



Original article

Protective role of scutellarin on LPS induced – Acute lung injury and regulation of apoptosis, oxidative stress and reduction of mitochondrial dysfunction

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ABSTRACT

Lung fluid accumulation was determined using wet/dry lung mass ratio. Rats subjected to LPS-induced acute lung injury (2.8 ± 0.33 , $P < 0.05$) presented with a significantly higher wet to dry lung weight ratio than sham rats (1.6 ± 0.23 , $P < 0.05$). These results demonstrate that acutely injured rats' lungs were oedematous. On the other hand, treatment with scutellarin alone and in combination with a JNK inhibitor, SP600125, both significantly attenuated pulmonary edema as shown via reduced wet/dry lung mass ratios (1.7 ± 0.09 and 1.8 ± 0.23 ; $P < 0.05$, respectively). These results showed that the interventions were effective against LPS-induced edema of the lungs. However, the difference between treatment groups' weight ratios was not statistically significant ($P > 0.05$). In the sham control rats, the levels of ROS and SOD production were maintained at a low and at a high concentration, respectively ($P < 0.05$). However, following LPS infusion, the ROS levels skyrocketed while that of SOD decreased significantly relative to the control rats ($P < 0.05$). Furthermore, we noted that pre-treatment with scutellarin reduced the ROS levels in LPS-injured rats while the SOD was increased to near control levels ($P < 0.05$). Moreover, the combined effect of scutellarin and JNK inhibitor SP600125 on the levels of ROS and the SOD activity followed a similar trend to that of scutellarin alone albeit with a lower magnitude of change. Our results also showed that the combinatorial treatment was not significantly different from scutellarin alone in terms of influence on the levels of ROS production and SOD activity ($P > 0.05$). The effect of Scutellarin on broncho-alveolar lavage fluid (BALF) cytokine secretion The expression of interleukins-1 β , -18 and -6 in the broncho-alveolar lavage fluid were significantly upregulated by LPS infusion ($P < 0.05$). The rise was, however, attenuated via pre-treatment with scutellarin only or in conjunction with SP600125, a JNK inhibitor (all $P < 0.05$). On the contrary, we observed that LPS injection caused a reduction of interleukins -4 and -10 secreted in the BALF. Pre-treatment with scutellarin alone ($P < 0.05$) and not in combination with SP600125 or SP600125 was able to significantly reverse this noted down-regulation (all $P > 0.05$).

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1. Introduction

Acute lung injury is a constellation of vascular endothelium and alveolar epithelium barrier injuries that are associated with PaO₂/

FiO₂ values between 200 and 300 mmHg as well as significant morbidity and mortality (Ferguson et al., 2002; Rubenfeld et al., 2005). The disorder, primarily inflammatory, is associated with excessive immune cyto-activation, pro-inflammatory mediator secretion and disruption of membrane functions (Matthay and Zimmerman, 2005). Its clinical management involves mechanical ventilation as well as pharmacological strategies. To date, the most successful interventions have been mechanical ventilation of acute lung injury patients (The Acute Respiratory Distress Syndrome Network, 2000). Numerous clinical trials revealed that pharmacotherapeutic modalities hold promises but have largely failed to mitigate against morbidity and mortality in these patients. As the advances in the understanding of pathophysiological processes underlying lung injury come ashore, efficacy testing of several

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drugs has fallen by the wayside (Steinberg et al., 2006; Morris et al., 2008; Liu et al., 2008) thereby warranting continued search for pharmacological agents that would mitigate cellular damage, reverse acute lung injury, and increase survival among patients.

The region of interest in acute lung injury is the site of gaseous exchange comprising the surfactant layer sitting on the alveolar type 1 cells which are separated from the vascular endothelial cells by respective basement membranes and an interstitial space. Several stress, including viral infections (coronavirus, influenza, and adenovirus), idiopathic, severe sepsis, burns, smoke inhalation and drug poisoning will invariably cause cellular and fluid liberation within this region of interest. The infiltrating neutrophils and alveolar macrophages will invade the interstitium. The alveolar macrophages exudate includes cytokines such as interleukins-1, -6, -8, -10, and TNF- α , that are chemoattractant and stimulatory to neutrophils and fibroblasts (Matthay et al., 2002). In turn, neutrophils liberate copious amounts of oxidizing agents, peptidases, leukotrienes, and other inflammation promoting molecules. The mitochondria have featured as leading site of subcellular pathological processes that have a strong bearing towards cytoprotection or cytotoxicity (Yu et al., 2016; Lee et al., 2017). During acute lung injury, the mitochondria may lose the ability maintain normal calcium ion and reactive oxygen species (ROS) concentration leading to excessive mitochondrial free radical leakage, mitochondrial DNA damage and out-of-control ROS production (de Prost et al., 2011; Du et al., 2014). The overall outcome of these pathological processes would be unmitigated mitochondrial dysfunction, oxidative stress and apoptosis of the cellular components of the inflamed lung tissue.

Scutellarin is a natural bioactive flavonoid extracted from *Eriogon breviscapus*. It has been shown to have antiinflammatory and antioxidative activity, antiapoptotic, antihyperglycemic, neuroprotective, and anti-tumorigenic effects (Hong and Liu, 2004; Luo et al., 2008; Ma et al., 2008; Wang et al., 2011; Yang et al., 2017; Hu et al., 2018) in several efficacy studies. Therefore, scutellarin suggests appear to be a versatile molecule that has immense potential for clinical applications in treating disorders such as acute lung injury whole hallmarks include oxidative stress, inflammation, and mitochondrial dysfunction. Thus, we examined the effect of scutellarin on acute lung injury in an LPS-induced pulmonary injury model.

2. Materials and methods

2.1. Animal husbandry and experimental modeling

After getting approval to conduct the experiments from the, 42 two-month-old male Sprague-Dawley rats weighing between 185 and 220 g were bought from the. The rats were accorded unbridled access to standard rat chow and water. They were allowed to get accustomed to the new environment with a 12-hour light and darkness cycle for 7 days. Before the experimentation, these rats were randomly subdivided into six groups (n = 6 each) namely: (i) control (saline vehicle), (ii) LPS-group (6 mg/kg, dissolved in normal saline), (iii) LPS plus Scutellarin, (iv) LPS plus SP600125 (a specific inhibitor of JNK), (v) LPS plus Scutellarin plus SP600125, (vi) LPS plus normal saline. To induce acute lung injury, the rats were anesthetized by isoflurane inhalation and then a 24-gauge cannula inserted into the jugular vein for the administration of LPS. Scutellarin (dose) or SP600125 (15 mg/kg) was administrated per intraperitoneal an hour before LPS infusion. An equal volume of sterile normal saline acted as a control vehicle. The rats were allowed to survive and sacrificed 3 days after LPS application.

2.2. Extracting lung tissue

All lungs (left side) tissue was carefully removed following *trans*-cardiac perfusion with precooled normal saline, and then kept at -80°C awaiting quantitative real-time PCR (RT-qPCR), Western Blotting, measurement of myeloperoxidase, mitochondrial bioactivity, SOD and ROS generation.

2.3. Lung edema evaluation

Whole lungs (right) were rapidly dissected out and cleaned of extra-pulmonary connective tissue. The lungs' wet mass was obtained and then they were placed in an oven to dry at 65°C over 4 nights. They were weighed again to obtain dry mass. The wet to dry weight ratio was computed as an indicator of fluid accumulation inside lungs.

2.4. RT-qPCR

Extraction of RNA and DNA was carried out using the reagent Trizol (Life technologies, USA) according to accompanying instructions. The primer set used in this assay were constituted using Primer3 suite and are listed in Table 1. The expression of targeted genes was compared to 18S rRNA or GAPDH, as applicable, and $\Delta\Delta\text{Ct}$ log-fold-change method used for quantitation (n = 6). The general RT-qPCR procedures followed were in line with previously published protocols (Zhu et al., 2017).

2.5. Reactive oxygen species (ROS) and SOD evaluation

The frozen lungs were broken to tiny pieces and homogenized in lytic enzymes for 1 h at 37°C . The homogenate was then filtered, centrifuged and its supernatant eluted. A 2, 7-Dichloro-dihydro fluorescein-diacetate solution was mixed with the supernatant and put in incubator at 37°C for half an hour. A resulting solution then rinsed thrice with serum free medium, and then suspended in phosphate buffer saline. Commercial kits purchased from (Nanjing Jincheng Bioengineering Institute, China) were used to assess ROS production and SOD activity following recommended protocols (n = 6 mice/group).

2.6. Western blot

Lung samples were homogenized and protein obtained using Abcam's 10X RIPA lysis buffer (Abcam, UK). The concentration of protein in samples was determined using a BCATM protein assay kit (Pierce, USA) according protocol given by the manufacturer. 30 μg per lane total protein was electroseparated and electrotransferred onto polyvinylidene-difluoride membranes (BioRad, USA). 5% skimmed milk was used as a blocking buffer for 1 h at room temperature. The following primary antibodies were used: mitofusin-1 protein (Mfn 1), c-jun, phosphor-c-jun, cytochrome c (Cyt C) (All 1: 1000; Cell Signaling Tech, USA), cleaved caspase-3 (1: 2000; Santa Cruz Biotech, USA), IL-1 β , IL-6 (all 1: 1500; Cell Signaling Technology, USA), and GAPDH (1: 1500; Santa Cruz Biotech, USA) overnight at 4°C . The next day, the strips of membrane were incubated with an HRP conjugated secondary antibody (. For optical density quantitation, each immunoband intensity was standardised to GAPDH (n = 6 mice/group).

2.7. Myeloperoxidase (MPO) activity: Bronchoalveolar lavage fluid (BALF)

MPO activity was used as a sign of pulmonary invasion by macrophages as well as neutrophils (Loria et al., 2008). This was measured at 450 nm according to instructions contained in com-

Table 1
RT-qPCR primers.

Gene	Forward primers(5'-3')	Reverse primers(5'-3')
IL – 1 β	ATGAAAGACGGCACACCCAC	AAGGCAGAGTCTTCGGTGAG
IL – 6	CAACCAAGAGGTGAGTGCTTC	GGTGTCTCTTTCCACACTG
IL – 18	GGAAGACCAGAGACATCCACTG	ACACTAGACCAAAGGCTTG
IL – 4	CTGTAGGGCTTCCAAGGTGC	CTCTCATGTGCCAGGTCAC
IL – 10	GCCAGTTAGAAAGCCACCAC	GGTTCAGCTGTTTCCCAAC
Mfn1	TGGGGAGGTGCTGTCTCGGA	ACCAATCCCCTGGGGAGGA
ACTIN	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAAACAATGCCATGT

mercial kits bought at Wuhan Huamei Biotechnology CO., PR China.

2.8. BALF cytokines levels

Rat BALF concentration of cytokines, interleukins-1 β , –18, –6, –4, and –10 we determined using commercial ELISA kits as dictated in the manufacturer's manual. The reading were obtained using amount of light absorbed at 450 nm on an ELx800 Absorbance Microplate Reader (Life Sciences, USA).

2.9. Statistical analysis

The data are represented by Mean \pm Standard Error of the Mean. The statistical significance of differences between various experimental and control groupings were computed using one-way analysis of variance supplemented with Tukey's post-hoc testing where appropriate. For scenarios whereby data does not follow a normal distribution, the statistical significance of the difference between groups were checked with one-way ANOVA followed by Dunn's post-hoc method. All p-values < 0.05 were taken as statistically significant differences.

3. Results

3.1. Scutellarin attenuates fluid accumulating in lungs

Lung fluid accumulation was determined using wet/dry lung mass ratio. Rats subjected to LPS-induced acute lung injury (2.8 ± 0.33 , $P < 0.05$) presented with a significantly higher wet to dry lung weight ratio than sham rats (1.6 ± 0.23 , $P < 0.05$, Fig. 1). These results demonstrate that acutely injured rats' lungs were edematous. On the other hand, treatment with scutellarin alone and in combination with a JNK inhibitor, SP600125, both significantly attenuated pulmonary edema as shown via reduced wet/dry lung mass ratios (1.7 ± 0.09 and 1.8 ± 0.23 ; $P < 0.05$, respectively). These results showed that the interventions were effective against LPS-induced edema of the lungs. However, the difference between treatment groups' weight ratios was not statistically significant ($P > 0.05$).

3.2. Scutellarin protects against LPS-induced pulmonary edema.

Pulmonary edema was assessed using wet/dry mass ratios at 3 days after LPS induction. *denotes significance at $P < 0.05$. Data presented is Mean \pm SEM; $n = 6$.

3.3. The effect of scutellarin on MPO activity

The MPO activity is a known biomarker of neutrophil and macrophage pulmonary infiltration (Loria et al., 2008). As Fig. 2 shows, LPS infusion resulted in a robust rise in MPO activity when compared with that seen in saline infused rats ($P < 0.05$). In addition, the rise was significantly inhibited by scutellarin pre-

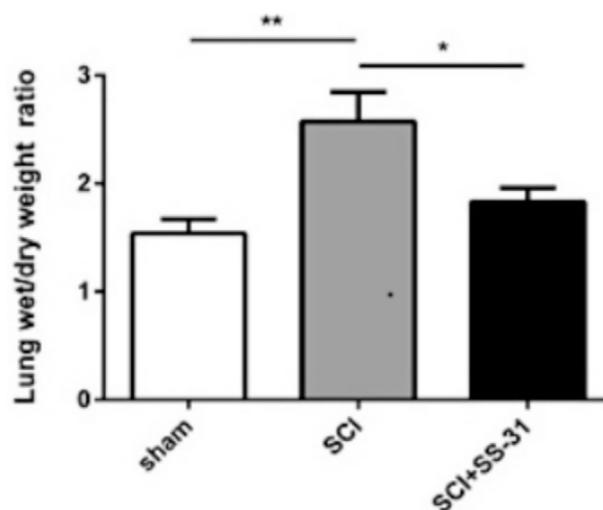


Fig. 1. Scutellarin attenuates fluid accumulating in lungs.

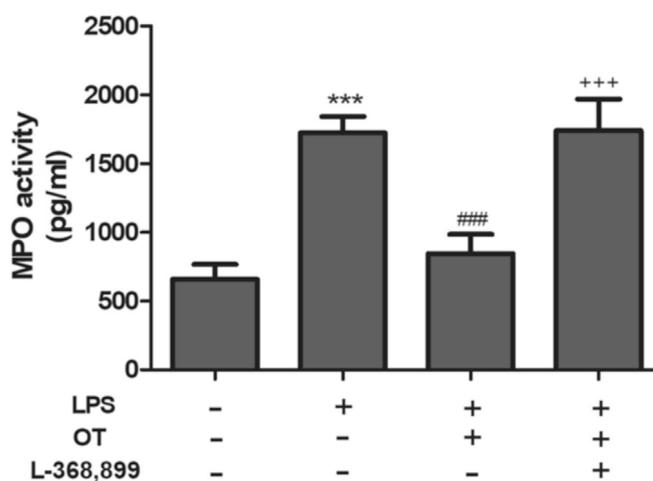


Fig. 2. Effect of Scutellarin on MPO activity.

treatment ($P < 0.05$). Furthermore, JNK inhibitor SP600125 given an hour prior to LPS insult restored MPO activity to near normal levels ($P < 0.05$). There was no evidence of synergism between scutellarin and SP600125 treatments as the difference between the scutellarin only and the scutellarin plus SP600125 was not statistically significant.

3.4. The effects of scutellarin on pulmonary MPO activity

The data is represented by Mean \pm standard error of the mean. The * denotes $P < 0.05$ for significant differences. LPS-induced acute lung injury vs normal saline sham control. LPS + Scutellarin vs LPS and LPS + scutellarin + SP600125. $n = 6$

3.5. Effect of scutellarin on ROS content and SOD activity

In the sham control rats, the levels of ROS and SOD production were maintained at a low and at a high concentration, respectively ($P < 0.05$, Table 2). However, following LPS infusion, the ROS levels skyrocketed while that of SOD decreased significantly relative to the control rats ($P < 0.05$; Table 2). Furthermore, we noted that pre-treatment with scutellarin reduced the ROS levels in LPS-injured rats while the SOD was increased to near control levels ($P < 0.05$, Table 1). Moreover, the combined effect of scutellarin

Table 2
ROS production and SOD activity following LPS-induced acute lung injury Oxidative stress measurements.

Parameters	Control	LPS	LPS + Scutellarin	LPS + Scutellrin + SP600125
ROS	109.11 ± 1.11	152.3 ± 4.11*	129.12 ± 2.95*	149.99 ± 4.21
SOD	60.77 ± 1.88	51.25 ± 1.44*	66.23 ± 2.01*	52.78 ± 1.85

ROS production and SOD activity following experimental acute lung injury caused by LPS plus treatment with SP600125 or scutellarin. LPS treatment robustly augmented the levels of ROS generation while decreasing SOD activity. However, after scutellarin significantly downregulate the levels of ROS and augmented SOD activity (*P < 0.05, 2)

and JNK inhibitor SP600125 on the levels of ROS and the SOD activity followed a similar trend to that of scutellarin alone albeit with a lower magnitude of change. Our results also showed that the combinatorial treatment was not significantly different from scutellarin alone in terms of influence on the levels of ROS production and SOD activity ($P > 0.05$, Table 1).

The effect of Scutellarin on broncho-alveolar lavage fluid (BALF) cytokine secretion

The expression of interleukins-1 β , -18 and -6 in the broncho-alveolar lavage fluid were significantly upregulated by LPS infusion ($P < 0.05$). The rise was, however, attenuated via pre-treatment with scutellarin only or in conjunction with SP600125, a JNK inhibitor (all $P < 0.05$). On the contrary, we observed that LPS injection caused a reduction of interleukins -4 and -10 secreted in the BALF. Pre-treatment with scutellarin alone ($P < 0.05$) and not in combination with SP600125 or SP600125 was able to significantly reverse this noted down-regulation (all $P > 0.05$). In addition, the trend of the levels of expression of these cytokines was also concordant with RT qPCR and Western blot assays shown in Fig. 3. Fig. 4.

The gene assay: IL-1 β (i), IL-18 (ii) IL-6 (iii), IL-4 (iv), and IL-10 (v). The confirmatory protein expression immunoblotting of selected IL-1 β ; IL-6 normalised to β -Actin levels (vi). The data represented by mean \pm standard error of the mean. The * denotes when $P < 0.05$, statistically significant; $n = 6$.

As depicted in Fig. 5, LPS insult significantly increased c-jun, c-jun phosphorylation and cleaved caspase 3 expression than observed in the control groups ($P < 0.05$). However, scutellarin pre-treatment significantly lowered the expression of these proteins relative to that observed in the LPS induction-only rats ($P < 0.05$). Furthermore, SP600125 pre-treatment alone or in combination with scutellarin also significantly reduced c-jun cleaved caspase 3 and phospho-c-jun protein overexpression relative to the controls. The results were corroborated by densitometry evaluation on the western blots.

3.6. The mechanism of scutellarin recovery in LPS-induced acute lung injured rats

The Western blot analysis revealed overexpression of pro apoptotic and JNK signaling pathways. Pre-treatment with scutellarin slowed JNK signaling and also reduced pro-apoptotic cleaved caspase 3 expression * denotes $P < 0.05$. The data depicts Mean \pm standard error of the mean for 6 rats per group.

3.7. Scutellarin restrains mitochondrial dysfunction

Our results show that LPS induced acute lung injury presents with significantly reduced mitochondrial DNA amount ($P < 0.05$), ATP synthases activity ($P < 0.05$), as well as heightened cytosolic liberation of Cyt C ($P < 0.05$) relative to controls. We also observed that pre-treatment with scutellarin restores mitochondrial DNA amount, ATP synthases function, as well as inhibits cytoplasmic Cyt C release at day three after LPS-induced lung injury. LPS infusion also reduced mitofusin-1 protein (Mfn1) expression which as then significantly reversed by scutellarin treatment.

3.8. Scutellarin attenuates mitochondrial dysfunction.

Fig. 6 A shows RT-qPCR assays of ATP-synthases and mt DNA genes at day 3 after LPS induced lung-injury. Fig. 6B represents cytochrome c and mitofusin-1 protein (Mfn1) expression western blots at day 3 after LPS-induced lung injury.* depicts $P < 0.05$. The data represents mean \pm standard error of the mean; $n = 6$.

4. Discussion

Clinically, acute lung injury is a serious condition accounting for the majority of acute respiratory failure among seriously ill patients worldwide. Its management remains largely supportive (The Acute Respiratory Distress Syndrome Network, 2000), creating an ongoing need for continued research. In the present study, we used an LPS injection lung injury model to demonstrate scutellarin's role in mitigating against oxidative stress, inflammation and apoptosis which collectively underpin severe adult respiratory distress seen in patients with acute lung injuries. In particular, our results indicated that the role of scutellarin is through interfering with the downstream JNK signalling, reduction of oxidative stress as well as improving antioxidant SOD activity and being antiapoptotic. Scutellarin also worked against LPS-induced decline in the number of the mitochondria. Furthermore, a JNK kinase inhibitor, also abrogated LPS-induced oxidative stress, inflammation and apoptosis in the injured lungs. Therefore, we posited that prevention of JNK dependent c-jun phospho-activation partially mediates the attenuation of acute injury to lungs *in vivo*.

Insult to the lungs kicks starts a series of pathological reactions, chief among them being the alveolar macrophage chemotaxis of neutrophils to the alveolar interstitium (Barnes, 2009; Grommes and Soehnlein, 2011). Once at the site of injury, neutrophils along with alveolar macrophages and fibroblasts secrete cell damaging oxidizing agents, peptidases, leukotrienes and cytokines that creates a positive feedback leading to setting in of local edema should inflammation fail to resolve quickly (Abraham, 2003). As observed in the present study, the biochemical markers of pulmonary infiltration by neutrophils and macrophages were all elevated in injured lung tissue (Loria et al., 2008). The injured lung wet to dry weight ratio was also skewed in favor of heightened pulmonary edema in the LPS-injured rats. These results created replicated a standard previously reported in human patients (Matthay et al., 1984) and animal model studies (Matsuyama et al., 2008). Interestingly, our present study showed that pretreatment with scutellarin reversed the severity of these biomarkers setting it as a good candidate for managing acute lung injuries as well as their clinical sequelae acute respiratory distress syndrome. Scutellarin appears to reduce neutrophil infiltration as shown by dwarfed myeloperoxidase activity, a feat that has been touted as a plausible therapeutic target in mice (Abraham et al., 2000) and being pursued in clinical trials (Eggers et al., 2016). Intriguingly, JNK inhibitor SP600125 also significantly attenuated these parameters, suggesting that the beneficial effects maybe mediated partially by blockage of c-jun phosphorylation and activation. JNK substrate, c-jun, is an immediate-early gene that is responsive to lung injury

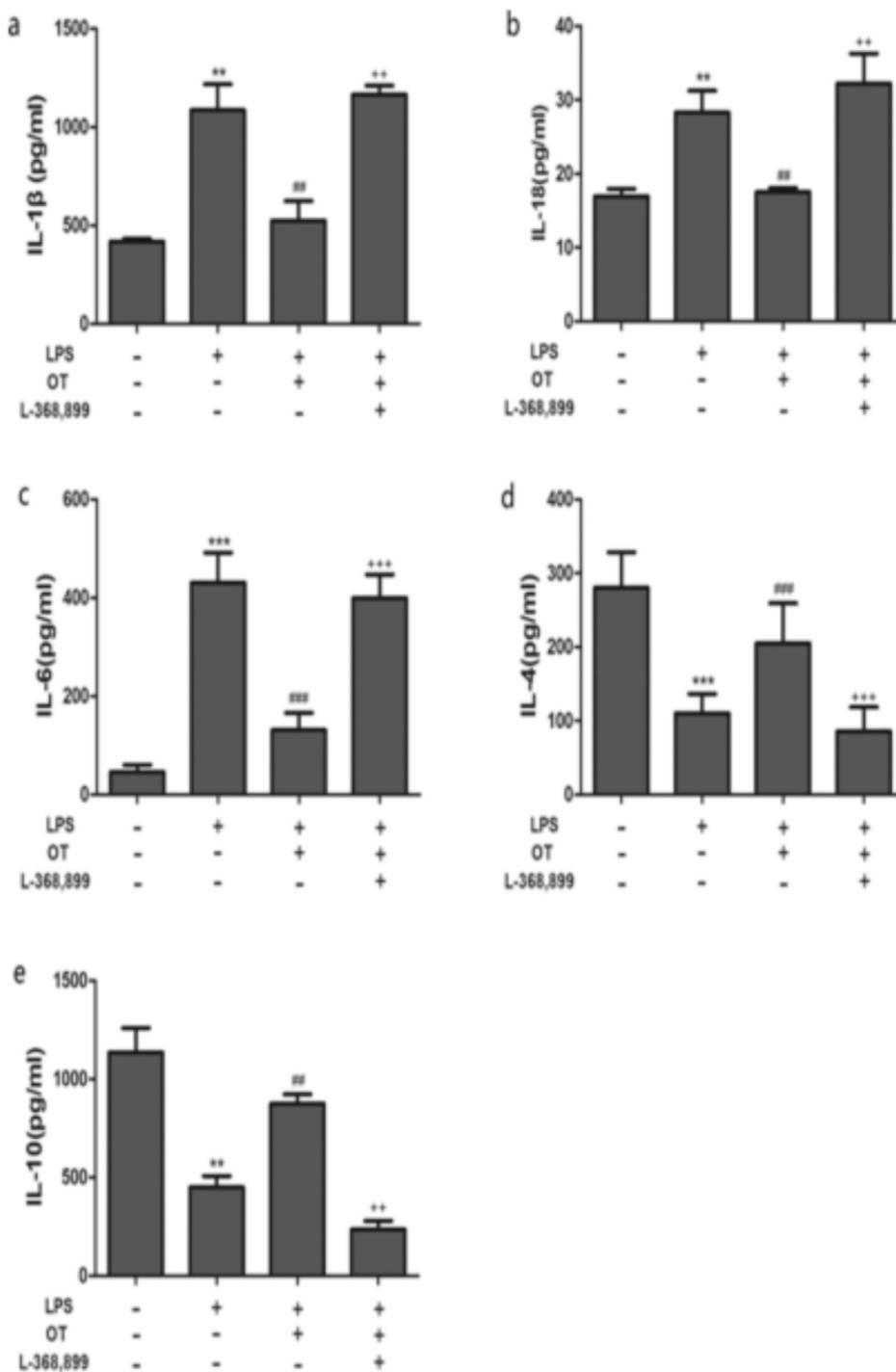


Fig. 3. The effects of scutellarin treatment on inflammatory cytokine expression in BALF of rats suffering from LPS-induced acute lung injury. The BALF expression of interleukins IL-1 β (i), IL-18 (ii), IL-6 (iii), IL-4 (iv), and IL-10 (v) determined by ELISA. Data is expressed as mean \pm standard error of the mean. The * denotes P < 0.05 as significant; n = 6.

and its phosphoactivation has been associated with pulmonary cytotoxicity, among other functions (Bennett, 2006).

Following acute lung injury, the recruited monocytes and macrophages along with neutrophils secrete copious amounts of cytokines such as interleukins-1 β , -6, and -18 which intercede the ensuing inflammatory response (Matthay et al., 2002). The expression of these pro-inflammatory interleukins in the broncho-alveolar lavage fluid were significantly upregulated by LPS infusion while anti-inflammatory IL-4 and IL-10 were down regulated in the current study. These trends are concordant with

the previous reports by An et al., (2019) and (Sawada et al., 2013) in mice and rats, respectively. IL-18, a known pro-inflammatory cytokine, recruits other inflammation mediating factors (Jordan et al., 2001) and was dysregulated in this study. We investigated the effects of scutellarin on and found that it reverses LPS-induced interleukin dysregulation. In addition, JNK inhibitor SP600125, showed a similar but weaker trend. These results suggest that scutellarin acts by promoting anti-inflammatory cytokine expression while suppressing pro-inflammatory ones. These dichotomous roles of scutellarin have been observed before in

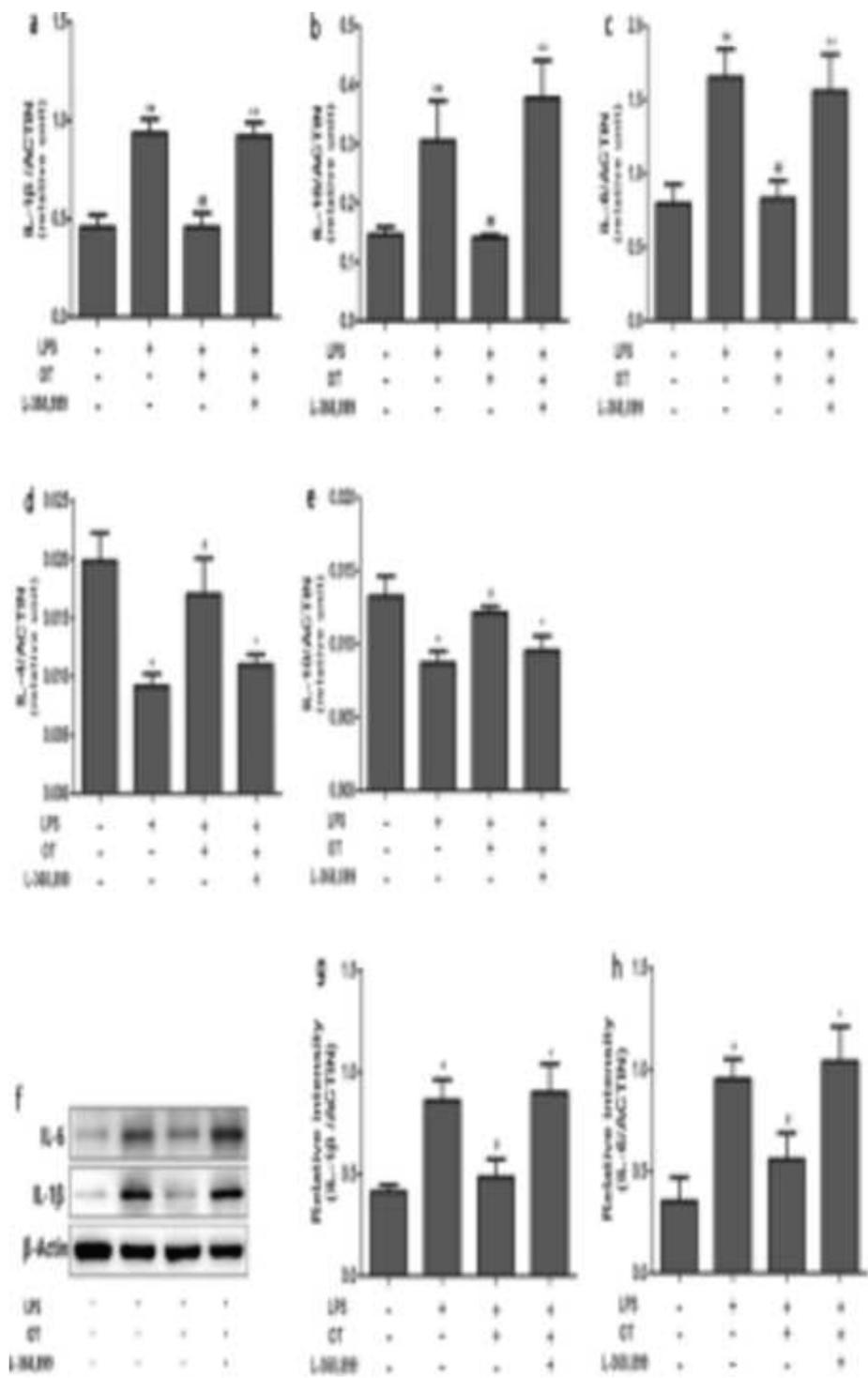


Fig. 4. The effect of scutellarin on the cytokine expression in the lungs of LPS-induced rats (RT-qPCR and Western blot).

stroke (Ma et al., 2008) and neurodegeneration (Hong and Liu, 2004), diabetes (Luo et al., 2008), nervous injury (Wang et al., 2011), and cancer (Yang et al., 2017).

The ultimate result of alveolar cellular infiltration and subsequent secretion of pro-inflammatory molecules in the alveolar interstitium offsets the endogenous scavenging capacity leading to rapid generation of reactive oxygen and nitrogen species. These oxidizing agents can destroy lipid, proteins and DNA leading to apoptosis. Manganese superoxide dismutase (Mn SOD is antioxidant and retrieves superoxide anions that would drive cytotoxic

processes in the injured lungs (Hart et al., 2015). As observed currently, LPS induced high ROS production as well as apoptosis while dwindling the endogenous SOD scavenging mechanism. These results are in line with prior observations in mice, rats and ARDS patients. Pretreatment with scutellarin reversed these LPS induced changes suggesting that its antioxidant functions are at play in injured lungs. The mitochondria is a metabolic center as well as integrator of cell death or survival via the mitochondrial apoptotic pathway. In this study, LPS injection dwindled mitochondrion numbers while pretreatment with scutellarin rescued mitochondrion

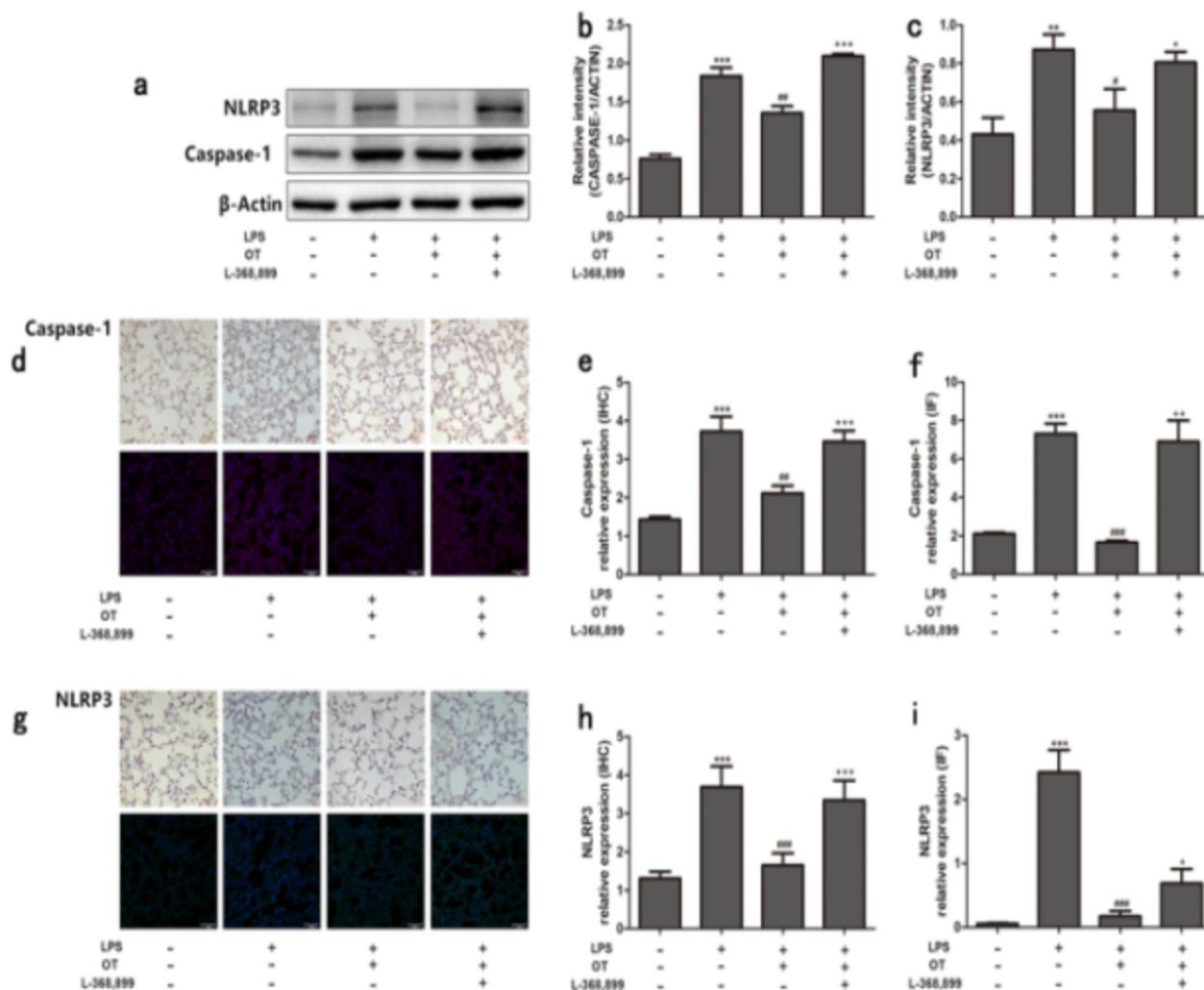


Fig. 5. The mechanism of Scutellarin recovery in LPS-induced acute lung injured rats.

drial loss. In addition, inhibition of cytochrome c liberation and mitofusin-1 protein overexpression where mediated by scutellarin

pretreatment. In response to injury, the mitochondria shifts the dynamic equilibrium between fission and fusion in order to preserve mitochondrial functions (Ni et al., 2015).

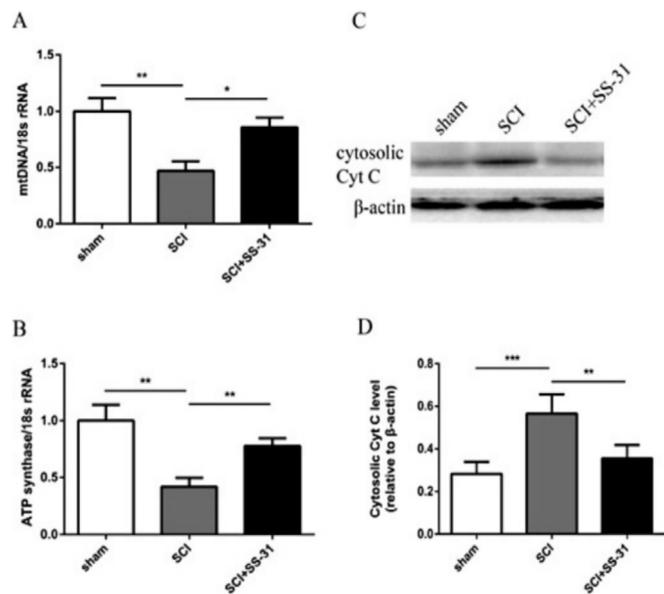


Fig. 6. Scutellarin attenuates mitochondrial dysfunction.

5. Conclusions

In conclusion, the current study has revealed that scutellarin alone and in combination with JNK inhibitor SP600125 is protective against acute lung injury induced by LPS by inhibiting inflammation, apoptotic as well as oxidative stress in a rat model. The mechanism appears to encompass inhibition of the JNK/c-jun/Phospho-c-jun/cleaved caspase 3 signaling pathway. These results sets scutellarin as a novel therapeutic strategy in the management of acute lung injuries.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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