Myocardial ischemic post-conditioning protects the lung against myocardial ischemia/reperfusion-induced damage by activating GSK-3β

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Abstract

Purpose: To investigate whether modulating GSK-3β could attenuate myocardial ischemia reperfusion injury (MIRI) induced acute lung injury (ALI) and analyze the underlying mechanism.

Methods: Male SD rats were subjected to MIRI with or without myocardial ischemic post-conditioning in the presence or absence of GSK-3β inhibitor. GSK-3β inhibitor was injected peritoneally 10min before MIRI. Lung W/D weight ratio, MPO, PMNs, histopathological changes, TUNEL, Bax, Bcl-2, IL-6, IL-8, IL-10, GSK-3β, and caspase-3 were evaluated in the lung tissues of all rats.

Results: After MIRI, lung injury was significantly increased manifested as significant morphological changes and increased leukocytes in the interstitial capillaries, Lung W/D ratio, MPO, and PMN in BALF, which was associated with enhanced inflammation evidenced by increased expressions of IL-6, IL-8 and reduced expression of IL-10. MIRI significantly increased cell apoptosis in the lung as increased levels of apoptotosis, Bax, cleaved caspase-3, and reduced expression of Bcl-2 was observed, which was concomitant with reduced p-GSK-3β. All these changes were reversed/prevented by ischemic post-conditioning, while these beneficial effects of ischemic post-conditioning were abolished by GSK-3β inhibition.

Conclusion: Myocardial ischemia reperfusion injury induces acute lung injury by induction of inflammation and cell apoptosis. Ischemic post-conditioning protects the lung from ALI following MIRI by increasing p-GSK-3β.

Key words: Myocardial Ischemia. Myocardial Reperfusion. Post-conditioning. Acute Lung Injury. Rats.
Introduccion

Acute lung injury (ALI) is usually caused by increased vascular permeability and inflammatory response. Myocardial ischemia reperfusion injury (MIRI) can prompt the accumulation of inflammatory mediators (e.g., IL-6, IL-8 and IL-10) and enhance cell apoptosis (e.g., Bax and Bcl-2)\textsuperscript{1,2}, which may lead to ALI, resulting in increased morbidity and mortality in patients with cardiac surgery\textsuperscript{3,4}. Myocardial ischemic post-conditioning alleviates MIRI by reducing inflammation and apoptosis\textsuperscript{5}, however, whether myocardial post-conditioning has beneficial effect on the lung remains unknown.

GSK-3β was first discovered in the process of sugar metabolism, which exists ubiquitously in the human body\textsuperscript{6}. In recent year, studies have shown that GSK-3β plays very important roles in embryonic development, cell differentiation, tumor formation, protein synthesis, and cell movement in general, specifically in cell survival\textsuperscript{7}. In cell survival, GSK-3β has a close relationship with oxidative stress, cell apoptosis and inflammatory reaction\textsuperscript{8}. Some researches found that in myocardial ischemia reperfusion injury, GSK-3β performed an important regulatory effect by signaling pathway, inflammatory reaction and oxidative stress\textsuperscript{9}. However, it is unclear whether GSK-3β can affect ALI induced by MIRI.

Present study aims to explore whether MIRI induced ALI by triggering inflammation and apoptosis, and whether myocardial ischemic post-conditioning can alleviate the ALI. At the same time, we explore whether GSK-3β plays a protective effect by alleviating the oxidative stress and inflammatory reaction in MIRI-induced ALI.

Methods

This study was approved by the ethics committee of the Renmin Hospital of Wuhan University, Wuhan, China. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals.

Male Sprague-Dawley (SD) rats weighing 250–300g were maintained on sterile, standard laboratory chow and water \textit{ad libitum} in individual ventilated cages under specific pathogen-free (SPF) conditions in the animal facility of the Experimental Research Centre of Wuhan University.

Experimental protocols

Fifty animals were randomly assigned into five equally numbered groups: sham-operated (S) group, myocardial ischemia/reperfusion (IR) group, myocardial ischemia/reperfusion with GSK-3β inhibitor (IRI) group, ischemic post-conditioning (IPost) group, and ischemic post-conditioning with GSK-3β inhibitor (IPostI) group.

Myocardial ischemia reperfusion model

Animals were anesthetized intraperitoneally with pentobarbital sodium (50mg/kg) followed by a tracheotomy and an artificial ventilation (TV: 6ml/kg, F: 80bpm, DW-2000, Jiapeng Keji, China). A fourth-intercostal space thoracotomy was performed, and the pericardium was excised to expose the heart. The left anterior descending coronary artery (LAD) was ligated 2mm above the left auricle by a 6-0 silk suture. A small polypropylene tube was placed between the ligature and the LAD. The artery was occluded for 30min by tightening the ligature. After 30min ischemia, the ligature was loosened to allow reperfusion for 2h. The sham group underwent the same surgical procedures, apart from tying the 6-0 silk suture. IPost was achieved by 3 cycles of 10s reperfusion followed by 10s ischemia immediately at the onset of reperfusion. GSK-3β

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inhibitor, 0.5% LiCl (Lithium chloride, 3mmol/kg), was injected intraperitoneally 10min before receiving MIRI in group IRI and group IPostI. Normal saline, with the same volume as the lithium was injected intraperitoneally in S group, IR group and IPost group.

The TTC staining for myocardial infarct size

To detect the success of myocardial ischemia reperfusion injury model, at the end of 2h of reperfusion, the rats were given heparin (1U/g, intraperitoneally). The coronary artery was reoccluded, and 1ml of 1.5% solution of Evans blue dye was injected via the left femoral vein to identify the ischemic risk area. 2ml of 10% potassium chloride solution was injected into the femoral vein to stop the heart, and then, the heart was excised. The presence of Evans blue dye indicated nonischemic area and its absence indicated area at risk (AAR). The heart was cut into five transverse slices from the apex to the base. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 25min, and they were then photographed with a digital camera (Cannon, Japan). The area lacking Evans blue staining (AAR) and the area lacking TTC staining (infarct area, IA) were determined using a light electron microscope (Olympus, Japan).

Histopathology of lung tissue

The anterior lobe of right lung was fixed with 10% formaldehyde solution for 48h, embedded in paraffin and cut into 4µm pieces by microtome, and stained with haematoxylin and eosin stain (H&E). All histopathological changes were detailed in each lung tissue, including intra-alveolar haemorrhage, disruption, capillary congestion, and leucocyte infiltration.

Histopathological evaluations was scored by a blinded and experienced laboratory pathologist using a five-point scale according to combined assessments of alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation: 0, minimum damage; 1+, mild damage; 2+, moderate damage; 3+, severe damage; 4+, maximum damage.

Lung wet-to-dry weight ratio (W/D)

The left lung was weighed and then dried in an oven at 60°C for 72h. Lung W/D weight ratio was assessed pulmonary oedema formation.

Myeloperoxidase (MPO) assay

1.0g of posterior lobe of right lung tissue was homogenised in 0.05M potassium phosphate buffer at pH5.5 and centrifuged at 3000rpm for 10min at 4°C. The pellet was redissolved in 10ml of 0.05M potassium phosphate buffer at pH 5.5 containing 0.5% hexadecyltrimethyl ammonium bromide. An aliquot of the supernatant was assayed by measuring the H2O2-dependent oxidation of tetramethyl benzidine in sodium phosphate buffer. Absorbance at 450nm of visible light was measured and the MPO activity was calculated in units per gram of lung tissue (U/g).

Polymorphonuclear neutrophils count in bronchoalveolar lavage fluid

Bronch-olveolar lavage fluid (BALF) was prepared by washing the lungs for three times with 4.0 ml phosphate-buffered saline (PBS). All three BALs was pooled and then centrifuged at 1000G for 10 min at 4°C. The supernatant of the BALs was used for polymorphonuclear neutrophils (PMNs) analysis.
**Immunohistochemical staining of Bax, Bcl-2, IL-6, IL-8, and IL-10**

Immunohistochemistry was performed to detect Bax, Bcl-2, IL-6, IL-8, and IL-10 expressions in the lung. Paraffin-embedded sections were dewaxed and rehydrated. After immersion in equilibration buffer 3 times, sections were incubated with 3% (v/v) H$_2$O$_2$ and 10% (v/v) methanol in PBS (pH 7.4) at room temperature in a humidified chamber for 10min to block endogenous peroxidase activity. Sections were then incubated in stop/wash buffer and subjected to antigen retrieval by boiling sections in 10mM sodium citrate buffer (pH 6.0) in a microwave for 20min. After cooling sections to room temperature, slides were washed with PBS (pH 6.5). After blocking in normal goat serum (1:10, Boster Biotech Inc. China) for 10min at room temperature, sections were incubated overnight at 4°C with rabbit anti-IL-6, IL-8, IL-10 (1:200, Boster Biotech Inc. China), Bax and Bcl-2 (1:100, ZSGB Biotech Inc. China) polyclonal antibodies respectively. After being washed with PBS, sections were incubated for 10min with biotinylated goat anti-rabbit IgG (1:10; Maixin Biotech Inc. China) and washed again with PBS, followed by incubation in a streptavidin-peroxidase complex (Maixin Biotech Inc.) for 10min at room temperature. Staining was visualized using diaminobenzidine (Maixin Biotech Inc.) as a coloring substrate. The number of positive cells was quantified under a BX51 microscope (×400) using six randomly selected fields of view from five sections (Olympus Inc, Japan).

**Western blot analysis of GSK-3β and caspase-3**

The expressions of phosphorylated GSK-3β (p-GSK-3β), total GSK-3β, caspase-3, and cleaved caspase-3 in lung tissue were determined by Western blot. After 2h reperfusion, lung tissues were sampled (100mg) and homogenized in 1ml lysis buffer. The homogenate was centrifuged at 12,000g at 4°C for 15min; equivalent amounts (50μg/lane) of total protein extracts were loaded into each lane and separated by 10% SDS gels, then transferred onto PVDF membrane. The membrane was blocked with 5% Bovine Serum Albumin (BSA) for 1h and then probed against the following primary rabbit monoclonal antibodies: p-GSK-3β (at Ser9), total GSK-3β, caspase-3 and cleaved caspase-3 (1:1000; Cell Signaling Technology, USA) diluted in 5% w/v BSA, and incubated overnight at 4°C, respectively. After washing with TBS-T buffer 3 times, the membrane was incubated with fluorescent tag Goat anti-Rabbit polyclonal IgG (1:10000, LI-COR, USA) for 1h at room temperature followed by additional washing.
GADPH was chosen as loading control to further assure the same volume for all the samples.

Statistical analysis

Data was presented as mean±SD and analyzed using SPSS17.0 software. The differences associated with main sources of variation were tested using one-way analysis of variance (ANOVA). When the F statistic was significant for ANOVA comparisons, the differences between individual means were tested for significance using Bonferroni tests. $P<0.05$ was considered statistically significant.

Results

Determination of the infarct size

The representative images of AAR and IA from each group were shown in Figure 1, which expressed as percentage of AAR/LV and IA/AAR. IR increased cardiac infarction compared to S group. The infarct size was lower in IPost group compared to IR and IRI groups ($P<0.01$). However, GSK-3β inhibitors significantly attenuated the protective action of post-conditioning against IR-induced cardiac damage in rats.

Figure 1 - The surgical procedure and determination of the infarct size. The representative images of AAR and IA from each group were shown, which expressed as percentage of AAR/LV and IA/AAR. IR increased cardiac infarction compared to S group. The infarct size was lower in IPost group compared to IR and IRI groups ($P<0.01$). However, GSK-3β inhibitors significantly attenuated the protective action of post-conditioning against IR-induced cardiac damage in rats ($^*P<0.01$, vs. S Group; $^+P<0.01$, vs. IR Group; $^-P<0.01$, vs. IPost Group).

MIRI-induced ALI was prevented by myocardial ischemic post-conditioning, but GSK-3β inhibition abolished the beneficial effect

After 2h reperfusion, the lung HE staining revealed normal lung parenchyma in the S group. intra-alveolar haemorrhage, alveolar structure disruption, capillary congestion and neutrophil infiltration in the interstitial were observed in IR and IRI groups. In contrast, neutrophil infiltration and interstitial edema in the lungs were significantly inhibited by myocardial ischemic post-conditioning in IPost group. In IPostI group, the protective effect of IPost was eliminated by GSK-3β inhibition (Figure 2).
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Figure 2 - Morphological changes of acute lung injury (ALI). The pulmonary histopathology represented by haematoxylin and eosin-stained sections (H&E, ×400). Microscopic observation showed inflammatory cells infiltration, intra-alveolar haemorrhage, high levels of intra-alveolar exudates and interstitial oedema in myocardial ischemia/reperfusion (IR) group, myocardial ischemia/reperfusion+GSK-3β inhibitor (IRI) group and ischemic post-conditioning+GSK-3β inhibitor (IPostI) group. The ischemic post-conditioning (IPost) group displayed a comparatively mild inflammatory cellular infiltration and intra-alveolar haemorrhage.

**Lung wet-to-dry weight ratio (W/D)**

Lungs exposed to IR and IRI group had significantly higher W/D compared with the S group. Post-conditioning significantly decrease the W/D ratio when compared with IR group. GSK-3β inhibitors administration significantly produced a marked increase in W/D compared with IPost group ($P<0.01$) (Table 1).

**Myeloperoxidase activity**

The activity of MPO in whole lung tissue was examined as a further marker of neutrophil activation. The MPO level in lung tissue was significantly higher in IR and IRI group. Post-conditioning can decrease the level of MPO, GSK-3β inhibitors treatment produced a marked increase in MPO activity ($P<0.01$) (Table 1).

**PMN Count in BALF**

PMN reflecting the inflammation status of the lungs were identified by using coulter counter. The leukocytes in the S group were mostly macrophages and a small quantity of neutrophils. All injury groups had significantly larger numbers of PMNs in BALF compared with S group ($P<0.01$). However, after the post-conditioning, PMNs in BALF was remarkably lower than in IR rats ($P<0.01$) (Table 1).

**Table 1 - Changes of Lung W/D, MPO, PMN in BALF (n=10, mean±SD).**

<table>
<thead>
<tr>
<th>Group</th>
<th>S</th>
<th>IR</th>
<th>IRI</th>
<th>IPost</th>
<th>IPostI</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/D</td>
<td>3.54±0.42</td>
<td>4.88±0.39*</td>
<td>4.90±0.33*</td>
<td>4.14±0.36*</td>
<td>4.87±0.41*</td>
</tr>
<tr>
<td>MPO(U/g)</td>
<td>0.73±0.09</td>
<td>1.33±0.16*</td>
<td>1.31±0.14*</td>
<td>0.98±0.11*</td>
<td>1.30±0.12*</td>
</tr>
<tr>
<td>PMN(%)</td>
<td>14.4±2.29</td>
<td>67.4±3.52*</td>
<td>66.8±2.96*</td>
<td>41.3±2.79*</td>
<td>67.1±3.03*</td>
</tr>
</tbody>
</table>

* $P<0.01$, vs. S Group; ** $P<0.01$, vs. IR Group; † $P<0.01$, vs. IPost Group.

**Expressions of Bax, Bcl-2, IL-6, IL-8, IL-10 in the lung**

Bax, IL-6 and IL-8 expressions were significantly increased while the expressions of Bcl-2, IL-10 were significantly decreased in the lung of IR group ($P<0.01$ vs. S group). The variation tendency in IRI group was same with IR group. All these changes were reversed by
post-conditioning ($P<0.01$ vs. IR group). In IPostI group, all 5 proteins’ expressions showed no significant difference in comparison to IR group (Figure 3).

**Figure 3** - Immunohisto-staining in the lung (×400). Bcl-2-associated X protein (Bax), Interleukin 6 (IL-6) and Interleukin 8 (IL-8) expressions were significantly increased while the expressions of B-cell lymphoma 2 (Bcl-2) and Interleukin 10 (IL-10) were significantly decreased in the lung of myocardial ischemia/reperfusion (IR) group and myocardial ischemia/reperfusion+GSK-3β inhibitor (IRI) group ($P<0.01$). In contrast, Post-conditioning reversed these changes in protein expressions following IR ($P<0.01$). In ischemic post-conditioning+GSK-3β inhibitor (IPostI) group, all 5 proteins’ expressions showed no significant difference in comparison to IR group ($P<0.01$, vs. S Group; $^*P<0.01$, vs. IR Group; $^+P<0.01$, vs. IPost Group).
Ischemic post-conditioning reduced cell apoptosis in the lung

TUNEL staining was used to identify cell apoptosis in the lung. The number of TUNEL positive cells in the lung tissue was higher in IR, IRI and IPostI groups than that of S and IPost groups ($P<0.01$), while there was no significant difference in the number of TUNEL positive cells between IR, IRI and IPostI groups (Figure 4).

Figure 4 - The pulmonary terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis (×400). TUNEL-positive cells expressed in lung tissue were higher in myocardial ischemia/reperfusion (IR), myocardial ischemia/reperfusion+GSK-3β inhibitor (IRI) group and ischemic post-conditioning+GSK-3β inhibitor (IPostI) group than other two groups ($P<0.01$) (*$P<0.01$, vs. S Group; †$P<0.01$, vs. IR Group; ‡$P<0.01$, vs. IPost Group).

Effect of ischemic post-conditioning on GSK-3β and Caspase-3

Pulmonary GSK-3β, p-GSK-3β, caspase-3 and cleaved-caspase-3 expressions were examined by western blot. As shown, there were no detectable differences in the expressions of total GSK-3β and caspase-3 in five groups. The p-GSK-3β level was markedly decreased in IR group, IRI group and IPostI group ($P<0.01$ vs. S group, IPost group). Post-conditioning increased the level of p-GSK-3β significantly compared to S, IR, IRI and IPostI groups respectively ($P<0.01$). The expression of cleaved-caspase-3 displayed an opposite trend to the expression of p-GSK-3β in five groups (Figure 5).
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Figure 5 - Effect of ischemic post-conditioning on GSK-3β and Caspase-3. There were no noticeable differences between the expressions of total GSK-3β and caspase-3 in four groups. The p-GSK-3β level was decreased in myocardial ischemia/reperfusion (IR), myocardial ischemia/reperfusion+ GSK-3β inhibitor (IRI) and ischemic post-conditioning+GSK-3β inhibitor (IPost) group when compared with other groups (P<0.01). Post-conditioning increased the level of p-GSK-3β significantly (P<0.01). The expression of cleaved-caspase-3 displayed an opposite trend to the expression of p-GSK-3β in groups IR, IRI, IPost and IPostI (*P<0.01, vs. S Group; #P<0.01, vs. IR Group; ‖P<0.01, vs. IPost Group).

Discussion

In the present study, we demonstrated that MIRI, by enhancing inflammation and cell apoptosis, led to ALI, which was attenuated by myocardial ischemic post-conditioning. We provided evidence that myocardial ischemic post-conditioning protected the lung from ALI by increasing p-GSK-3β.

ALI significantly affects postoperative recovery in patients with cardiac surgery. ALI is usually caused by a various of factors that increase vascular permeability and inflammatory responses. The migration and accumulation of inflammatory factors and apoptotic factors can produce large amount of oxygen free radicals and protease, resulting in pulmonary capillary endothelial cell and alveolar epithelial cell injuries and ultimately leads to cell apoptosis, causing ALI. Previous studies showed that many pro-inflammatory cytokines such as IL-8 and IL-6 contributed to the induction of ALI. In the present study, we provided additional evidences that MIRI, on one hand, increased cell apoptosis in the lung; on the other hand, increased pro-inflammatory cytokines release (IL-6 and IL-8) and reduced anti-inflammatory cytokine (IL-10) release, resulting in enhanced inflammation in the lung, and caused ALI.

Though ischemic pre- and post-conditioning both have the function of organic protection, however, post-conditioning is better than per-conditioning because of its operability.
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and practicability. In some cardiac surgery, the operators loosen aorta clamp slowly to imitate a similar process of post-conditioning. Post-conditioning can alleviate the myocardial IRI injury, here we demonstrated that it can also effectively attenuate the pulmonary inflammation responses and reduce the lung injury. This suggests that myocardial ischemic post-conditioning not only protects the heart against MIRI but also protects organs that are distant from the heart during MIRI. That is different from our previous study which showed that lung ischemic post-conditioning (applied at the lung) protected the lung from ischemia reperfusion injury as lung ischemic post-conditioning is a repetitive trauma to the underlying vessel which might worsen endothelial dysfunction and contribute to restenosis. Our current study may provides an alternative strategy to protect the lung from ischemia reperfusion injury when patients were doing the cardiac surgery without affecting the pulmonary underlying vessels.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that has recently emerged as a key regulatory factor in modulating inflammatory response. Activation of GSK-3β, an isoform of GSK-3, plays a pivotal role in the pathophysiology of organ injury/dysfunction following MIRI, possibly through regulating the opening of mitochondrial permeability transition pore (mPTP) channel. mPTP is a non-specific aperture on the mitochondrial inner membrane which allows entrance of active oxygen and free radicals when opening, resulting in organ damage. Inactivation of GSK-3β exacerbates ALI. While activation of GSK-3β prevents mPTP opening and attenuates MIRI in cardiomyocytes. In line with these results, in our current study, after MIRI, GSK-3β activation was significantly decreased in the lung that was associated with increased lung injury, suggesting that MIRI induced ALI by down-regulating p-GSK-3β. Interestingly, we further showed that myocardial ischemic post-conditioning increasing GSK-3β phosphorylation and attenuated MIRI-induced ALI, suggesting that GSK-3β may play a role in the protective effects of myocardial ischemic post-conditioning in MIRI-induced ALI.

Previous studies reported that ischemic pre- and post-conditioning confer myocardial protection by decreasing cell apoptosis, which in turn phosphorylates GSK-3β and increases the expression of anti-apoptosis proteins such as Bcl-2, blocking cell apoptosis. To further clarify whether apoptosis was involved in the protective effects of myocardial ischemic post-conditioning in MIRI-induced ALI, we examined expressions of anti-apoptotic protein Bcl-2, and pro-apoptotic proteins Bax and caspase-3. We showed that myocardial post-conditioning increased the level of Bcl-2 and reduced expressions of Bax and caspase-3 in the lung, which were initially inverted by MIRI. We further confirmed this results by showing that myocardial post-conditioning diminished TUNEL-positive cells, an index of cell apoptosis, in lung. In addition, activating GSK-3β has been found to prevent apoptosis by blocking activation of the caspase cascade, which can partially attribute to the observed down-regulation of pro-apoptotic proteins Bax and caspase-3, and the up-regulation of anti-apoptotic Bcl-2. As a result, our data provided evidence supporting the notion that myocardial ischemic post-conditioning protects the lung against MIRI-induced ALI by reducing apoptosis.

In our study, inhibition of GSK-3β by lithium, cancelled the protection of myocardial ischemic post-conditioning in MIRI-induced ALI. Previous literatures reported that GSK-3β exerted cardioprotective effect to an extent similar to that achieved with either pre- or post-conditioning in MIRI. It has been demonstrated that lithium can inhibit GSK activity both directly or indirectly by increasing inhibitory phosphorylation with unclear relativity between these modes of inhibition. However, recent literatures indicated that
lithium regulates GSK-3 not only directly but also through more complex network affecting multiple molecular targets at a time\textsuperscript{25}, which complicates its effect on GSK-3\textbeta{} even more on top of the bifunctional role of GSK-3\textbeta{} in cell apoptosis\textsuperscript{26}. On the other hand, in our study, lithium was given before MIRI, which cancelled the protective effects of myocardial ischemic post-conditioning. This together with the fact that inhibition of GSK-3\textbeta{} before MIRI can eliminate pre-conditioning protective effects\textsuperscript{27,28}, suggesting that the role of GSK-3\textbeta{} on ischemia reperfusion injury depends on the duration and the degree of its Binding site. However, the detailed mechanism remains to be defined.

\section*{Conclusion}

Myocardial ischemic post-conditioning protected MIRI-induced ALI by down-regulating inflammation and apoptosis in the lung through activating p-GSK-3\textbeta{}.

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