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Original Article

The regulation of glycogen synthase kinase-3β on the mesenchymal stem cell therapy in the lypopolysaccharide induced acute lung injury in rat

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Abstract: Acute lung injury (ALI) is a one of severe manifestation and remains a major cause of morbidity and mortality in critically ill patients. Mesenchymal stem cell (MSCs) based therapy is a promising and novel treatment, it has been considered to be a potential treatment of acute lung injury. Glycogen synthase kinase-3β (GSK-3β) has been associated with the pathogenesis of several diseases, that can be a potential therapeutic target. In the current study, we were focused on the possible enhancement, regulatory mechanism of MSCs therapy, what improves the outcome of cell treatment, so that we explored the role of inhibiting GSK-3β with a selective inhibitor of LiCl during treatment of MSCs in the acute lung injury, in vivo. Experiments were carried out on 40 adults, Sprague-Dawley (SD) rats, those were randomly classified into 4 groups: 1. Control group (treated with normal saline (NS) 5 mg/kg), n = 10. 2. Acute lung injury (ALI) group (treated with lypopolysaccharide (LPS) 15 mg/kg), n = 10. 3. ALI+ MSCs group (treated with MSCs via tail vein, after LPS administration 2 h) n = 10. 4. ALI+ MSCs + Lithium chloride (LiCl) group (treated with MSCs and LiCl 20 mg/kg after LPS administration 2 h). The blood serum was collected at 12 h to determine inflammatory cytokines, lung tissues were harvested and analyzed on the 7th and 14th day of study. It was revealed that MSCs with inhibition of GSK-3β (LiCl) treatment group was more effectively suppressed systemic inflammation, inflammatory cell infiltration in lung tissue, pulmonary edema, pathological impairment and protect pulmonary epithelial cells in the acute phase of acute lung injury. In conclusion, in the present study suggested that inhibition of GSK-3β likely to play an enhancement role to improve outcome of MSCs treatment in the acute phase of acute lung injury in vivo.

Keywords: Mesenchymal stem cells, acute lung injury, glycogen synthase kinase-3β, wnt/β-catenin signaling pathway, lithium chloride, lypopolysaccharide

Introduction

Acute lung injury is a syndrome characterized by acute hypoxemic respiratory failure with bilateral pulmonary infiltrates that is associated with pulmonary or non pulmonary risk factors [1]. Most cases of ALI associated with pneumonia or sepsis, although, gastric aspiration, massive transfusion and trauma are said to be contributory factors [2]. The occurrence of ALI is nearly 200,000 cases every year in the United State with an in-hospital mortality rate of nearly 40% [1]. IL-1β, IL-6, IL-8, TNF-α are major cytokines, those release from damaged alveolar epithelial cells and activated systemic immune cells that play an major roles contribute to the pathological process, including pulmonary edema, alveolar capillary damage and development of acute lung injury and strongly affects to mortality [3]. Although, mechanical ventilation is a current treatment of ALI that provides essential life support of critically ill patients, it may aggravate alveolar damages [4]. In several studies demonstrated that mesenchymal stem cells (MSCs) multipotent capacity able to proliferate, migrate, engraft to

injured sites and differentiate into injured alveolar cells, moreover, inhibit inflammatory response and pathological impairment and involve healing process, in vivo [5-7]. Although, MSCs attenuate and participate in the development of new lung tissue following injury through various of mechanism, the regulation of differentiation and immunomodulation effect of MSCs is still controversial field.

The canonical Wnt/β-catenin, which most critical and widely studied signaling pathway. It plays an important role in tissue repair, wound closure, fibrosis and tissue remodeling. Breafly, WNT ligand binding to cell surface receptors such as Frizzled (Fz) and co-receptor low density lipoprotein related protein (LRP) 5/6 lead to cytosolic stabilization and nuclear translocation of β-catenin may express the target gene for contributing cell proliferation, differentiation, organ development and healing process on injured any organ. In contrast, absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated by Glycogen synthase kinase-3β (GSK-3β) and casein kinase 1 (CK1) belonging to the Axin complex [8].

The glycogen synthase kinase-3β (GSK-3β) is a not only involve intracellular transduction pathway, but also plays major role process of the inflammatory response. Glycogen synthase kinase-3β (GSK-3β) activates during several diseases, including type II diabetes and Alzheimer's disease and acute lung injury, acute pancreatitis [9-12]. So that it would be a potential drug target. Lithium chloride (LiCl), a widely prescribed drug for bipolar mood disorder, has been reported that acts as a selective inhibitor of the GSK-3 family of protein kinase, to possess anti inflammatory properties [13, 14]. In a previous study elucidated that novel role of Lithium, which not only strengthen MSCs transplantation therapy efficacy through accelerated MSCs proliferation and differentiation in vitro but also suggested an alternative therapeutic agent for against bacterial infections and certain kind of disorders resulting from suppression of Wnt/β-catenin pathway [11, 15, 16].

However, the mechanisms through which GSK-3β inhibition promotes stem cell therapy outcome and provide lung protection remains unclear. In the current study, we sought to explore the role of inhibiting GSK-3β with a selective inhibitor of Lithium chloride, whether to improve MSCs treatment outcome in the lypopolysaccharide (LPS) induced acute lung injury, that was for demonstrating the possible enhancement, regulatory mechanism of stem cell therapy, in vivo. To demonstrate in detail, we had investigated four main features of experimental acute lung injury, including systemic inflammatory response, histological changes and collagen deposition of lung tissue, alteration of the alveolar epithelial barrier, regulation of β-catenin, GSK-3β in vivo. Those experiments performed at the three time points when the 12th hour (Acute phase within 48 h), 7^{th} day (Proliferative phase >7 days) and 14^{th} day (Fibrotic phase >14 days).

Materials and methods

Experimental animals and study design

Healthy Sprague-Dawley (SD) male rats were donated from the Specific Pathogen Free Laboratory Animal Center of Dalian Medical University, China. All experiments were performed according to institutional guidelines, the study was approved by the Institutional Animal Care Committee of Dalian Medical University. Total 40 healthy SD male rats (250 g, 6-8 weeks) were randomly classified into four groups: 1. Control group (n = 10) treated with normal saline 5 mg/kg via intraperitoneal injection. 2. ALI group (n = 10) treated with LPS 15 kg/kg (extracted from Escherichia coli serotype 0111: B4 Sigma-Aldrich, St Louis, MO, USA) via intraperitoneal injection. 3. ALI+ MSCs group (n = 10) treated with MSCs 1×10^6 via tail vein, after LPS administration 2 h. 4. ALI+ MSCs+ Lithium chloride (LiCl) group (n = 10) treated with MSCs 1×10^6 and LiCl 20 mg/kg (GSK-3β selective inhibitor) via intraperitoneal injection, after LPS administration 2 h. LiCl was injected at every 24 hours to maintain blood concentration during the experiment.

Sample collection and management

All blood samples were collected from the tail vein of each group of rats (n = 24) after LPS administration at 12 h of the experiment and centrifuged immediately (4°C, 2000 rpm/min for 15 min) for collecting serum and stored at -80°C until for identifying inflammatory cytokines. Thereafter, rats were sacrificed under the anesthesia condition with intraperitoneal

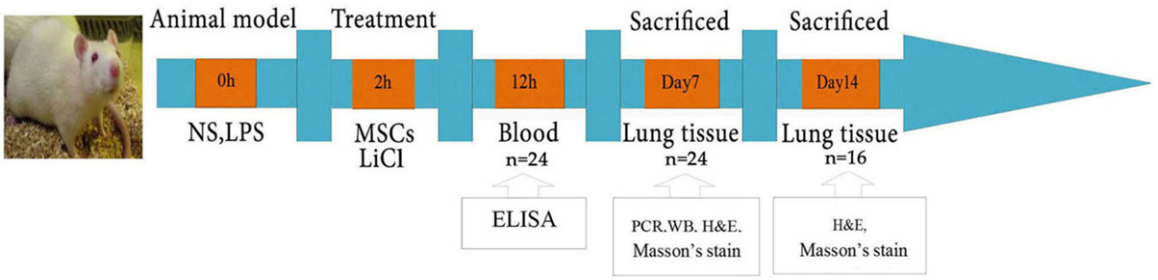

Figure 1. Experimental design. MSCs, mesenchymal stem cells; LPS, lypopolysaccharide; LiCl, lithium chloride; NS, normal saline; ELISA, Enzyme-linked immunosorbent assay; H&E staining, hemotoxylin and eosin staining; WB, western blotting; RT-PCR, Reverse transcription-polymerase chain reaction.

injection of 10% chloral hydrate aldehyde (4 ml/kg body weight) on the 7th and 14th day of the experiment. The 7th day of lung samples were collected to perform WB, RT-PCR, H&E staining, Masson's trichrome staining and the 14th day of lung samples were collected to perform H&E, Masson's staining for comparing the pathological changes with previous experiment. All of the samples stored at -80°C until assayed (**Figure 1**).

Isolation and culture of bone marrow derived MSCs

The bone marrow adherence selection method was used to isolate and culture the MSCs as previous described [16]. Healthy SD, 4-5 weeks, 80-100 g body weight male rat was sacrificed by cervical dislocation under anesthesia with 10% chloral hydrate aldehyde (4 ml/kg body weight) via intraperotoneal injection and long bones (tibia and femur) of the limbs were aseptically dissected. The bones were washed extensively with sterile phosphate-buffered saline (PBS) (Sigma, USA) with 1%-penicillin-streptomycin for decontamination. The bone marrow was repeatedly flushed out from the cavities using 1% L-Glutamine-Basal SD rat MSCs special medium supplemented with 10% Fetal bovine serum (FBS) (Sigma, USA), Penicillin 100 units/ml, Streptomycin 100 μg/ml (PS 1%) (Sigma, USA). The collected cells were filtered through a 70 μm cell strainer and centrifuged 1500 rpm for 8 minutes, at room temperature. The cells were subsequently cultured with complete medium into 25 cm^2 uncoated flask, incubated at 37°C in an atmosphere of 5% CO_2 with saturated humidity. At confluence 80-90%, following 7-10 days incubation at optimum condition, cells were routinely washed twice with sterile PBS and trypsinised (Sigma, USA) for 3 min, at 37°C to passage. Following centrifugation at 1500 rpm for 10 min, at room temperature cells were resuspended in complete medium and diluted at a ratio of 1:2 at each passage. We had used for all experiments passage 3 cells.

Characterization of MSC by flow cytometry analysis

The cell morphology, growth and proliferation had observed by optic microscopy. Following, MSCs were then analyzed of the surface marker expression by flow cytometry assay as previously described [17]. Breafly, cultured passage 3 cells were collected, washed with ice cold phosphate-buffered saline (PBS). 1×10^5 cells were incubated with each of primary antibody which Phycoerythrin (PE) Hamster Anti-Rat CD29 (BD Biosciences, USA), mouse fluorescein isothiocyanate (FITC)-conjugated Anti Rat CD34 (Santa Cruz Biotechnology, INC), mouse Phycoerythrin (PE) Anti-Rat CD90 (BioLegend, INC), fluorescein isothiocyanate (FITC)-conjugated mouse Anti Rat CD45 (BD Biosciences, USA) at 4°C for 30 min in the dark, respectively. After washing 3 times with PBS, cells were analyzed using a FACSVantage SE flowcytometer machine and Paint-A-Gate software (Becton Dickinson, Co., USA).

Detection of inflammatory cytokines by Enzyme-linked immunosorbent assay (ELISA)

The concentration of TNF-α, IL-6, Endotoxin (ET) were measured using an ELISA-kit (R&D System, Minneapolis, MN, USA) in blood serum. The blood samples were obtained from the tail vein following LPS administration at 12 h and centrifuged at 4°C, 2000 rpm for 15 min for collecting serum. Briefly, plates were blocked

Table 1. Primer sequences for RT-PCR

Product	5'-primer	3'-primer	TM (°C)
β-catenin	CCACTCCAGGAATGAAGG	AGCAGTCTCATTCCAAGC	56
GSK-3β	CAACAGCCACCCCAAGA	GATCCCATCTTGGTCATCC	58
SP-A	TAACTAAGTGCTGCCCTCTGA	ACTAGGCTTTCCCAGAAATCC	58
SP-C	AGCCCACCGGATTACTCGAC	AGGACTACCACCACAACCACG	64
β-actin	AGATCCTGACCGAGCGTGGC	CCAGGGAGGAAGAGGATGCG	66

RT-PCR, Reverse transcription-polymerase chain reaction; GSK-3β, glycogen synthase kinase-3β; SP-A, surfactant protein-A.

and incubated for 1 h at room temperature, then samples were added 40 μl/well and subsequently added Biotinylated antibodies 10 μl/well and streptavidin-horseradish-peroxidase 50 μl mixed gently, incubated at 37°C for 1 h according to the manufacturer's instructions. Following, treated with color developing agent A and B 50 μl/well, incubated at 37°C for 10 min. The reaction was stopped by the addition of stop solution 50 μl, respectively. Optimal density (OD) values were obtained using a wavelength 450 NM plate reader in the each sample. Concentrations of cytokines were calculated with CurveExpert 1.4 standard curves.

Reverse transcription-polymerase chain reaction (RT-PCR)

Gene expression of β-catenin, GSK-3β, SP-A, C and β-actin were detected by RT-PCR which performed as previously described [17]. Total RNA was extracted from each group of lung tissues on the 7th day of the experiment. After grinding the lung tissues to small fragments, homogenization phase was achieved using a 1 ml Trizol solution. RNA was separated using 200 μl of chloroform and then precipitated with 500 μl of isopropanol alcohol according to the manufacturer's instructions. cDNA was synthesized from 5 μg of total RNA using Random Hexamer primer according to the Thermo scientific RevertAid First Strand cDNA synthesis Kit (#K1622) manual. Following, 2 μl cDNA was mixed with Fermentas life sciences DreamTaq™ PCR Master Mix (2X) (#K1071) 25 μl, Forward primer 0.1-1.0 μM, Reverse primer 0.1-1.0 μM, Water, nuclease-free 20 μl of the PCR reaction system and placed in MyGene™Series Peltier Thermal Cycler to react. The denaturation, annealing and extension temperatures were 95°C, TM-5 and 72°C, respectively. The reaction product 5 μl was subjected to 1% agarose gel electrophoresis at 100 V for 20 min and analyzed by gel scanning system. The primer sequences are shown in **Table 1**.

Western blotting assay detection of β-catenin protein level in lung tissue

Total protein extracted from each group of lung tissues on the 7th day of the experiment as previous described [18]. Breafly, 100 mg tissues were taken and lysed in 1 ml of ice cold Lysis buffer, containing 1 μl of protease inhibitor, 5 μl of phosphatase inhibitor cocktail (KeyGENBioTECH, KGP250-2100). Lysates were centrifuged at 4°C, 14000 rpm for 15 min, and then supernatants were heated to 95°C for 10 min in order to achieve complete denaturation, as previously described. The proteins were then resolved by 10% sodium dedocyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were washed with TBST and blocked with 5% non-fat dry milk for 1 h at room temperature, the membranes were incubated with primary antibody (1:5000; Rabbit anti rat β-Catenin Polyclonal Antibody; Protein tech, Mouse anti rat β-actin Monoclonal Antibody; Santa Cruz Biotechnology, Inc) at 4°C, overnight. After washing, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:5000; Goat anti-Rabbit/Mouse IgG; Abbkine, Inc., USA) shaking gently, for 2 h at room temperature. Immunoreactive protein bands were detected by using electrochemiluminescence (ECL) scanning system.

Detection of lung pathological changes and collagen deposition by hematoxylin & eosin and Masson's trichrome staining

It was performed according previously described of detecting the histologic structure integrity of lung tissue [4, 19]. Tissue samples were taken on the 7th and 14th day of the experiment. In brief, extracted lung tissues were fixed in 10% paraformaldehyde at 4°C for overnight and embedded in liquid paraffin in optimal cutting temperature. The tissues were cut transversely at a thickness of 5 μm and following dewax and dehydrating step, it was stained with hematoxylin & eosin (H&E) and Masson's trichrome staining in according to the manufacturer's recommendations. Observations were

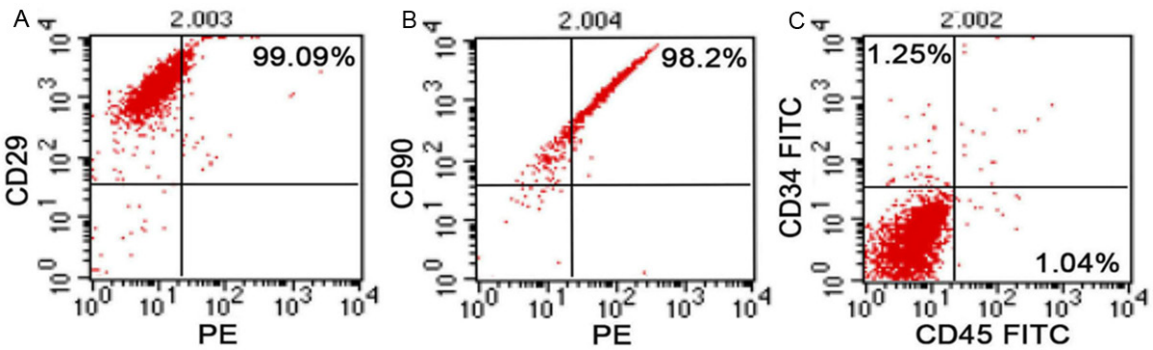

Figure 2. Flow cytometry analysis of MSCs. Cell markers of (A) CD29 (99.09%) and (B) CD90 (98.2%) expressions were identified positive, but (C) CD 34 (1.25%) and CD45 (1.04%) expressions were identified extremely low, respectively.

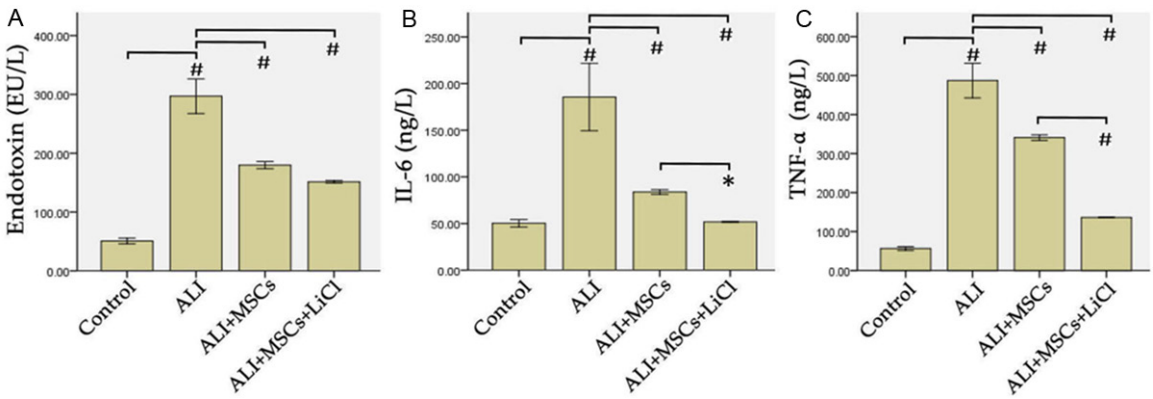

Figure 3. ELISA assay. The serum concentrations of ET (A), IL-6 (B) and TNF-α (C) were decreased in the MSCs and MSCs with inhibition of GSK-3β (LiCl) treatment groups compared to the ALI group, after LPS administration at 12 h ($P<0.01$). The decrease in the concentration of those cytokines were significantly greater in the MSCs with inhibition of GSK-3β (LiCl) treated group than those in the MSCs alone treated group ($P<0.05$). Particularly, TNF-α statistically significantly suppressed than IL-6 and ET in the MSCs with inhibition of GSK-3β (LiCl) group ($P<0.01$). The data represented as the mean ± SD, *$P<0.05$, #$P<0.01$. LPS, Lypopolysaccharide; ALI, Acute lung injury; MSCs, Mesenchymal stem cells; LiCl, Lithium chloride; ET, Endotoxin; TNF-α, Tumor necrosis factor-α; IL-6, Interleikin-6.

performed under a Nikon Optophot light microscope (Tokyo, Japan) and photographed with Nikon Digital camera at 100, 200 magnifications. The pulmonary histological score was obtained using the histologic ALI scoring system incorporating criteria what proposed by the American Thoracic Society [20]. We chose 5 main parameters include neurophils in the air space and the interstitial space, formation of hyaline membranes, proteinaceous debris filling in the air spaces and alveolar septal thickening, those indicate alveolar and interstitial damage. Each of histological findings was scored from 0 to 4 (none, mild, moderate, severe, very severe) as previously described [4, 20]. Lung fibrosis score was obtained using the method of Ashcroft, as previous described [21]. Briefly, pulmonary fibrosis was scored on a scale from 0 to 5 (normal lung = 0; Minimal fibrotic thickening of alveolar or bronchial walls = 1; Moderate thickening of the walls without obvious damage to the lung architecture = 2; increased fibrosis with defining damage to the lung structure and formation of fibrous bands or small fibrous masses = 3; Severe distortion of the structure and large fibrous area "honeycomb lung" formation = 4; total fibrous obliteration in the field = 5).

Morphometric analysis

We used the Masson's trichrome staining technique for obtaining area percent of collagen fibers in each group. With this technique, collagen fibers were stained blue color, smooth muscle and elastic fibers of the bronchioles

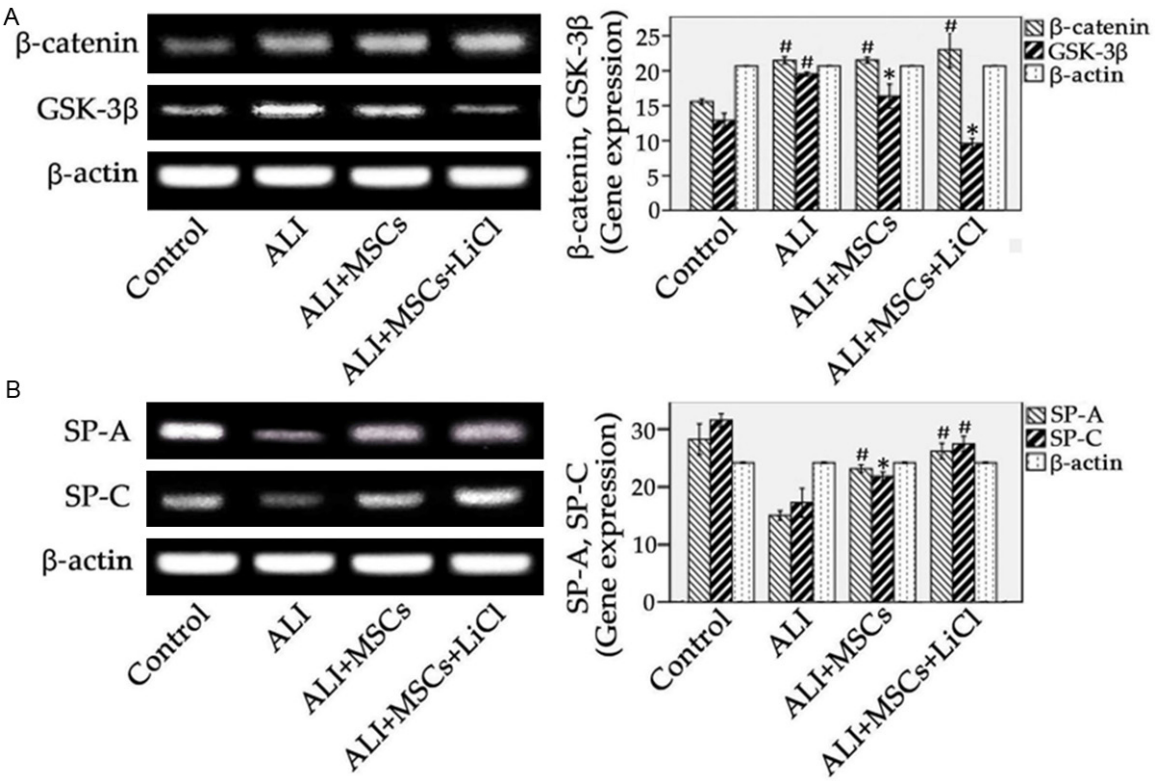

Figure 4. RT-PCR result. Gene expression of special components of Wnt/β-catenin signaling pathway and alveolar epithelial type II cell markers in lung tissue on the 7th day of experiment. A. Gene expression of β-catenin was increased in ALI, ALI+MSCs and ALI+MSCs with inhibition of GSK-3β (LiCl) groups compared to the control group ($P<0.01$). GSK-3β overexpressed in ALI group compared to the control group ($P<0.01$). Although, it was downregulated in MSCs treatment group, more efficacious downregulated in the MSCs with inhibition of GSK-3β (LiCl) group ($P<0.05$). B. Gene expression of SP-A, SP-C were overexpressed in the MSCs with and without inhibition of GSK-3β (LiCl) groups compared to the ALI group ($P<0.01$). SP-C mRNA expression was revealed significantly higher in MCSs with inhibition of GSK-3β (LiCl) group compared to the MSCs alone treated group ($P<0.05$). β-actin used for internal control. The data represented as the mean ± SD, *$P<0.05$, #$P<0.01$. ALI, Acute lung injury; MSCs, Mesenchymal stem cells; LiCl, Lithium chloride; SP, Surfactant protein; GSK, Glycogen synthase kinase.

were stained red color, cell nuclei was stained purple color. Collagen fibers were measured using "Image Pro Plus 6" image analyzer computer system. Five randomly selected high power fields in each slide were analyzed by a histopathologist who was blinded to this experiment.

Statistical analysis

All statistical analysis was performed by SPSS 21.0 software. Data are presented as the mean ± standard deviation (SD). After establishing that data were normally distributed, One-way analysis of variance (ANOVA) was used to compare the control and experimental groups. $P<0.01$ and $P<0.05$ were considered statistically significant.

Results

Chracteristics of MSCs by flow cytometry assay

Cultured passage 3 MSCs showed plastic adherent, highly proliferative, typical fibroblast-like and spindle-shaped morphology by optic microscope. Moreover, it was identified with positive expression of CD29 (99.09%), CD90 (98.2%), and extremely low expression of CD34 (1.25%) or CD45 (1.04%) cell markers by flow cytometry analysis (**Figure 2A-C**). Those features indicated that cultured cells with high purity.

ELISA assay

The serum concentrations of TNF-α, IL-6 and Endotoxin were measured (487.28 ± 24.5 ng/l;

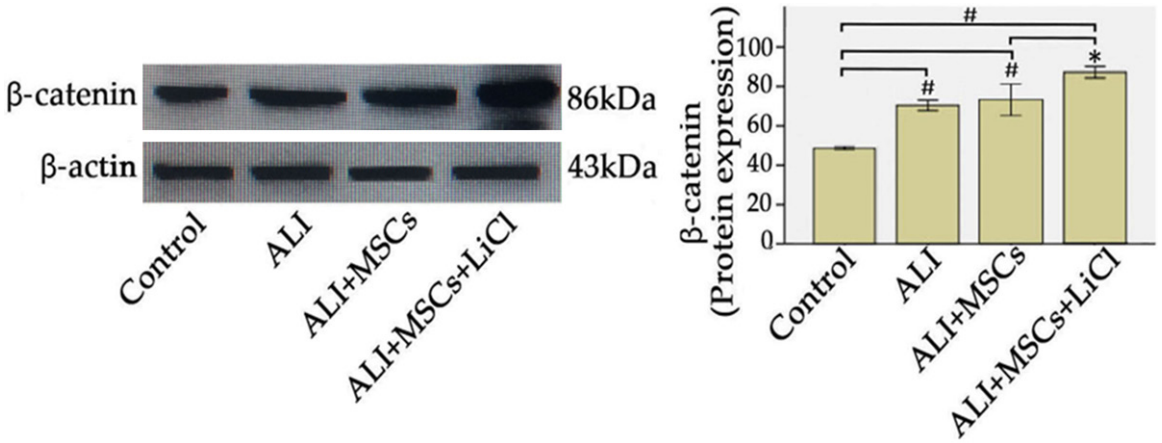

Figure 5. Western blotting assay. Activation of Wnt/β-catenin signaling pathway in lung tissue on the 7th day of experiment. The protein level of β-catenin was increased in ALI, ALI+MSCs and ALI+MSCs with inhibition of GSK-3β (LiCl) groups compared to the control group (P<0.01). It was highly activated in MSCs with inhibition of GSK-3β (LiCl) group compared to the MSCs alone treated group (P<0.05). The data represented as the mean ± SD, *P<0.05, #P<0.01. ALI, Acute lung injury; MSCs, Mesenchymal stem cells; LiCl, Lithium chloride.

193.75 ± 17.09 ng/l; 297 ± 15.54 EU/L) higher in the ALI group than those in control group (56.62 ± 7.6 ng/l; 50.34 ± 6.3 ng/l; 50.83 ± 7.72 EU/L). Those were decreased in MSCs treatment group (341 ± 11.2 ng/l; 83.85 ± 9.3 ng/l; 179.86 ± 9.7 EU/L) and MSCs with inhibition of GSK-3β (LiCl) treatment group (136.13 ± 9.6 ng/l; 57.77 ± 7.5 ng/l; 151.39 ± 8.2 EU/L) compared to the ALI group, after LPS administration at 12 h (P<0.01). The decrease in the concentration of those cytokines were significantly greater in the MSCs with GSK-3β inhibition group (LiCl) than those in the MSCs alone treated group (P<0.05). In particular, TNF-α was significantly suppressed compared to the IL-6 and ET in the MSCs with inhibition of GSK-3β (LiCl) treatment group (P<0.01) (**Figure 3**).

RT-PCR assay

RT-PCR result demonstrated that, intraperitoneal administration of LPS resulted in an activation of Wnt/β-catenin signaling in lung tissue on the 7th day of the study. Which indicated by overexpression of β-catenin mRNA in the ALI, ALI+MSCs and ALI+MSCs+LiCl groups compared to the control group (P<0.01). But it was no significant difference between MSCs alone and MSCs with inhibition of GSK-3β (LiCl) group (P>0.05). Moreover, mRNA of GSK-3β overexpressed in ALI group compared to control group. Although, it was downregulated in MSCs treatment group, more efficacious downregulated in the MSCs with inhibition of GSK-3β (LiCl) group (P<0.05). That result indicated that GSK-3β activated in injured lung tissue what could be involves progress of the pathological processes of ALI. Treatment of MSCs were insufficient suppressed the activation of GSK-3β, but it was promoted by a selective inhibitor ofLiCl (**Figure 4A**). Surfactant protein-A and C mRNA expression was decreased in ALI group. But it was overexpressed in MSCs with and without inhibition of GSK-3β (LiCl) groups compared to ALI group (P<0.01). Expression level of SP-C mRNA was revealed significantly higher in MCSs with inhibition of GSK-3β (LiCl) group compared to the MSCs alone treated group (P<0.05). β-actin used for internal control (**Figure 4B**).

Western blotting assay

Western blotting assay revealed that similar results with RT-PCR assay. The protein level of β-catenin was increased in ALI, ALI+MSCs and ALI+MSCs with GSK-3β (LiCl) groups compared to the control group (P<0.01). It was highly activated in the MSCs with inhibition of GSK-3β (LiCl) group compared to the MSCs alone treated group (P<0.05), which indicated that inhibition of GSK-3β lead to excessive activation of Wnt/β-catenin signaling on the 7th day of the experiment. β-actin used for internal control (**Figure 5**).

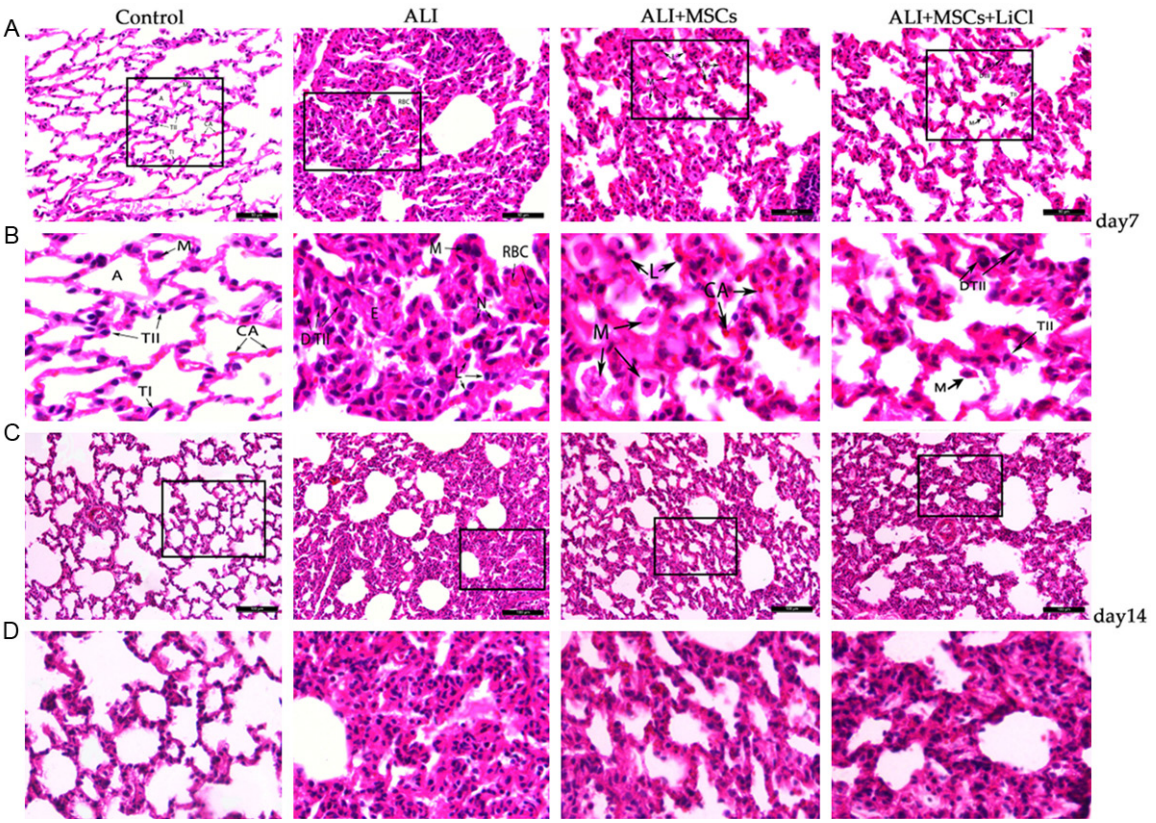

Figure 6. H&E stained lung sections. Pathological changes on the 7th day of the lung sections (original magnification ×200). Control group was observed normal appearance (none = 0). ALI group was observed very several alveolar walls thickening, inflammatory cell infiltration and red blood cells in alveolar and interstitial space, obliterating of alveolar architecture with atelectasis and proteinaceous debris filling in air space (very severe = 4). The MSCs treatment group was observed several levels of alveolar walls thickening and reduced proteinaceous debris filling, but still revealed infiltration of inflammatory cells and capillary vessel dilation in the interstitial space (severe = 3). MSCs with inhibition of GSK-3β (LiCl) group was observed mild level of alveolar walls thickening and interstitial edema, dramatically decreased proteinaceous debris filling in air space and inflammatory cell infiltration (mild = 1) (A, B). Pathological changes in the 14th day of the lung sections. MSCs with inhibition of GSK-3β (LiCl) group, however, was observed inflammatory cell infiltration less than ALI and ALI+ MSCs groups, somehow more observed collections of irregular cell nucleus and fibrous degeneration (C, D). A, air space; TI, type I cell; TII, type II cell; D TII, divided type II cell; CA, capillary vessel; M, alveolar macrophage; RBC, red blood cell; L, lymphocyte cell; E, excudate.

Histological result

H&E stained lung sections

Microscopic observation: Pathological changes on the 7th day of lung sections. Control group was observed normal lung architecture (none = 0). ALI group was observed very several lost lung architecture, inflammatory cell infiltration and red blood cells in alveolar and interstitial space, obliterating of alveolar architecture with atelectasis and proteinaceous debris filling in air space (very severe = 4). The MSCs treatment group was observed several level of alveolar walls thickening and reduced proteinaceous debris filling compared to the ALI group, but still revealed infiltration of inflammatory cells, especially neutrophil macrophage cells and capillary vessel dilation in the interstitial space (severe = 3). MSCs with inhibition of GSK-3β (LiCl) group was observed mild level of alveolar walls thickening and interstitial edema, dramatically decreased proteinaceous debris filling in air space and inflammatory cell infiltration (mild = 1) (**Figure 6A**, **6B**). Pathological changes on the 14th day of lung sections. Control group was (none = 0), ALI group was (very severe = 4), MSCs group was observed (moderate = 2). MSCs with inhibition of GSK-3β (LiCl) treatment group was observed inflammatory cell infiltration less than ALI and ALI+ MSCs groups, but more observed collection of irregular cell nucle-

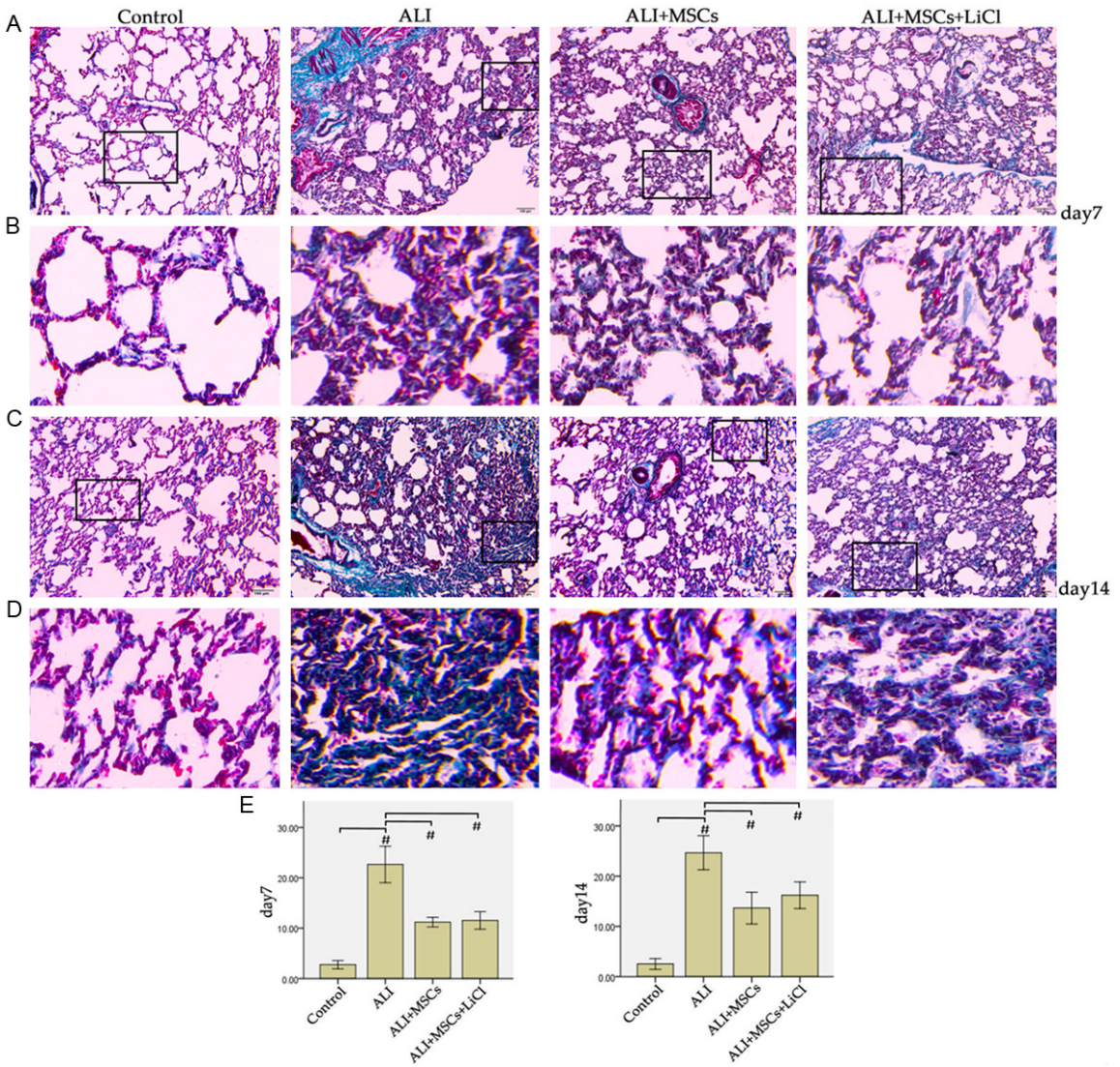

Figure 7. Masson's trichrome staining result. Microscopic observation (original magnification ×100). Collagen fibers on the 7th day of lung sections. Control group revealed normal collagen fibers. ALI group revealed extensive collagen fiber deposition. ALI+MSCs group revealed moderate collagen fibers. MSCs with inhibition of GSK-3β (LiCl) group revealed minimal collagen fiber deposition compared to ALI group, respectively (A, B). Collagen fibers on the 14th day lung section. A section of the ALI group revealed a severe distortion of structure with large collagen deposition, ALI+MSC group revealed contiguous fibrotic masses in the alveolar walls. In contrast, a section of MSCs with inhibition of GSK-3β (LiCl) treatment group revealed more fibrotic compared to MSCs alone treated group at day 14 (C, D). The blue color indicates the positive staining of collagen, mucus and cartilage of bronchioles. Red color indicates the smooth muscle and elastic fibers of bronchioles, pink color indicates the cell cytoplasm, red blood cells in blood vessels, purple color indicates the cell nuclei. (E) Morphometric analysis result. Mean area percentage of collagen fibers in the Masson's trichrome stained lung sections on the 7th and 14th day of the experiment. The data represented as the mean ± SD, *P<0.01.

us it might be type II cell proliferation and fibrous degeneration (moderate = 2) (**Figure 6C**, **6D**).

Masson's trichrome stained lung sections

Microscopic observation: Collagen fibers on the 7th day of lung sections. Control group revealed normal collagen fibers within the lung interstitium. ALI group revealed extensive collagen fiber deposition. ALI+MSCs group revealed moderate collagen fibers in the lung interstenium. MSCs with inhibition of GSK-3β (LiCl) group revealed minimal collagen fiber deposition compared to ALI group, respectively (**Figure**

7A, **7B**). Collagen fibers in the 14th day lung section. A section of the ALI group revealed a severe distortion of structure with large collagen deposition, ALI+MSC group revealed contiguous fibrotic masses in the alveolar walls. In contrast, a section of MSCs with inhibition of GSK-3β (LiCl) treatment group revealed more fibrotic compared to MSCs alone treated group at day 14 (**Figure 7C**, **7D**). As shown in **Figure 7**, on the 7th day of lung sections revealed less fibrotic than on the 14th days of lung sections.

Mophometric analysis result: Mean area percentage of collagen fibers were measured in the Masson's trichrome stained lung sections. On the 7th day, control group was measured (2.75 ± 0.91). It was increased in ALI group (22.64 ± 4.05) compared to the control group. MSCs alone and MSCs+LiCl treated groups were measured (11.2 ± 1.07) & (11.54 ± 1.95), those were significantly less than ALI group, respectively ($P<0.05$). On the other hand, there were no significant difference between MSCs alone and MSCs with inhibition of GSK-3β (LiCl) treatment groups in the 7th day of experiment ($P>0.05$). On the 14th day, control group was measured (2.52 ± 1.19) and ALI group was (24.66 ± 3.78) significantly higher compared to the control group. The MSCs alone and MSCs with inhibition of GSK-3β (LiCl) treated group were measured (13.65 ± 3.53) & (16.25 ± 2.97), those were still significantly less than ALI group ($P<0.05$). However, percent of collagen fibers were increased in the MSCs with inhibition of GSK-3β (LiCl) group, it was no significant difference compared to the MSCs alone treated group ($P>0.05$) (**Figure 7E**). H&E stain and Masson's stain results indicated that, although, transplantation of MSCs group was revealed better that ALI group but did not significantly ameliorate ALI. It was observed that inhibition of GSK-3β (LiCl) significantly suppresses inflammatory cell infiltration, pulmonary edema, prevent histologically impairment and may improve MSCs treatment outcome in early phase (<7 days) of ALI. But excessive inhibition of GSK-3β may lead to aberrant activation of β-catenin what caused a type II cell proliferation and collagen deposition to promote fibrotic phase (>14 days) of ALI.

Discussion and conclusion

ALI causes by a variety of direct and indirect reason. Gram negative bacterial caused severe sepsis is a one of the most common reasons of human acute lung injury [1]. We established a murine model of experimental lypopolysaccharide-induced acute lung injury characterized by respiratory dysfunction, systemic inflammation, neutrophil infiltration in the lung tissue, interstitial edema, damage of alveolar wall epithelial and vascular endothelial cells and eventually remodeling of lung fibrosis.

Nowadays, mesenchymal stem cell based therapy is a promising and novel treatment, it has been considered to be a potential treatment of acute lung injury. In several studies demonstrated that exogenous administration of MSCs able to migrate, engraft to injured sitesand contribute to repair of the injured alveolar epithelium and vascular endothelium, although, main points of stem cell treatment remain to be controversial, including regulation of anti inflammatory, proliferation and differentiation mechanisms of MSCs. In this study, mesenchymal stem cells were harvested from bone marrow and cultured in vitro. It was observed with plastic adherent, high proliferative features and identified expression of certain cell surface markers including CD90 CD29, but not expression of CD34, CD45 by a flow cytometry assay. Those features indicated that their phenotype and multipotent differentiation potential, which are typically of MSCs and consistent with previous studies.

GSK-3β is a one of the target protein what belonging to the Axin complex of Wnt/β-catenin signaling pathway and LiCl is a selective inhibitor. In previous studies showed that inhibition of GSK-3β protects endotoxaemic induced acute renal failure by down regulating pro-inflammatory cytokines, renal cell apoptosis and it has a protective effect from the intragastric instillation of K. pneumonia induced liver injury and necrosis in vivo [22, 23]. Moreover, inhibition of GSK-3β reduced severity of tissue edema, inflammatory cell infiltration and serum level of amylase, lipase, myeloperoxidase activity in cerulean induced acute-pancreatitis and it has been suggested that an important target for stabilizating of the blood brain barrier in neuroinflammation [11, 24]. In this study, we showed that excessive activation of GSK-3β in the LPS treated ALI group and it was not efficiently suppressed transplantation of the MSCs treatment group by RT-PCR assay. So we sus-

pected that, however, administration of MSCs to suppress inflammatory processes or repair of the injured tissue, it probably requires enhancement of recruitment, what to improve cell treatment outcome. Mentioned above, however, the mechanisms through which GSK-3β inhibition promotes stem cell therapy outcome and provide lung protection remain unclear. In the current study, we were focused on the possible an enhancement, regulatory mechanism of stem cell therapy, so that we investigated the role of inhibiting GSK-3β during the treatment of MSCs in the acute lung injury, in vivo. Next experiments confirmed that administration of MSCs with inhibition of GSK-3β (LiCl) more efficiency suppressed systemic inflammatory by reducing the serum level TNF-α, ET, IL-6 after LPS administration at 12 h, resulting to significantly reduce the damage of alveolar wall epithelial cells, decreased inflammatory cell infiltration, septum widening, interstitial edema and the other histological impairment in the lung tissue.

Inhibition of GSK-3β lead to activate β-catenin protein what is also one of special component of the Wnt/β-catenin signaling pathway, which play a various fundamental organ development roles, including cell fate specification, proliferation, polarity and migration, survival during embryonic development [25-27]. Wnt signaling also involved in the regulation of tissue homeostasis, tissue damage and remodeling, injury termination, tissue repair or destruction. Aberrant activation of Wnt signaling may contribute to pathological changes such as retinopathies, pulmonary artery hypertension, pulmonary fibrosis, cancer, asthma, stroke and others [28, 29]. Previous study demonstrated that Wnt/β-catenin signaling pathway activated with Wnt3α and promote β-catenin protein nuclear translocation in MSCs after on day 14, which promote MSCs differentiate into fibroblast and myofibroblast cells those approved by increased of α-SMA, collagen 1A, Vimentin protein levels by Western blotting assay in vitro. However, the MSCs can decrease proinflammatory cytokine in blood serum but could not decrease the expression of fibroblast markers after HCl-induced ALI in vivo [17].

In the current study showed that protein expression of β-catenin activated in injured lung tissue after LPS administration on the 7th day of experiment and excessive activated in the MSCs with inhibition of GSK-3β (LiCl) treatment group by WB assay. In the histological result showed that irregular cell nucleus and mean area percentage of collagen fibers were revealed more on the 14th day than those on the 7th day of the experiment in the MSCs with inhibition of GSK-3β (LiCl) treatment group. However, additional evidence needed to be provided to support, those results demonstrated that activation of β-catenin may translocated into type II cell nucleus to regulate target gene expression, including SP-A, SP-C, indicating that activation of Wnt signaling not only involve type II cell proliferation but also may promote collagen deposition in vivo.

In conclusion from this result that inhibition of GSK-3β likely to play a facilitating role to improve outcome of MSCs treatment in the acute phase (<7 days) of acute lung injury in vivo. But excessive inhibition of GSK-3β may lead to aberrant activation of Wnt signaling, which contribute fibrosis phase (day 14<) of acute lung injury and accelerate irregular cell proliferation.

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Disclosure of conflict of interest

None.

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