ventilation associated with ventilation-induced lung injury[35].

4.2. Apoptosis stimulated with mechanical force

Tschumperlin DJ found primary epithelial type II cells, the percentage of dead cells increased significantly after a single deformation ranged from 0.5% to 72.0% over the range of deformations used[36]. Hammerschmidt S and his coworkers find in alveolar type II (AT II) cells, early apoptotic cells were significantly increased in S60-13 and S40-30(characterized by frequency [min(-1)]-increase in surface

area [%])[37]. In this study, we find the early apoptosis of A549 cells increased significantly in 15% and 30% stretch, but not 5%. So in clinic, reducing the tide volume during mechanical ventilation can induce a 22% reduction of mortality in patients suffering from ARDS[38]. Edwards and his coworkers found short-term cyclic stretch (22% enlongation , 3 cycles/min) induced the apoptosis in primary cultures of rat alveolar type II cells, including nuclear condensation, the generation of oligosomal DNA fragments[39]. Later they also reported the apoptosis cells in response to 30% cyclic stretch[40]. In their study apoptosis was detected by measurement of DNA condensation with the nuclear stain Hoechst 33258, which detects late stage apoptosis. In contrast, we use annexin Ⅴ binding, which detects apoptosis at early stage.

Tschumperlin and colleagues used ethidium homodimer-1 to detect the effect of apoptosis induced by mechanical stretch[41]. Ethidium homodimer-1 is excluded by the intact plasma membrane of viable cells, was used to mark dead cells. Here we use PI and annexin Ⅴ to detect the early apoptosis. They are two different detection systems.

4.3. Apoptosis and IL-8 stimulated with LPS and mechanical stretch

Vlahakis and colleagues found An additive effect between TNF-a and mechanical stretch on IL-8 production[42]. The immediate question that arises from this work is whether the addictive effect on apoptosis can be induced by mechanical strain and LPS. In the present experiment we find the additive effect. In the presence of mechanical stretch, with the increasing concentration of LPS, the addictive effect on the early apoptosis enhanced greatly. As to IL-8, mechanical stretch and LPS enhanced IL-8 levels both in mRNA and protein. So, clinical outcomes were similar whether lower or higher positive end-expiratory pressure levels were used[43], which is because the protocol proposed by the ARDS network, lacking solid physiologic basis, frequently fails to induce alveolar recruitment and may increase the risk of alveolar overinflation.

Using cytomechanic methods, we find an additive effect between LPS and mechanical stretch on apoptosis and IL-8 secretion in alveolar type II cells , which suggest us, in clinic, when ventilation is applied to patients with sepsis, bacterial infection, or other complications, this combined effect may enhance the apoptosis and IL-8 production, which may reversly influence the outcome of patients with ARDS. But there are still some limitations in my research. Our *in vitro* model for examining the effects of stretch on type II cells is an over-simplification which has eliminated the influence of other cell types and does not take into account spatial relationship of type II cells. Moreover, the pathways by which stretch regulates the apoptosis and cytokines release from lung cells also merit further investigations.

Conflict of interest statement

We declare that we have no conflict of interest.

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