# 行政院國家科學委員會專題研究計畫 期末報告

## 木犀草素對急性肺損傷保護作用與分子機制之研究(第 3 年)

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計 畫 主 持 人：關宇翔
共 同 主 持 人：林則彬、陳春榮、林則彬、陳春榮、林則彬
陳春榮
計畫參與人員：碩士級-專任助理人員：林附偉
碩士班研究生-兼任助理人員：陳楓宜
碩士班研究生-兼任助理人員：蔡欣辰

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中 華 民 國 102 年 10 月 21 日

中文摘要：急性肺損傷為常見且嚴重急症，當惡化後會形成急性呼吸窘迫症候群，伴有相當高的死亡率（40~60 %）。大部分病人並非死於呼吸衰竭，而死於因全身性發炎反應引發的多發性器官衰節。嗜中性球活化於細菌感染引起的急性肺損傷的病理過程中，扮演著極為重要的角色。因此若能增進對於細菌感染引起的急性肺損傷的機制認知，採用適當策略降低或抑制嗜中性球活化現象，對於疾病的預防治療應該可提供實質幫助。過去的研究結果發現嗜中性球浸潤、肺血管滲透性增加、肺水腫、肺壁透明膜形成等為細菌感染引起急性肺損傷重要的病理變化。相關研究更證實促發炎物質過度堆積、嗜中性球活化、肺血管內皮障壁喪失等傷害機制會惡化急性肺損傷。反之，則可以降低細菌所造成的急性肺損傷。不幸的，目前治療急性肺損傷只有呼吸器支持療法為主，仍無有效治療藥物可以運用。因此，若開發新的化合物以有效調控急性肺損傷的發炎現象及相關病理變化，將可有效的預防或治療急性肺損傷。金銀花（Lonicera japonica）於中醫藥已使用了數千年，具有許多的藥理活性抗發炎、抗血管新生、止痛等，近年來也被當做健康食品使用。金銀花製劑的單方或複方的萃取物目前已應用於癌症、呼吸道感染、糖尿病及風濕性關節炎，其中木犀草素（luteolin）為金銀花的活性成分之一。初步研究報告指出木犀草素會降低急性肺損傷，可能與抗氧化及抗發炎相關。經本研究計畫第一年的研究，主要在研究木犀草素的保護機制對於以氣管注射投予脂多醣引發急性肺損傷。我們發現木犀草素以濃度依存性的方式降低由脂多醣所引起的 (1)動脈血中增加的二氧化碳分壓與降低的氣氧分壓 (2)肺部病理切片的變化 (3) 肺泡沖出液中嗜中性白血球的侵潤與活化 (4) 肺組織中脂質的過氧化與 myeloperoxidase, catalase 與 superoxide dismutase 的活化 (5)釋放至肺泡中的 TNF-$\alpha$, keratinocyte-derived chemokine, 與 ICAM-1 (6) 於肺組織中活化的 mitogen-activated protein kinase (MAPK) 與 nuclear factor-岂B (NF 岂B) 路徑。總結來說，籍由本研究發現 luteolin 經由降低脂多醣所引發 Akt, MAPK 與 NF 岂B 的活化，而降低急性肺損傷的發炎現象。本研究計畫第二年的研究，主要在研究木犀草素的保護機制是否經由抑制巨噬細胞活化與降低內皮細胞滲透性。我們發現木犀草素以濃度依存性的方式降低由脂多醣所引起巨噬細胞 RAW 264.7 cells 活化，所增加的活性氧代謝物、一氧化氮與細胞素，如白三烯素。更進一步發現腫瘤壞死因子引起血管內皮細胞滲透度增加與白三烯素的釋放。總結來說，籍由本研究發現 luteolin 經由降低巨噬細胞活化與內皮細胞滲透度增加，而降低急性肺損傷的

發炎現象。本研究計畫第三年的研究，主要在研究木犀草素的保護機制是否經由抑制巨噬細胞活化與降低內皮細胞滲透性。我們發現木犀草素以濃度依存性的方式降低由 fMLP 或脂多醣所引起嗜中性球趨化或呼吸爆發作用，但並不影響 PMA 對嗜中性白血球活化的作用。而此種抑制作用的機轉主要是經由 MAPK/ERK kinase 1/2 (MEK), extracellular signal-regulated kinase (ERK), 與 Akt 磷酸化所致。綜合上述，木犀草素為一有效預防脂多醣所誘發的急性肺損傷疾病的保健食品物質。

中文關鍵詞： 急性肺損傷、木犀草素、嗜中性球、血管內皮細胞、黏附因子、細胞訊息傳遞

英文摘要： Acute lung injury (ALI) is a common disorder with significant impact on quality of life and enormous social cos. PMN play an important role and hallmarker in bacteria-induced ALI. Luteolin is recognized as a potential protective agent of ALI, its detailed protective mechanisms are understood. The study in first year was to investigate the protective mechanism of luteolin in IT of LPS-induced ALI in vivo. Luteolin suppressed the LPS-induced pressure of O2 decrement and pressure of CO2 increment in arterial blood； histopathological changes； PMN infiltration and activation； MPO, catalase and SOD activation； lipid peroxidation； secretion of TNF-$\alpha$, KC, and ICAM-1. Furthermore, luteolin reduced LPS-induced activation of MAPK and NFkB pathways. Collectively, luteolin effectively attenuates LPS-induced ALI via MAPK and NFkB pathways. This study in secondary year was to investigate the protective mechanism of luteolin, which is an anti-inflammatory polyphenolic compound, in intratracheal administration of LPS- induced RAW264.7 cells activation and TNF-$\alpha$ induced SVEC cells permeability. We found luteolin suppressed the LPS-induced expression of nitric oxide, intracellular reactive oxygen species, and IL1in RAW264.7 cells. Furthermore, we also demonstrated luteolin reduced TNF-$\alpha$ induced up-regulation of permeability and generation of IL1 in SVEC cells. In conclusion that luteolin effectively attenuates macrophage activation and increase endothelial barrier. Luteolin can be a

potential therapeutic anti-inflammatory compound in ALI therapy. In PMN studies in third year, luteolin attenuated the fMLP-induced PMN chemotaxis and respiratory burst, but had a negligible effect on superoxide anion generation during PMA stimulation. Furthermore luteolin effectively blocked MEK, ERK, and Akt phosphorylation in fMLP- and LPS-stimulated neutrophils.

英文關鍵詞： Acute lung injury, Luteolin, Polymorphonuclear leukocytes, Vascular Endothelial Cells, Adhesion Molecules, Cellular Signaling

# 行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

## 木犀草素對急性肺損傷保護作用與分子機制之研究

計畫類別：■個別型計畫　□整合型計畫
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計畫主持人：關宇翔
共同主持人：林則彬，陳春榮
計畫參與人員：林附偉，蔡欣辰，陳楓宜

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中　華　民　國 102 年 10 月 21 日

**中文摘要**

急性肺損傷為常見且嚴重急症，當惡化後會形成急性呼吸窘迫症候群，伴有相當高的死亡率 (40~60 %)。大部分病人並非死於呼吸衰竭，而死於因全身性發炎反應引發的多發性器官衰節。嗜中性球活化於細菌感染引起的急性肺損傷的病理過程中，扮演著極為重要的角色。因此若能增進對於細菌感染引起的急性肺損傷的機制認知，採用適當策略降低或抑制嗜中性球活化現象，對於疾病的預防治療應該可提供實質幫助。過去的研究結果發現嗜中性球浸潤、肺血管滲透性增加、肺水腫、肺壁透明膜形成等為細菌感染引起急性肺損傷重要的病理變化。相關研究更證實促發炎物質過度堆積、嗜中性球活化、肺血管內皮障壁喪失等傷害機制會惡化急性肺損傷。反之，則可以降低細菌所造成的急性肺損傷。不幸的，目前治療急性肺損傷只有呼吸器支持療法為主，仍無有效治療藥物可以運用。因此，若開發新的化合物以有效調控急性肺損傷的發炎現象及相關病理變化，將可有效的預防或治療急性肺損傷。金銀花 (*Lonicera japonica*) 於中醫藥已使用了數千年，具有許多的藥理活性抗發炎、抗血管新生、止痛等，近年來也被當做健康食品使用。金銀花製劑的單方或複方的萃取物目前已應用於癌症、呼吸道感染、糖尿病及風濕性關節炎，其中木犀草素 (luteolin) 為金銀花的活性成分之一。初步研究報告指出木犀草素會降低急性肺損傷，可能與抗氧化及抗發炎相關。經本研究計畫第一年的研究，主要在研究木犀草素的保護機制對於以氣管注射投予脂多醣引發急性肺損傷。我們發現木犀草素以濃度依存性的方式降低由脂多醣所引起的 (1)動脈血中增加的二氧化碳分壓與降低的氣氧分壓 (2)肺部病理切片的變化 (3) 肺泡沖出液中嗜中性白血球的侵潤與活化 (4) 肺組織中脂質的過氧化與 myeloperoxidase, catalase 與 superoxide dismutase 的活化 (5)釋放至肺泡中的 TNF-α, keratinocyte-derived chemokine, 與 ICAM-1 (6) 於肺組織中活化的 mitogen-activated protein kinase (MAPK) 與 nuclear factor-κB (NFκB) 路徑。總結來說，籍由本研究發現 luteolin 經由降低脂多醣所引發 Akt, MAPK 與 NFκB 的活化，而降低急性肺損傷的發炎現象。本研究計畫第二年的研究，主要在研究木犀草素的保護機制是否經由抑制巨噬細胞活化與降低內皮細胞滲透性。我們發現木犀草素以濃度依存性的方式降低由脂多醣所引起巨噬細胞 RAW 264.7 cells 活化，所增加的活性氧代謝物、一氧化氮與細胞素，如白三烯素。更進一步發現腫瘤壞死因子引起血管內皮細胞滲透度增加與白三烯素的釋放。總結來說，籍由本研究發現 luteolin 經由降低巨噬細胞活化與內皮細胞滲透度增加，而降低急性肺損傷的發炎現象。本研究計畫第三年的研究，主要在研究木犀草素的保護機制是否經由抑制巨噬細胞活化與降低內皮細胞滲透性。我們發現木犀草素以濃度依存性的方式降低由 fMLP 或脂多醣所引起嗜中性球趨化或呼吸爆發作用，但並不影響 PMA 對嗜中性白血球活化的作用。而此種抑制作用的機轉主要是經由 MAPK/ERK kinase 1/2 (MEK), extracellular signal-regulated kinase (ERK), 與 Akt 磷酸化所致。綜合上述，木犀草素為一有效預防脂多醣所誘發的急性肺損傷疾病的保健食品物質。

關鍵詞：急性肺損傷、木犀草素、嗜中性球、血管內皮細胞、黏附因子、細胞訊息傳遞

英文摘要

Acute lung injury (ALI) is a common clinical disorder with significant impact on quality of life and enormous social cost, with a mortality rate of approximately 40~60%. Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), are characterized by an acute inflammatory process in the airspaces and lung parenchyma. Respiratory failure is an uncommon cause of death in ALI patients, and a large proportion of patients with ALI died of sepsis-related multiorgan failure. Polymorphonuclear leukocytes (PMN) play an important role in the pathogenesis process of bacteria-induced ALI. It would be beneficial to the prevention and treatment of bacteria-induced ALI if the pathological mechanisms are clear. Accumulating evidence suggests that PMN infiltration, lung vascular permeability elevation, lung edema, and hyaline membrane formation are hallmarkers of bacteria-induced ALI. Among those pathological changes, proinflammatory mediators over accumulation, PMN activation, lung vascular endothelial barrier dysfunction amplified inflammatory changes leading to further tissue damage. On the contrary, reduction of these phenomenons protects against bacteria-induced ALI. Unfortunately, there is no effective therapeutic agent for the treatment of ALI. Therefore, to understand the regulatory mechanism of the inflammation and pathology phenomenon in ALI should provide more suitable targets to design and development of novel anti-ALI compounds. Honeysuckle flower, *Lonicera japonica*, has been used in Chinese's medicine for a long time and used as a healthy food in recent years. Honeysuckle flower is possessing several biological activities including antioxidant, anti-inflammation, analgesia, and anti-angiogenesis. Honeysuckle flower is currently used for the treatment of cancer, respiratory tract infection, diabetes, and rheumarthritis. Luteolin is an active component of honeysuckle flower. Studies show that luteolin is capable of reducing acute lung injury. The proposed protective mechanisms of luteolin include antioxidant and anti-inflammation. Although luteolin is recognized as a potential protective agent of ALI, its detailed protective mechanisms are poorly understood. This study in first year was to investigate the protective mechanism of luteolin, which is an anti-inflammatory polyphenolic compound, in intratracheal administration of lipopolysaccharide (LPS)-induced ALI in vivo. We found luteolin suppressed the LPS-induced pressure of oxygen decrement and partial pressure of carbon dioxide increment in arterial blood; histopathological changes of lung tissue; infiltration ($Ly6G^+/CD45^+$) and activation ($CD11b^+/CD45^+$) of neutrophils in bronchoalveolar lavage fluid (BALF); activation of myeloperoxidase, catalase and superoxide dismutase in, lipid peroxidation in lung tissue; secretion of TNF-α, keratinocyte-derived chemokine, and ICAM-1 in BALF in a concentration-dependent manner. Furthermore, luteolin reduced LPS-induced activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NFκB) pathways. Collectively, the present results support the conclusion that luteolin effectively attenuates LPS-induced ALI via MAPK and NFκB pathways. Luteolin can be a potential therapeutic anti-inflammatory compound in ALI therapy. This study in secondary year was to investigate the protective mechanism of luteolin, which is an anti-inflammatory polyphenolic compound, in intratracheal administration of lipopolysaccharide (LPS)- induced RAW264.7 cells activation and tumor necrosis factor-α (TNF-α) induced SVEC 4-10 cells permeability. We found luteolin suppressed the LPS-induced expression of nitric oxide, intracellular reactive oxygen species, and interleukin-1in RAW264.7 cells. Furthermore, we also demonstrated luteolin reduced TNF-α induced up-regulation of permeability and generation of interleukin-1 in SVEC 4-10 cells. Collectively, the present results support the conclusion that luteolin effectively attenuates macrophage activation and increase endothelial barrier. Luteolin can be a potential therapeutic anti-inflammatory compound in ALI therapy. In PMN studies in third year, luteolin attenuated the fMLP-induced neutrophil chemotaxis and respiratory burst (IC50 0.2±0.1 μmol/L and 2.2±0.8 μmol/L, respectively), but had a negligible effect on superoxide anion generation during phorbol myristate acetate stimulation. Furthermore

luteolin effectively blocked MAPK/ERK kinase 1/2 (MEK), extracellular signal-regulated kinase (ERK), and Akt phosphorylation in fMLP- and LPS-stimulated neutrophils.

Keywords: Acute lung injury, Luteolin, Polymorphonuclear leukocytes, Vascular Endothelial Cells, Adhesion Molecules, Cellular Signaling

## 1. Introduction

Lipopolysaccharide (LPS) as an endotoxin is the major outer wall membrane of the gram-negative bacteria and exists in high concentrations in tobacco and tobacco smoke (Hasdav et al., 1999). LPS is a potent trigger of septic shock and respiratory distress syndromes such as acute lung injury (ALI) and its most severe presentation, acute respiratory distress syndrome. However, ALI is strongly associated with mortality in critically ill patients and associated with a high morbidity of approximately 34 – 58%. No specific and effective treatment strategies for ALI exist (Rubenfeld et al., 2005).

The physiologic characterizations of ALI are that increase in alveolar-capillary permeability, plasma protein leakage, protein-rich hyaline membrane formation, leukocytes infiltration, pulmonary edema, haemorrhaging, (Bhatia and Moochhala, 2004; Zhou et al., 2005). Proinflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, nitric oxide (NO), and prostaglandin E2 (PGE2) plays a critical role in process of disease development in ALI (Bhatia and Moochhala, 2004). NO and PGE2 generated by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Expression of TNF-α, IL-6, iNOS, and COX-2 is regulated by transcription factor nuclear factor (NF)-κB activation (Tak and Firestein, 2001). In addition, protein kinase B (Akt/PKB) which is a serine/threonine kinase and a major downstream of phosphoinositide 3-kinases (PI3K), modulates NFκB activation in LPS-induced ALI (He et al., 2009).

Neutrophils are the large amount type in White Blood Cells and constitute the first line of host defence against microbial invasion. Through chemotaxis and phagocytosis, neutrophils migrated from the peripheral blood to the infection site and ingest invasion pathogen separately. Furthermore, pathogen was destructed by toxic reactive oxygen species (ROS) and proteolytic enzymes cause of respiratory burst and degranulation [5-7]. However, neutrophil excessive activation is also responsible for tissue impairment under inflammatory conditions. In serious pulmonary inflammatory disease, ROS and proteolytic enzymes induce the alveolar-capillary integrity disrupted. Let neutrophils migrate into the parenchyma and plasma protein leakage to the alveolar space [8]. It has been recognized that neutrophils play an critical role in LPS-induced acute lung injury [9, 10]. Mechanism of neutrophil activation is a complication and not completely identify. Subsequent reports have indicated that several kinases participate in neutrophil activation, including p38 mitogen-activated protein kinase (MAPK), extracellular regulated kinase (ERK) [11], Phosphoinositide 3-kinases (PI3K)-protein kinase B/ Akt [12]. The important point to notice is the compound could regulate neutrophil activation in order to ALI treatment efficiency.

Luteolin, a flavonoid, is widely distribution in vegetables, fruits, and herbs. In traditional Chinese medicine, plants rich in luteolin have been prescribed to treat hypertension, inflammatory diseases, and cancer. Luteolin reportedly has antiinflammatory, antioxidant, antiallergic, and antitumorigenic activities (López-Lázaro M, 2009). In macrophages, luteolin effectively inhibits LPS-induced pro-inflammatory cytokine expression and nitric oxide production (Harris et al., 2006). Moreover, luteolin significantly reduces PMN activation, including superoxide anion generation, leukotriene B4 secretion, migration, and chemotaxis (Lu et al., 2001; Kotanidou et al., 2002). Recent study showed that luteolin acts against PMN activation via the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) and PI3K/Akt pathways (Lee et al., 2010). The aim of this study is to determine how luteolin exerts anti-inflammatory and its mechanism of ALI after LPS administration in animal model.

## 2. Materials and methods

*2.1. Materials*

Mouse polyclonal antibodies to GAPDH, p38, ERK1/2, JNK, GAPDH, NFκB, Akt, iNOS, COX2, and IκB were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to phospho-p44/42 MAPK, phospho-p38 MAPK, phospho-Akt, and phospho-JNK were purchased from Cell Signaling Technology. Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, and calcein-AM were obtained from Invitrogen. Secondary antibodies were obtained from Jackson ImmunoResearch. Fluorescent-conjugated antibodies were obtained from BD Pharmingen. ELISA kits were obtained from R & D Systems. Catalase and superoxide dismutase activity assay kits were obtained from Cayman. Liu's stain kit was purchased from Tonyar Biotechnology (Taiwan). Malondialdehyde assay kit was purchased from ZeptoMetrix. FluoroBlok™ Insert System was obtained from BD Biosciences. Other chemicals were purchased from Sigma-Aldrich. The final volume of DMSO in the reaction mixture was <0.5%.

*2.2 Animals*

Male ICR mice (25 ± 3 g) were obtained from the Animal Department of BioLASCO (Taiwan). The mice were fed on a normal standard diet with tap water ad libitum and housed in a 12 h light/dark cycle in a temperature- and humidity-controlled animal facility.

*2.3 Murine model of LPS-induced lung inflammation*

Five groups of animals with 12 mice each were installed. To determine the pretreatment effects of luteolin on LPS-induced lung injury, the mice were injected intraperitoneally with different concentrations of luteolin (18, 35, and 70 μmol/kg) or vehicle for 30 min, followed by an intratracheal instillation of LPS (100 μg/50 μl) or saline. The control animals received vehicle followed by saline or LPS challenge. For the 3 experimental groups, animals received 18, 35 or 70 μmol/kg of luteolin followed by LPS challenge. After 6 hours, the mice were sacrificed by sodium pentobarbital (60 mg/kg, i.p.), and samples were collected.

*2.4. Blood gas in arterial blood analysis*

End of the experiment, blood gas levels were detected to verify the efficacy of intratracheal LPS instillation. Blood samples were collected from the abdominal aorta. Partial pressure of oxygen ($PaO_2$), carbon dioxide ($PaCO_2$), and acidity (pH) in arterial blood were measured immediately by Blood Gas Analyzer (Radiometer ABL 700 Blood Gas Analyzer, Copenhagen, Denmark.).

*2.5 Lung histopathological Studies*

Following euthanasia of the animals with pentobarbital, the lungs were removed immediately by thoracotomy, inflated-fixed with 4% paraformaldehyde, and the end of the trachea was tied off. Then the whole specimens were immersed overnight in 4% paraformaldehyde. To remove extrapulmonary structure, the lungs were cut into proper portions suitable for histological processing, embedded in paraffin, sectioned at 3 μm on microtome, and then stained with hematoxylin and eosin (H&E). Evaluation of lung injury was performed under the light microscope. The histopathology scoring values of lung injury range from 0 to 5 was accorded by Szarka et al. (1997), the histopathological changes at each value is as described below: (0) no reaction in the alveolar walls; (1) diffuse reaction in the alveolar walls, primarily neutrophilic, without thickening of alveolar walls; (2) diffuse presence of inflammatory cells (neutrophilic and mononuclear) in alveolar walls with slight thickening; (3) distinct (2-3 times) thickening of the alveolar walls due to presence of inflammatory cells; (4) the increasing thickness of the alveolar wall can add up to 25% more than the normal condition; and (5) the thickness of the alveolar wall is 50% more than the normal condition. Results were expressed as the means of the scoring values from 50 microscopic fields.

*2.6. Lung wet/dry weight ratio*

The ratios are representative of lung edema. The left lung was obtained and the wet weight was recorded. The lung was then baked in an incubator at 80 °C for 24 hours to determine dry weight. The wet/dry weight ratios were calculated by dividing the wet weight by the dry weight.

*2.7. Analysis of Leukocytes in Bronchoalveolar lavage fluid (BALF)*

Following euthanasia, trachea was exposed and intubated with a tracheal cannula. The lungs were lavaged repeatedly with 1 ml sterile saline for three times. The lavage fluids were pooled together, centrifuged at 500 *g* for 5 min at 4 ℃, and the supernatants were stored at -20 ℃ for cytokine, chemockine, adhesion molecule, nitrite, and protein assays. Cell pelletes were resuspended in 1.0 ml of PBS (pH 7.4). For population and activation of neutrophils, cells were counted by flowcytometry using rat monoclonal antibodies, which were phycoerythrin- conjugated anti-CD45 to stain for leukocytes; fluorescein isothiocyanate-conjugated anti-Ly-6G to stain for neutrophils; and fluorescein isothiocyanate-conjugated anti-CD11b to act as a marker for neutrophil activation. Cell samples were analyzed by a flowcytometry (FACSVantage; Becton Dickinson; Mountain View, CA) with software for data acquisition (CELL Quest). Data for 5,000 cells were acquired.

*2.8. Protein concentration in BALF*

Bronchoalveolar lavage was performed as previously described (Lee et al., 2010). After euthanasia, trachea was exposed and intubated with a tracheal cannula. BALF was performed by flushing the airways and lungs with 1 ml cold saline three times. The pooled BALF was collected on ice and then centrifuged at 500 *g* for 5 min at 4 ℃, and the supernatants were stored at -20 ℃ for further assay. Protein concentrations in the cell-free BALF were determined using Bio-Rad protein assay reagents. A standard curve was generated in the same fashion using bovine serum albumin.

*2.9. Cytokines analysis*

The levels of cytokines were measured on BALF using a commercially available ELISA kits (R & D Systems, Minneapolis, MN). These concentrations were interpolated from the standard curves for recombinant cytokines.

*2.10. Measurement of MPO*

The lungs from each group were isolated and homogenized in 0.1 M phosphate buffer (pH 7.0) contained 0.5% cetyltrimethylammonium bromide for MPO analysis. Briefly, the supernatant was added into a 1.5 ml phosphate buffer containing 0.2% cetyltrimethylammonium bromide in the presence of 10 mM guaiacol. Reaction was started by the addition of 0.01% $H_2O_2$. Arbitrary activity was expressed as the absorbance change was monitored at 470 nm for 1 min.

*2.11. Western blot analysis of Lung Tissue*

The lungs were harvested from post-treated mice. Whole tissue extracts were prepared from lung tissue by homogenization in tissue protein extraction solution (T-PER; Pierce, Rockford, IL) containing 1% proteinase inhibitor cocktail. After centrifugation, the protein concentration in the supernatant was determined by Bradford assay and 100 μg was loaded in each well, separated by SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried milk for 1 h at room temperature to reduce nonspecific binding, washed with PBS containing 0.1% Tween-20, and then probed with the indicated antibodies including IκB, GAPDH, phosphorylated and non-phosphorylated forms of ERK, JNK, and p38. After the membranes were washed again with PBST buffer, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents.

*2.12. NFκB activation*

The assay was measured in nuclear extracts from lung homogenates by a nuclear extraction kit (NE-PER

Nuclear and Cytoplasmic Extraction Reagents, Thermo Science) according to the manufacturer's instructions. The protein concentration was measured using a Bradford assay dye reagent (Bio-Rad). For NFκB activation, a NFκB p65 Transcription Factor Kit (Thermo Science) was used according to the manufacturer's instructions. 10 μg nuclear proteins were incubated with the consensus NFκB binding which element coated on a 96-well plate for 1h. After incubation with anti-p65 antibody, followed by a secondary antibody linked to horseradish peroxidase, plates were developed with a chemiluminescent substrate and read in a microplate reader.

*2.13. Cell Culture of RAW264.7 and SVEC 4-10*

The murine macrophage cell line RAW264.7 and endothelial cell lines SVEC4-10 (Bioresource Collection and Research Center, Hsinchu, Taiwan), was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) at 37℃ in 5 % $CO_2$-enriched air. The culture medium contained 10 % heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA) supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco Laboratories, Grand Island, NY, USA).

*2.14 Nitrite assay*

The nitrite concentration in the supernatant was measured as an indicator of NO production detected by Griess reaction. Briefly, $5 \times 10^4$ cells were seeded in 24-well plates for 24 h, treated with different concentrations of BisGMA or vehicle for additional 2 h. In addition, the cells were incubated with or without 3 μM BisGMA for the indicated periods. 50 μL culture media were mixed with an equal volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthyl-ethylenediamine dihydrochloride in distilled water; Sigma-Aldrich, St. Louis, MO, USA) and then shaken for 10 min at room temperature. The absorbance at 540 nm was determined with a microplate reader. The nitrite concentration was compared with a standard curve generated with known concentrations of sodium nitrite.

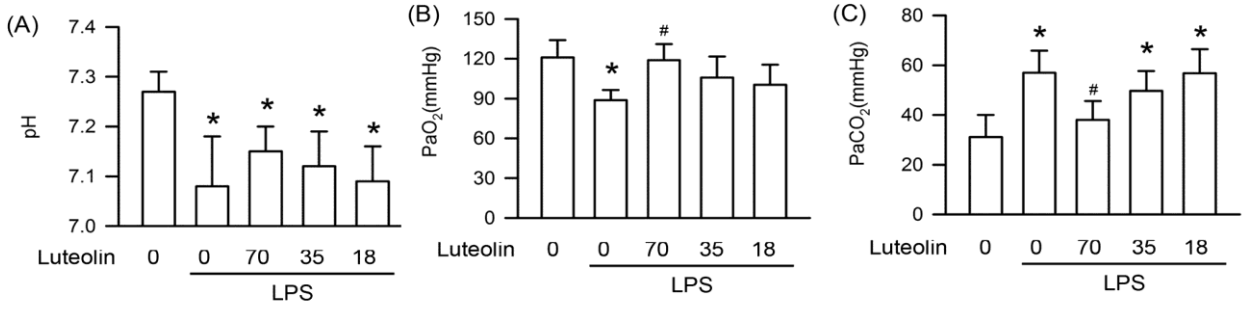
*2.15. Statistical analysis*

Statistical analyses were performed using analysis of variance followed by the Bonferroni *t* test for multigroup comparisons; $p<0.05$ was considered significant for all tests.

## 3. Results

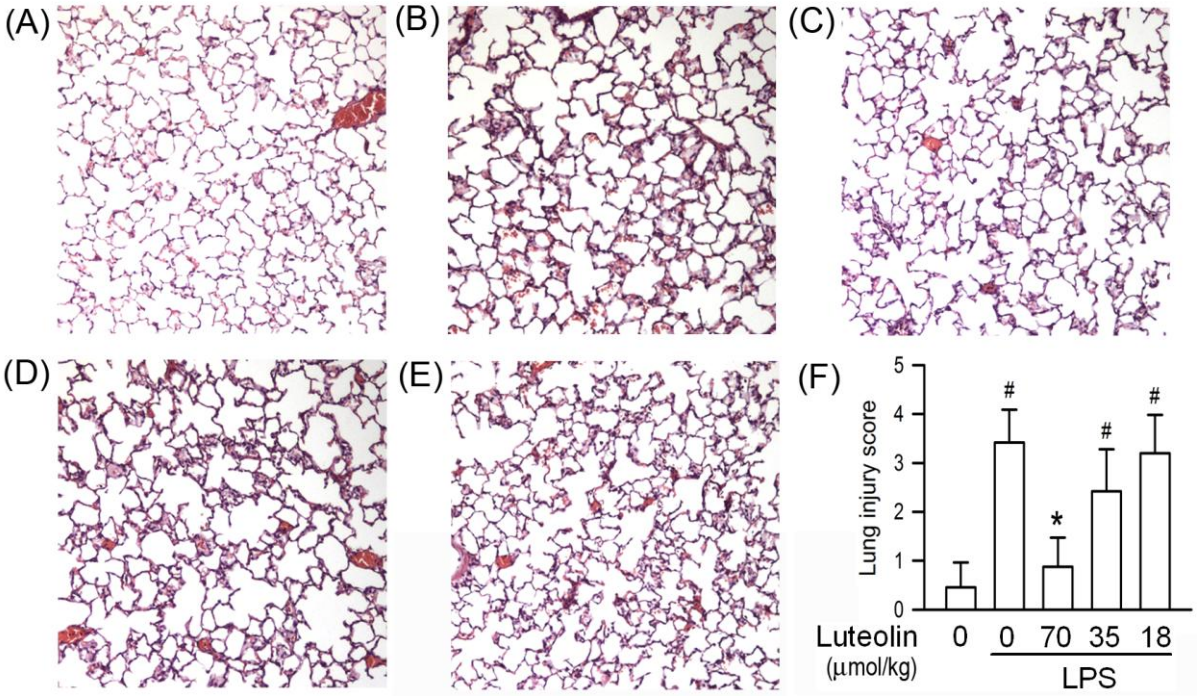
*3.1. Effects of Luteolin on blood gas in arterial blood in LPS-induced ALI*

To examine the effects of luteolin on LPS-induced pulmonary responses, the mice were treated with luteolin for 30 min before LPS administration. We found that LPS caused a significant decrease in the pH and $PaO_2$, and a notable increase in $PaCO_2$. Administration of 70 μmol/kg luteolin significantly reversed $PaO_2$ down-regulation and $PaCO_2$ augmentation. These results suggest that our ALI animal model was successful and luteolin can reduce the physiologic parameters of LPS-induced blood gas change.



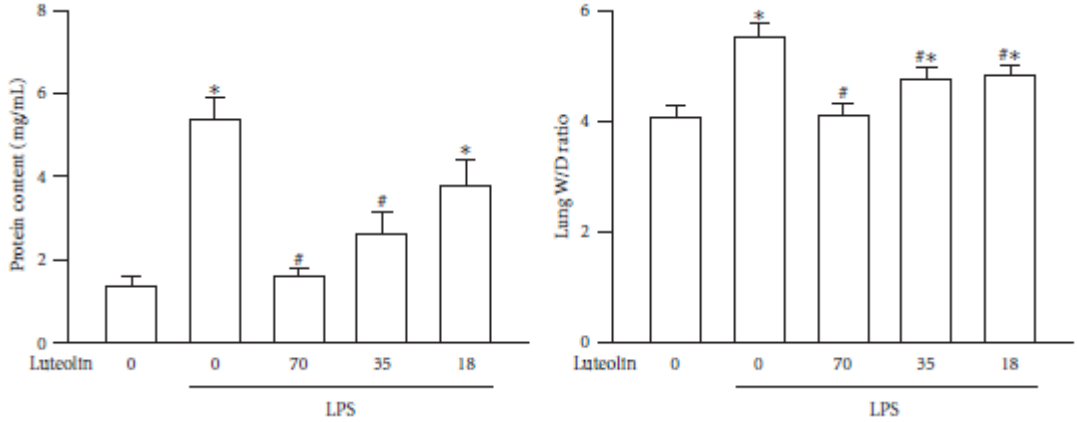
*3.2. Effects of luteolin on lung histopathology of LPS-induced ALI*

To correlate the inspected influence on physiologic parameters to morphology, we performed the histopathological examination. As expected, normal pulmonary structure and no histopathological changes was exhibited in the control group under the light microscope (A). When mice were instilled intratracheally with LPS, lung tissues were apparently impaired, the histopathological changes include haemorrhage, interstitial edema, thickening of the alveolar wall and infiltration of inflammatory cell into the lung parenchyma and alveolar spaces (B). The severities of the lung injury induced by LPS were attenuated by luteolin in a concentration-depended manner (C, D, and E). Furthermore, a similar inhibitory effect was obtained in the semi-quantitative analysis which allowed the measurement of the severity and the extent of inflammatory change was expressed in F. The results suggest that luteolin improved the histopathological conditions of lung in LPS-induced ALI.



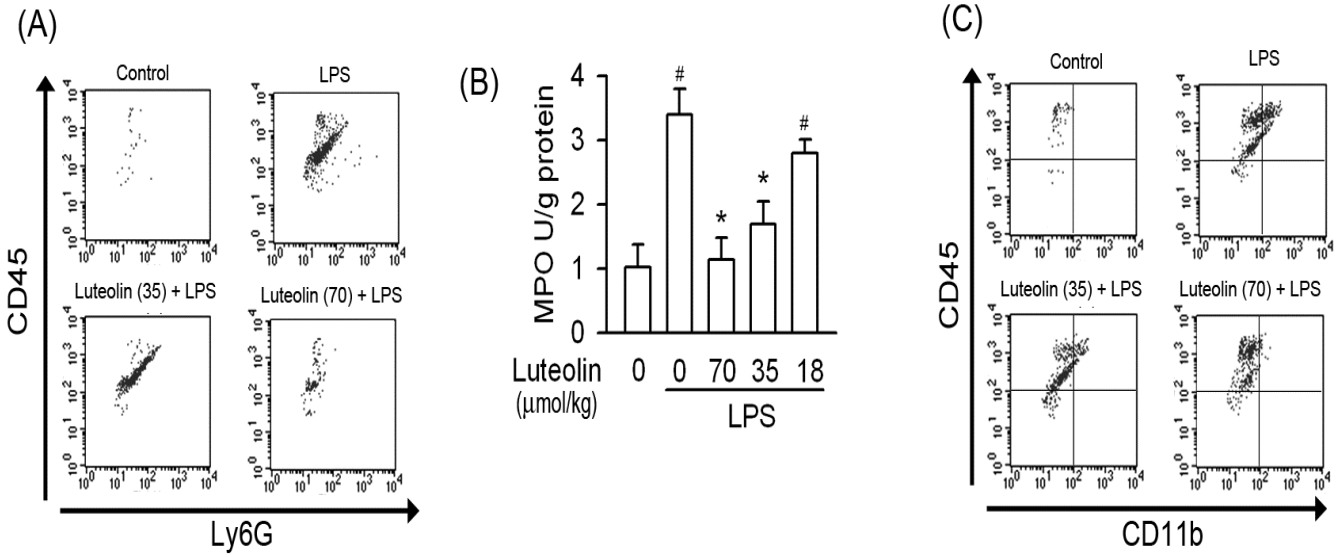

*3.3. Effects of Luteolin on LPS-Induced Vascular Permeability and Edema in Lung.*

The critical feature of LPS-induced ALI is the destruction of vascular integrity, and the subsequent upregulated permeability will result in protein leakage and pulmonary edema. Treatment with LPS alone was found to significantly increase protein leakage in BALF ($P < 0.05$) and edema in pulmonary parenchyma ($P < 0.05$) in comparison with those of the sham operation group (Figure 2), while with pretreatment of luteolin at 35 and 70 $\mu$ mol/kg significantly reduced protein leakage and pulmonary edema ($P < 0.05$). These results indicated luteolin prevented LPSinduced pulmonary permeability and edema.



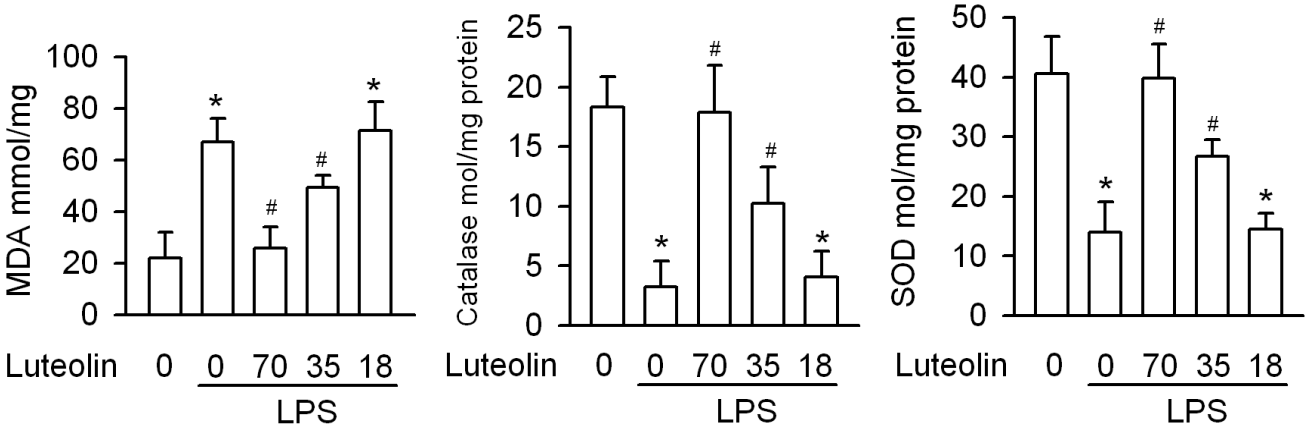
*3.4. Effects of luteolin on the infiltration and activation of PMNs in LPS-induced ALI*

Infiltration of PMNs into BALF in LPS-induced ALI was assessed using dual expressions of CD45, a common leukocyte antigen, and Ly6G, a specific surface marker of neutrophils. Infiltration of PMNs ($CD45^+/Ly6G^+$) into the lung in LPS-induced ALI increased significantly compared to the vehicle administration. The response was attenuated by luteolin in a concentration-depended manner (A). We next investigated the ability of luteolin to affect MPO activity, which plays as an index of neutrophil sequestration. ELISA analysis showed that MPO activity was markedly up-regulated by the LPS challenge in the lung tissue. Pretreatment of animals with luteolin prevented the up-regulation of MPO activity (B). Furthermore, activation of PMNs was assessed using dual expressions of CD45 and CD11b, which are granular membrane constituents of neutrophils and their expression were increased when activated. Control animals expressed little CD11b on the cellular membrane in BALF, whereas LPS administration promoted a significantly increased expression. Luteolin inhibited the LPS-stimulated expression of CD11b in a concentration-depended manner (C).



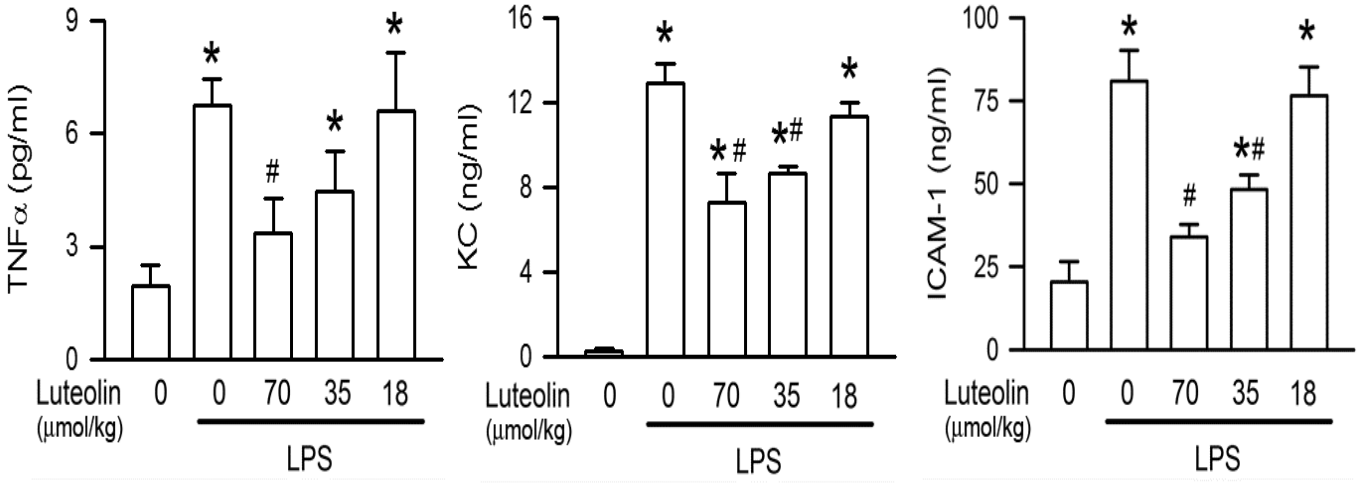

*3.5. Effects of luteolin on MDA formation, and catalase and SOD activity in LPS-induced ALI*

Oxidative damage resulted in the lipid peroxidation of membrane phospholipids and culminated in the MDA formation, and the inactivation of antioxidant enzymes, which include catalase and SOD. These results suggest that luteolin reduced the oxidative stress in LPS-induced ALI.



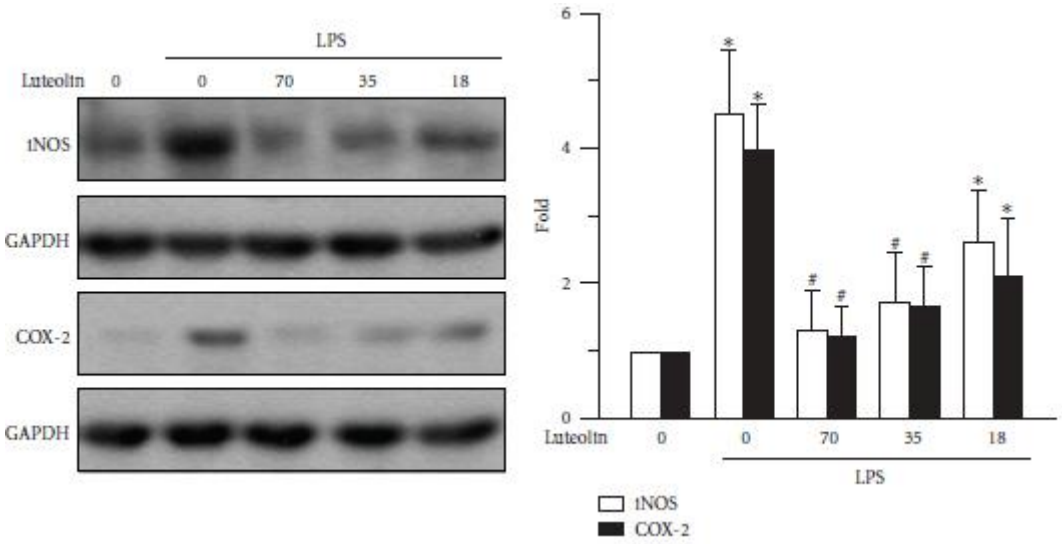
*3.6. Effects of luteolin on TNF-α, KC, and ICAM-1 production in LPS-induced ALI*

A treatment with LPS led to the increase level of TNF-α and KC in BALF. The response was attenuated by luteolin in a concentration-depended manner. These results show that luteolin reduce the chemokine and cytokine expression for treated ALI.



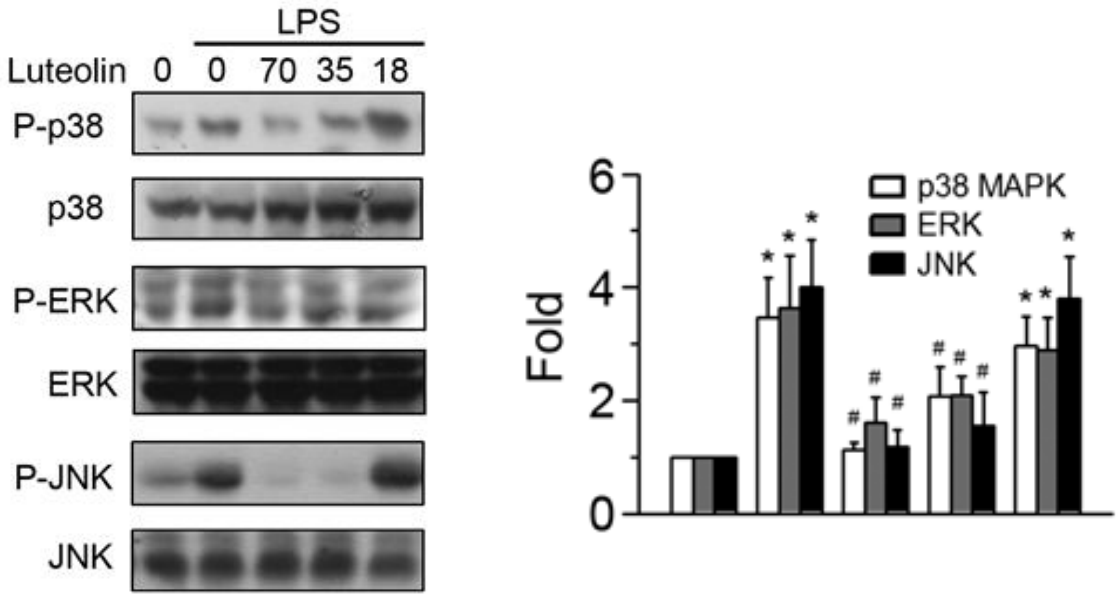

*3.7. Effects of Luteolin on LPS-Induced iNOS and COX-2 Expressions.*

iNOS and COX-2 play critical roles in the pathology of LPS-induced ALI. The effect of luteolin on iNOS and COX-2 expressions in lung tissue was analyzed using a Western blot assay. The LPS significantly increased iNOS and COX-2 expressions compared with those of the control group ($P < 0.05$). Pretreatment with luteolin reduced LPS-induced expressions of iNOS and COX-2 in a concentration-dependent manner, both 35 and 70 μmol/kg significantly attenuated the expression of the two proteins ($P < 0.05$).



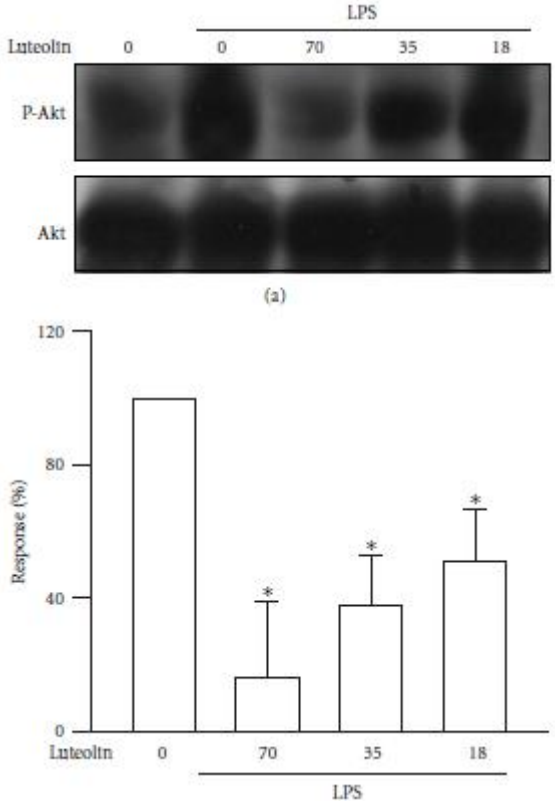

*3.8. Effects of luteolin on activation of MAPK in LPS-induced ALI*

To examine the effect of luteolin on MAPK, the phosphorylation of MAPK in LPS-induced ALI was analysed by western blotting. Luteolin suppressed the LPS-induced phosphorylation of MAPK pathways in a concentration-depended manner. These results suggest that luteolin relieves LPS-induced ALI via inhibition of MAPK pathways activity.



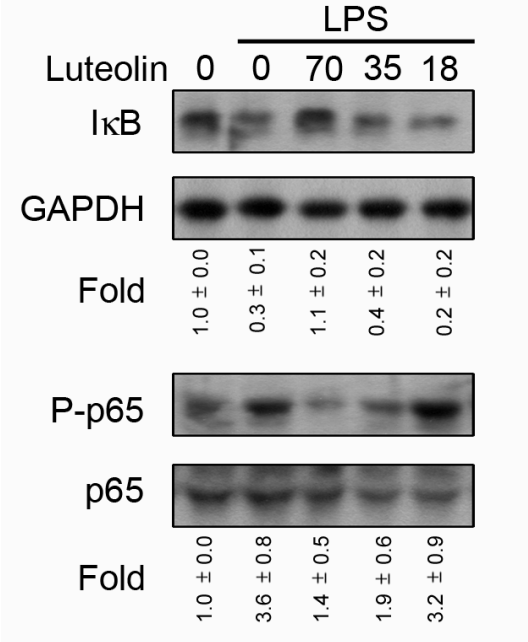

*3.9. Effects of Luteolin on LPS-Induced Akt Activation.*

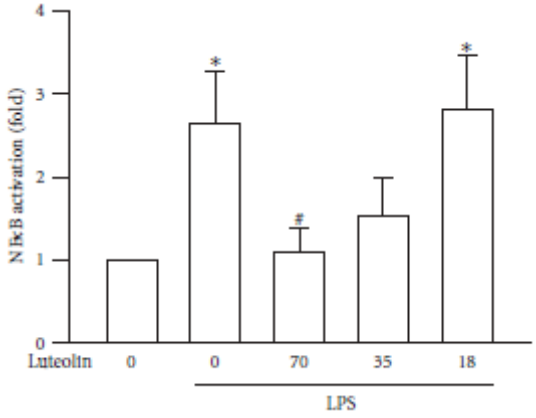
As an upstream factor in NF$\kappa$B activation, Akt participates in LPS-induced ALI. Phosphorylation on Akt S473 represents its maximal activation. Therefore, the effect of luteolin on Akt activation in lung tissue was assessed by phosphorylation of Akt at the site of S473 via western blot analysis. Administration of LPS on mice significantly increased Akt phosphorylation compared with that of the control group ($P < 0.05$), and luteolin reduced LPS-induced Akt phosphorylation in a concentration-dependent manner with an IC50 value of 30.5 $\pm$ 17.8 $\mu$mol/kg. These results suggested that luteolin reduced the severity of LPS induced ALI via inhibition of the PI3 K/Akt pathway.



*3.10. Effects of luteolin on NFκB activation and IκB degradation in LPS-induced ALI*

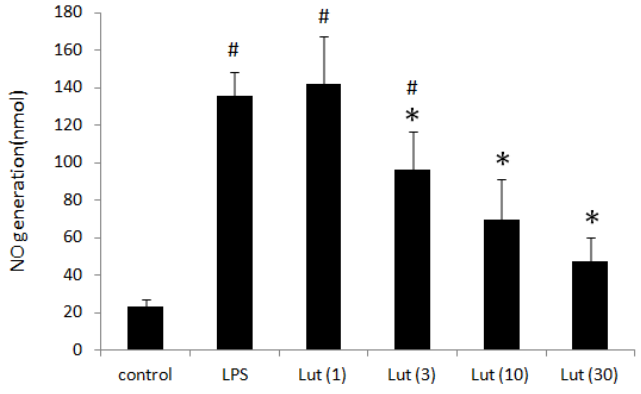
Evidences show LPS induced NFκB activation and tyrosine phosphorylation through IκB degradation in lung tissue are associated with inflammatory responses. After LPS administration, the tyrosine phosphorylation of NFκB p65 markedly increased. The tyrosine phosphorylation of NFκB p65 was significantly suppressed by luteolin pretreatment in a concentration-depended manner. In parallel with tyrosine phosphorylation of NFκB p65, the effect of luteolin was also investigated in IκB degradation. Like tyrosine phosphorylation of NFκB p65, LPS administration induced elevated degradation of IκB. The increase degradation was significantly attenuated by luteolin.

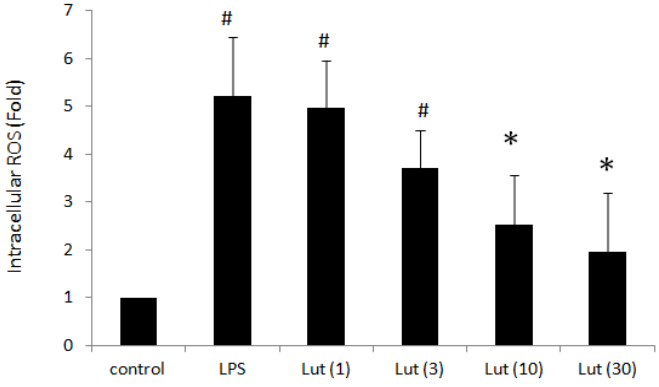



*3.11. Effects of luteolin on generation of NO and ROS in LPS-stimulated RAW264.7 cells*

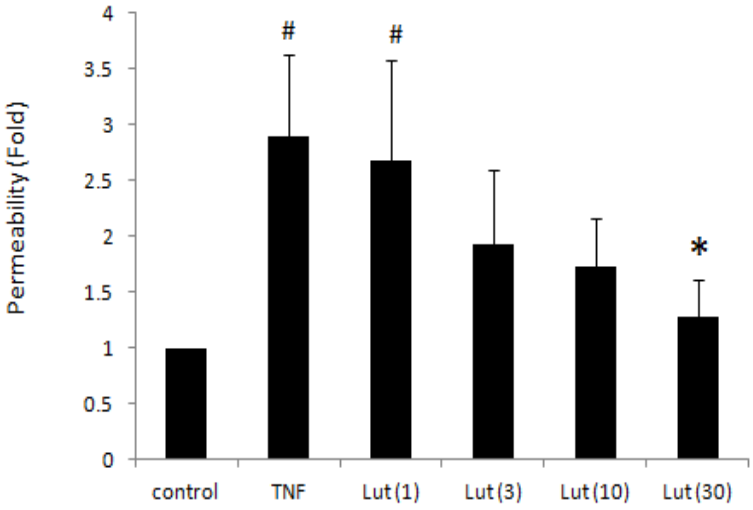
After LPS administration, the generation of NO and ROS markedly increased. The ROS and NO generation was significantly suppressed by luteolin pretreatment in a concentration-depended manner.



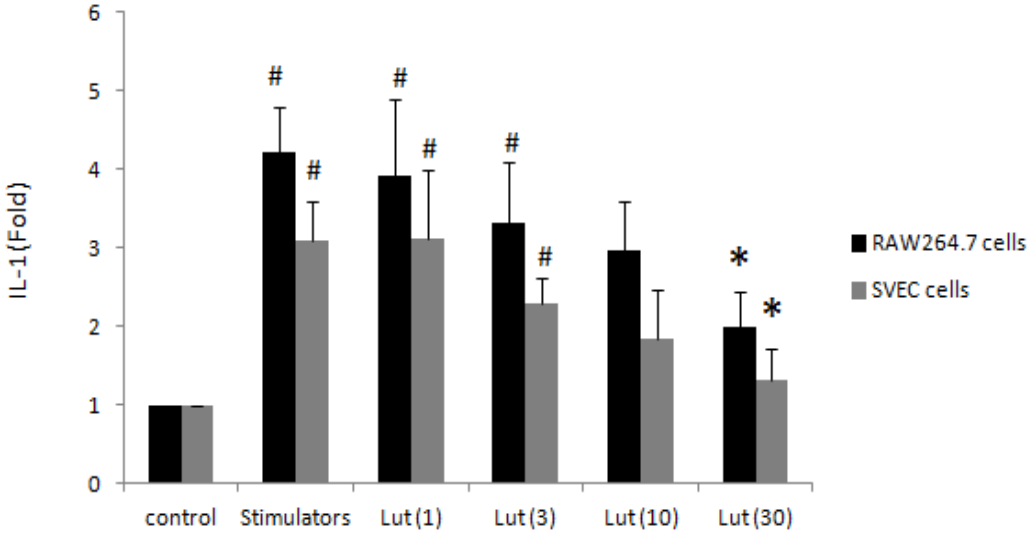

*3.12. Effects of luteolin on permeability in TNF-α-stimulated SVEC 4-10 cells*

After TNF-α administration, the permeability of SVEC 4-10 cells markedly increased. The permeability was significantly suppressed by luteolin pretreatment in a concentration-depended manner.



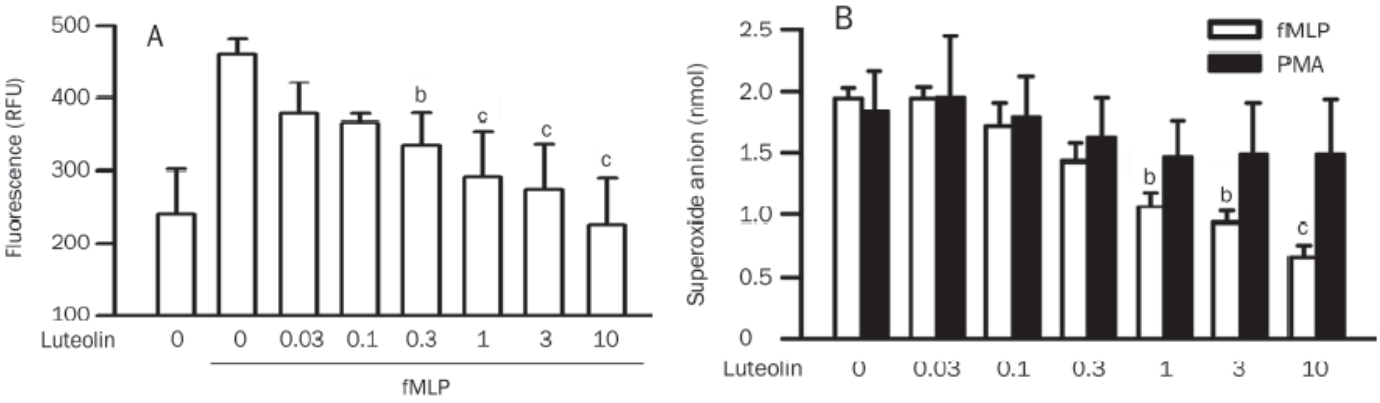

*3.13. Effects of luteolin on IL-1 generation in TNF-α-stimulated SVEC 4-10 cells and LPS-stimulated RAW264.7 cells*

After TNF-α administration, the IL-1 of SVEC 4-10 cells markedly increased. After LPS administration, the IL-1 of RAW264.7 cells markedly increased. The IL-1 was significantly suppressed by luteolin pretreatment in a concentration-depended manner.



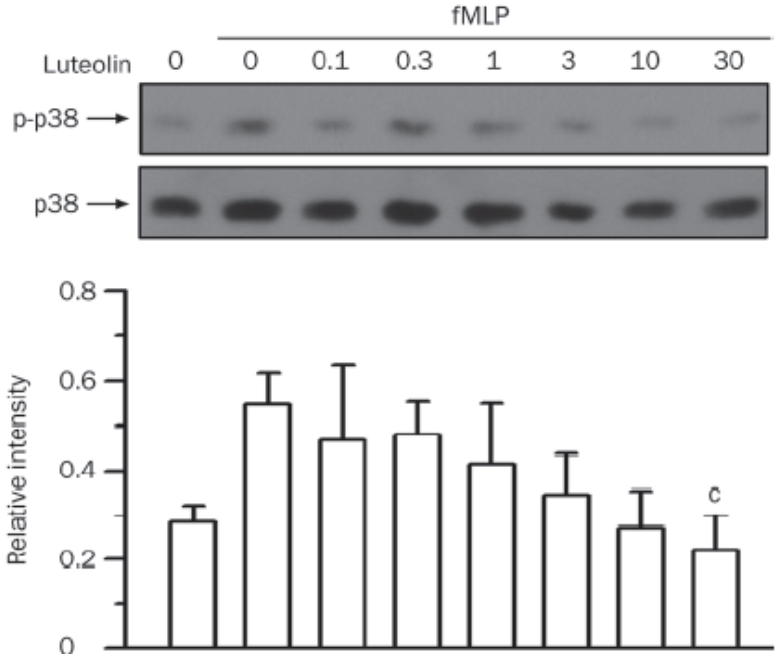

*3.14 Effects of luteolin on neutrophil chemotaxis and the respiratory burst*

To obtain further insight into the protective effects of luteolin against ALI, a mechanistic study was conducted using a cultured neutrophil model. fMLP caused an increase in neutrophil chemotaxis in vitro ($P<0.01$). This response was attenuated by luteolin in a concentrationdependent manner with an IC50 value of 0.2±0.1 μmol/L (Figure A). During the assay, cell viability was >95% in the treatment with testing different concentrations of luteolin (assessed by lactate dehydrogenase efflux, data not shown). Stimulation of neutrophils with 1 μmol/L fMLP and 10 nmol/L PMA evoked superoxide anion generation. Luteolin had an inhibitory effect against fMLP-induced superoxide anion generation, with an IC50 value of 2.2±0.8 μmol/L. However, no effect on PMA-induced superoxide anion generation was observed (Figure B). The results showed that luteolin was capable of inhibiting chemoattractant-induced neutrophil chemotaxis and the respiratory burst.



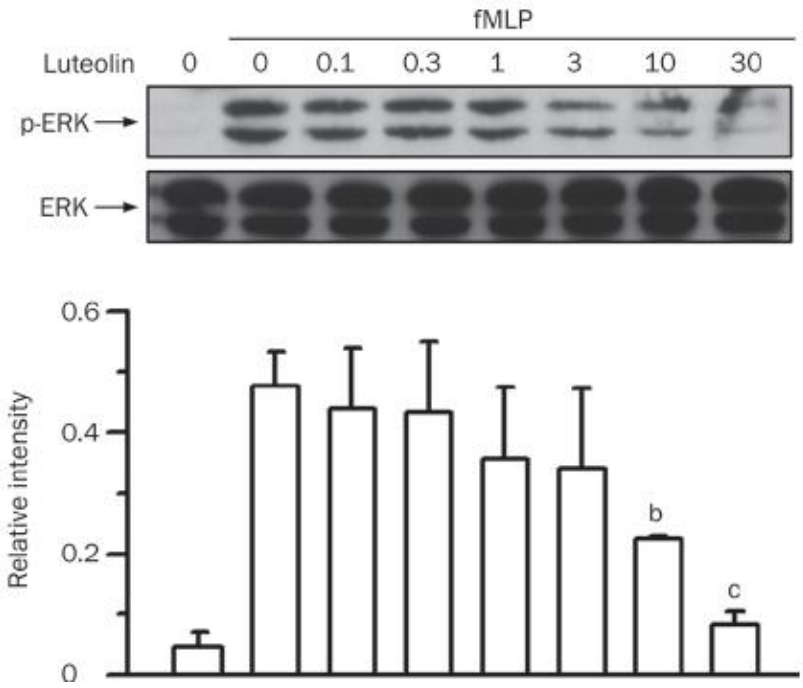

*3.15. Effects of luteolin on fMLP-stimulated p38 MAPK phosphorylation*

To examine the effect of luteolin on p38 MAPK, the phosphorylation of p38 MAPK in fMLP-stimulated neutrophils was analyzed by Western blotting. fMLP stimulation induced an increase in p38 MAPK phosphorylation (1.9±0.2 fold, $P<0.01$). The inhibitory effect of luteolin on fMLPincreased p38 MAPK phosphorylation was observed only at concentrations of luteolin up to 30 $\mu$mol/L (61% inhibition). The IC50 values were 100- and 10-fold higher than those required for chemotaxis and for the respiratory burst, respectively.



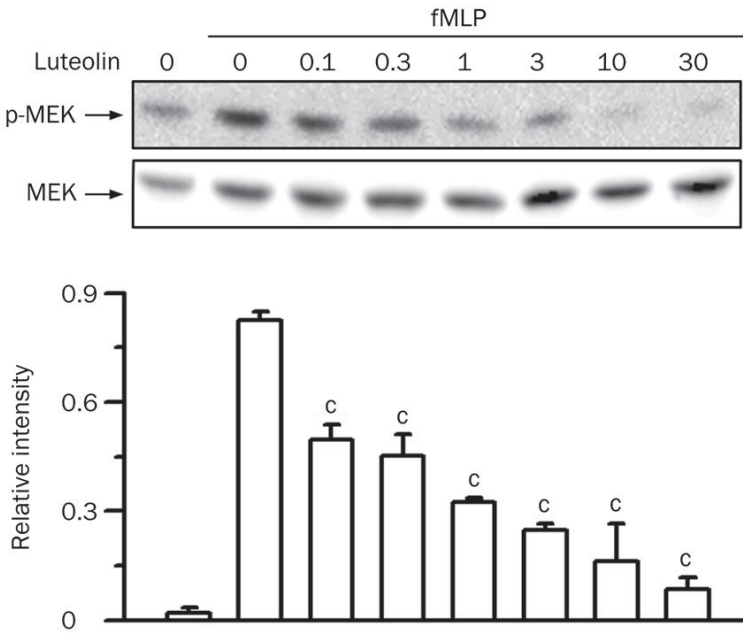

*3.16 Effects of luteolin on fMLP-stimulated ERK pathway*

The ERK pathway participates in chemoattractant-induced neutrophil chemotaxis and the respiratory burst and in LPS-induced ALI. Stimulation of neutrophils with fMLP resulted in an increase in the phosphorylation of ERK ($P<0.01$). Luteolin reduced the fMLP-induced ERK phosphorylation in a concentration-dependent manner, with an IC50 value of 5.4±2.2 $\mu$mol/L.



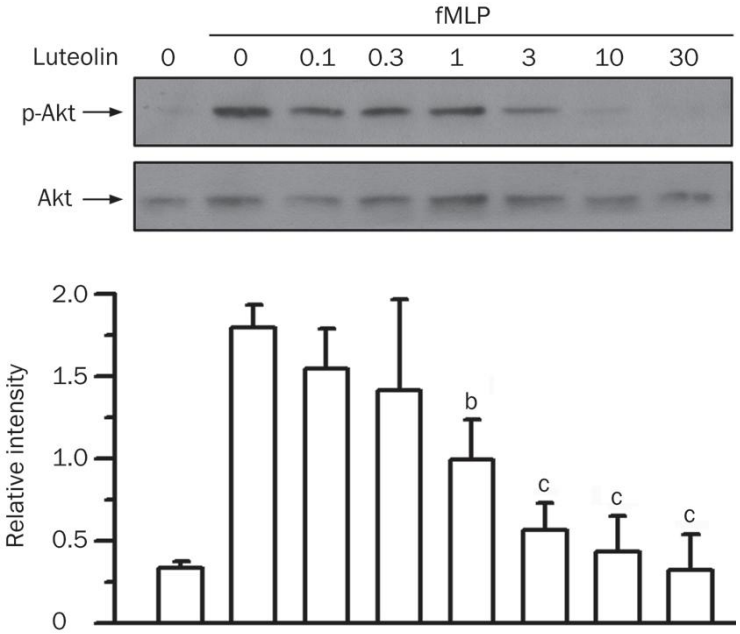

*3.17 Effects of luteolin on fMLP-stimulated MEK pathway*

Activation of ERK is mediated through its phosphorylation by MEK in response to various stimuli. Therefore, we next examined the effect of luteolin on the phosphorylation of MEK. As shown in Figure 6, MEK phosphorylation was weak in vehicle-treated cells, whereas a visible band was detected in response to fMLP stimulation ($P<0.01$). Luteolin decreased the fMLP-induced MEK phosphorylation in a concentration-dependent manner, with an IC50 value of 0.1±0.1 $\mu$mol/L. These results suggest that the MEK and ERK-related pathways play key roles in luteolin-mediated attenuation of fMLP-induced neutrophilic responses.



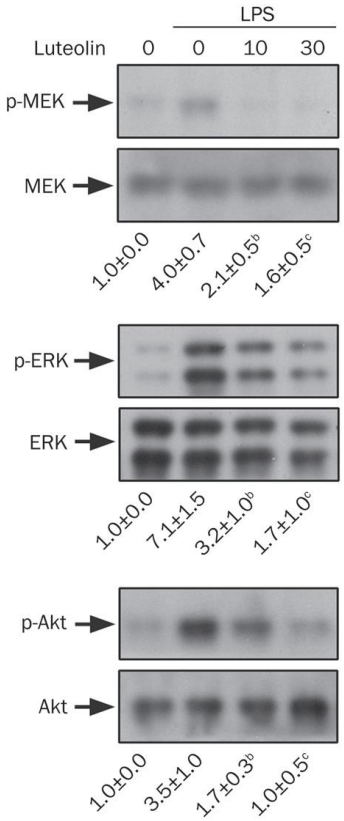
*3.17 Effects of luteolin on fMLP-stimulated Akt pathway*

Protein kinase B/Akt has been implicated in LPS-induced ALI and plays a critical role in neutrophil chemotaxis and the respiratory burst. fMLP stimulation of neutrophils led to an increase in Akt phosphorylation ($P<0.01$). Luteolin attenuated the Akt phosphorylation in a concentration-dependent manner, with an IC50 value of 1.8±1.0 $\mu$mol/L. These results suggest that the Akt-related pathways might actively participate in luteolin-mediated attenuation of fMLP-induced neutrophilic responses.




*3.18 Effects of luteolin on LPS-stimulated MEK, ERK, and Akt phosphorylation*

In parallel with LPS-induced injury, the effects of luteolin were also investigated in LPS-stimulated neutrophils. Like fMLP, LPS stimulation induced elevated phosphorylation of MEK (4.0±0.7 fold, $P<0.01$), ERK (7.1±1.5 fold, $P<0.01$), and Akt (3.5±1.0 fold, $P<0.01$). The increased phosphorylation was significantly attenuated by luteolin.



## 4. Discussion

Experimental results obtained by this study demonstrate that luteolin contributed to a preventive effect on I.T. administration of LPS-induced inflammatory responses such as alveolar congestion, haemorrhaging, leukocytes infiltration, alveolar wall thickness, protein leakage, and lung edema in the lungs. We found the protection mechanism of luteolin against LPS-induced ALI via suppression of TNF-α and IL-6 productions, iNOS and COX-2 expression, NF $\kappa$ B and Akt activation.

LPS, an important virulence macromolecule, consists of the following three regions: lipid A, which is enclosed in the outer membrane; the core oligosaccharide, which exists as a bridge between lipid A and O-antigen; and, the O-antigen, which is the outermost region. The toxic segment of LPS is lipid A and the immunogenic but nontoxic portion is the core oligosaccharide and O-antigen. LPS is a primary trigger of innate immunity and acute proinflammatory responses. Therefore, LPS, a potent inducer of lung injury, can be employed to investigate ALI. To ensure that a real dose is delivered to the lungs of each animal, LPS was administrated directly into the lungs via the trachea (Driscoll et al., 2000). Administration of LPS I.T. evokes acute inflammatory responses resulting in ALI. So far, there is no effective pharmacological intervention for treating ALI (Matute-Bello et al., 2008). Notably, LPS-induced inflammatory response in the lung is the primary cause of ALI-associated mortality, and is an important factor in sepsis development. Gas exchange disturbance is one of the major ALI features (Wheeler and Bernard, 2007). Respiratory acidosis and hypoxaemia via arterial blood gas analysis in LPS-treated animals were indicative of lung respiratory dysfunction with clinical symptoms and pulmonary lesions under ALI. Luteolin improved acidosis and hypoxaemia by significantly decreasing arterial $PaCO_2$ and increasing the arterial $PaO_2$ concentration. These results indicated the protective effect of luteolin in treating ALI.

Exposure to LPS injures lung vascular integrity, which is the most important initial cause of ALI, results in hemorrhage, protein leakage, and leukocyte infiltration. These changes contribute to the development of hyaline membrane and congestion of alveolar spaces (Burnham EL, 2008). In addition, LPS increases secretion of proinflammatory cytokines, including TNF-α, IL-8, and IL-6, in alveolar macrophages and bronchial epithelial cells (Khair et al., 1996; Wang et al., 2010). These proinflammatory cytokines are crucial to leukocytes activation and recruitment into the infected site (Bhatia and Moochhala, 2004). Leukocytes activation produces reactive oxygen species and protease leads to alveolar barrier disruption, permeability increase and direct tissue injury (Abraham E., 2003a). In previous studies have demonstrated that pretreatment with luteolin abolished the LPS induced accumulation of leukocytes in the airspace (Lee et al., 2010; Kotanidou et al., 2002). Oral administration of luteolin reduced bleomycin-induced total cells and neutrophils proportion in BALF (Chen et al., 2010). At present, we also found I.P. administration of luteolin suppressed LPS-induced leukocytes influx into alveolar space. Moreover, luteolin prevent alveolar congestion, hemorrhage, alveolar septum thickness, and hyaline membrane formation caused by LPS. Furthermore, we demonstrated luteolin also inhibited LPS-induced pulmonary permeability and edema. These results indicate that luteolin might have beneficial anti-inflammatory effects on LPS-induced ALI animal model.

The level of MPO, a PMNs marker enzyme, in lung represents influx of PMNs to lung parenchyma or alveolar space (Borregaard and Sorensen, 2007). Pretreatment with luteolin significantly reduced the MPO level in LPS-induced ALI. And then, to characterize the type of leukocytes in the alveolar space, immunostaining for CD45 and Ly6G was performed on cells from BALF (Khandoga et al., 2009), and the results indicate that LPS-induced PMNs infiltration was prevented by luteolin. Finally, we estimated degranulation, a critical response of PMNs activation (Wheeler and Bernard, 2007). Four different types of granules, azurophil, specific, gelatinase, and secretory granules, exist in PMNs. CD11b is a membrane

constituent of specific, gelatinase, and secretory granules, whereas CD45 resides primarily in the membrane of secretory granules. Secretory granules have the highest appetite for extracellular discharge, followed by gelatinase, specific, and azurophilic granules (Borregaard and Sorensen, 2007). Thus, mild stimulation results in rapid and few secretions of secretory granules without significant release of other granules. However, more powerful stimulators lead to release of gelatinase, specific, and azurophilic granules (Faurschou and Borregaard, 2003). In the alveolar space, PMNs can be activated by manual interference and express CD45; this is in contrast to CD11b, which is expressed by LPS administration. Therefore, CD45 and CD11b expressions were high and low respectively in the control group. In LPS-stimulated groups, PMNs activation promoted CD45 and CD11b expression considerably. Luteolin primarily attenuated LPS-induced CD11b expression, with moderate effects on CD45 expression. The analytical results had shown that luteolin prevented LPS-induced ALI by decreasing the infiltration and activation of PMNs.

The early-stage of LPS-induced lung damages via responses of PMNs, included respiratory burst and degranulation. Respiratory burst leads PMNs to produce reactive oxygen species (ROS), such as $O_2^{\cdot -}$ and hydrogen peroxide ($H_2O_2$), for killing pathogen. Degranulation leads PMNs to secrete MPO, which catalyzes the reaction between chloride and $H_2O_2$ to form hypochlorous acid, which is also classified as an ROS. However, excessive ROS can damage tissue and cause inflammation (Borregaard and Sorensen, 2007). We found that luteolin suppressed the generation of $O_2^{\cdot -}$ in fMLP-stimulated PMNs (Lee et al., 2010) and the level of MPO in lung of LPS-treated mice. Major attacking targets of ROS on cell membrane are the polyunsaturated fatty acids, which results in the formation of lipid peroxidation products such as MDA, and leads to tissue damage (Del Rio et al., 2005). We found pretreatment with luteolin prevented the formation of MDA in the lung of LPS-induced ALI.

In addition, ROS are eliminated by the enzymatic antioxidant system, involveing SOD and catalase, where SOD converts $O_2^{\cdot -}$ into $H_2O_2$, and catalase metabolizes $H_2O_2$ into hydrogen oxide and oxygen (Ueda et al., 2008). Administration of LPS significantly decreased the activities of SOD and catalase in lung tissue; this finding is in agreement with their decreased activity by inflammatory responses in the ALI patients (Ueda et al., 2008). Experimental data demonstrated that SOD and catalase activities increased in mice pretreated with luteolin when compared with those in the LPS group. These results support the hypothesis that luteolin could effectively reduce the production of oxyradicals during inflammatory response in LPS-induced ALI.

Proinflammatory cytokines TNF-$\alpha$ and IL-6 play critical roles and contribute to lung injury severity in ALI/ARDS patients (Giebelen et al., 2007). The earliest and primary endogenous mediator in the inflammatory process is TNF-$\alpha$, which primarily originates from alveolar macrophages. TNF-$\alpha$ binds with receptors in lung tissue leads to lysosomes leaking, directly resulting in disruption of pneumoangiogram vascular endothelial cells and increasing their permeability. Moreover, TNF-$\alpha$ stimulates alveolar epithelial cells to generate another proinflmmatory cytokine such as IL-6 (Marsh and Weavers, 1996). Both TNF-$\alpha$ and IL-6 induce adhesion molecule expression in vascular endothelial cells, resulting in recruitment of leukocytes into inflammatory site. Therefore, TNF-$\alpha$ and IL-6 serve as predictive markers for ALI severity. Many sequelae associated with ALI result from persistent elevation of proinflammatory cytokines in serum and BALF (Agouridakis et al., 2002). Previously, luteolin inhibited LPS-induced TNF-$\alpha$ and IL-6 release from alveolar macrophage (Chen et al., 2007; Kotanidou et al., 2002). In mice model studies show that bleomycin stimulated production of TNF-$\alpha$ and IL-6 is reversed by luteolin (Chen et al., 2010). Experimental data in this study demonstrate pretreatment with luteolin downregulated expressions of TNF-$\alpha$ and IL-6 in the BALF at 6 h after the LPS challenged mice. It is imply that luteolin confers protection to mouse ALI induced by LPS through reduced the production of TNF-$\alpha$ and IL-6 in mice.

Expression of iNOS, which generate NO, and COX-2, which generate prostaglandins and thromboxanes, contributes to the pathophysiological progression of ALI (Bhatia and Moochhala, 2004, Farley et al., 2006; Speyer et al., 2003). In mouse alveolar macrophages, luteolin pretreatment suppressed expression of iNOS and COX-2 after LPS treatment (Chen et al., 2007). An *in vivo* assay indicates that luteolin reversed the expression of iNOS and COX-2 are pretreated by bleomycin (Chen et al., 2010). According to experimental results, we conclude with fair certainty that luteolin inhibited LPS-induced increases in iNOS and COX-2 expression in lung.

The molecular signal transduction of inflammatory responses in LPS-induced ALI is regulated at the transcriptional level via the MAPK and NF$\kappa$B pathways (Fan et al., 2001; Liu et al., 2008; Lee et al., 2004; Schuh et al., 2009). After LPS administration, toll-like receptors initiate a series of NF$\kappa$B and MAPK cascades and result in expression of cytokines, chemokines, and adhesion molecules; generation of ROS; and secretion of MPO. The MAPK are serine/threonine kinases required by dual phosphorylation (threonine and tyrosine) for enzymatic activation and contain three major families (ERK, p38 MAPK, and JNK) in mammals (Kim and Choi, 2010). In previous study, luteolin effectively attenuated phosphorylation of MEK1/2 and ERK1/2 but not p38 MAPK in fMLP- and LPS-stimulated PMNs *in vitro* (Lee et al., 2010). However, data obtained from this study indicate that luteolin attenuated activation of ERK, p38 MAPK, and JNK in the lung tissue of mice with LPS-induced ALI. These inconsistent results could be due to the differences in the application between primary PMNs culture model (*in vitro*) and the lung tissue in animal model (*in vivo*). In addition, under rest NF$\kappa$B associated with I$\kappa$B resulting in its inactivated form in cytoplasm. Upon NF$\kappa$B activation, I$\kappa$B is ubiquitinatedand degraded by proteasomes. And then, liberated NF$\kappa$B can translocate into the nucleus, where it binds to specific sequences in the promoter or enhancer regions of genes (Senftleben and Karin, 2002). This study demonstrted that NF$\kappa$B was activated and I$\kappa$B was degraded after LPS administration, and luteolin pretreatment prevented these manifestations. These results suggest that inhibition of NF$\kappa$B activation and I$\kappa$B degradation by luteolin may be due to inhibition of activated MAPK pathways. The preventive mechanism of luteolin is also similar to penehyclidine, which is derived from hyoscyamine and has good curative effects for chronic obstructive pulmonary disease (Wang et al., 2005), except that penehyclidine has no influence on JNK phosphorylation (Shen et al., 2009).

In lung, the expression of iNOS, COX-2, TNF-$\alpha$, and IL-6 are regulated by NF-κB activation, which participates in the regulation of the expression of multiple immediate early genes involved in the acute inflammatory responses (Baeuerle and Henkel, 1994, Martin and Frevert , 2005). NF-$\kappa$B activation is stimulated by LPS through activation of Akt (Ardeshna et al., 2000). Akt is the major downstream molecule of phosphoinositide 3-kinases and full activation requires S473 phosphorylation in a hydrophobic motif (Wymann et al., 2003). Therefore, Akt and PI3K participate in LPS-induced ALI (He et al., 2009). Luteolin prevents Akt phosphorylation and NF-$\kappa$B activation in LPS-stimulated alveolar macrophages (Chen et al., 2007). In LPS-induced ALI animal model, we obtained luteolin prevents Akt phosphorylation and NF-$\kappa$B activation in whole lung. These analytical results indicate that the PI3K/Akt pathway mediated NF$\kappa$B activation in luteolin protect LPS-induced inflammatory responses of ALI.

In conclusion, pretreatment with luteolin in mice markedly attenuated pulmonary inflammation in ALI caused by LPS via I.T. administration. The manifestations of pulmonary inflammation are as follows: (1) pathological change in lung damage, such as increased in alveolar congestion, hemorrhage, leukocytes infiltration into alveolar space, alveolar septum thickness, and hyaline membrane formation; (2) evaluation of lung permeability and edema; (3) production of TNF-α and IL-6; (4) iNOS and COX-2 expressions in the lung. The molecular mechanism by which luteolin protects the lung from injury is by inhibiting NF-$\kappa$B

activation via the PI3K/Akt pathway. In addition, luteolin can effectively attenuate LPS-induced ALI by inhibiting infiltration of PMNs into the lung and activation of PMNs in the BALF, and via production of oxidative stress, proinflammatory cytokines, chemokines, and adhesion molecules in the lung. The protective effect of luteolin involves inhibition of NF $\kappa$ B activation and I $\kappa$ B degradation via inhibition of MAPK pathways. These experimental results suggest that luteolin is a potential therapeutic anti-inflammatory compound forALI therapy. Experimental findings support the potential use of luteolin as a therapeutic agent for prevention of ALI associated with direct infection by gram-negative bacteria.

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# 國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■ 達成目標

□ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

□ 其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 □無

技轉：□已技轉 □洽談中 □無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

經由第一年的研究發現 luteolin 經由降低脂多醣所引發 Akt, MAPK 與 NFκB 的活化，而降低急性肺損傷的發炎現象。經由第二年度的計畫發現 luteolin 經由降低巨噬細胞活化與內皮細胞滲透度增加，而降低急性肺損傷的發炎現象。經由第三年度的計畫發現木犀草素以濃度依存性的方式降低由 fMLP 或脂多醣所引起嗜中性球趨化或呼吸爆發作用，但並不影響 PMA 對嗜中性白血球活化的作用。而此種抑制作用的機轉主要是經由 MAPK/ERK kinase 1/2 (MEK), extracellular signal-regulated kinase (ERK), 與 Akt 磷酸化所致。綜合上述，木犀草素為一有效預防脂多醣所誘發的急性肺損傷疾病的保健食品物質。

# 國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/21

| 國科會補助計畫 | 計畫名稱: 木犀草素對急性肺損傷保護作用與分子機制之研究 |
|---|---|
| | 計畫主持人: 關宇翔 |
| | 計畫編號: 99-2320-B-040-012-MY3　　學門領域: 保健營養 |

無研發成果推廣資料

# 99 年度專題研究計畫研究成果彙整表

| 計畫主持人：關宇翔 | 計畫編號：99-2320-B-040-012-MY3 |
|---|---|
| 計畫名稱：木犀草素對急性肺損傷保護作用與分子機制之研究 | |

| 成果項目 | | | 量化 | | | 單位 | 備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等） |
|---|---|---|---|---|---|---|---|
| | | | 實際已達成數（被接受或已發表） | 預期總達成數(含實際已達成數) | 本計畫實際貢獻百分比 | | |
| 國內 | 論文著作 | 期刊論文 | 0 | 0 | 100% | 篇 | |
| | | 研究報告/技術報告 | 0 | 0 | 100% | | |
| | | 研討會論文 | 0 | 0 | 100% | | |
| | | 專書 | 0 | 0 | 100% | | |
| | 專利 | 申請中件數 | 0 | 0 | 100% | 件 | |
| | | 已獲得件數 | 0 | 0 | 100% | | |
| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（本國籍） | 碩士生 | 2 | 2 | 100% | 人次 | |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 1 | 1 | 100% | | |
| 國外 | 論文著作 | 期刊論文 | 5 | 3 | 100% | 篇 | |
| | | 研究報告/技術報告 | 0 | 0 | 100% | | |
| | | 研討會論文 | 0 | 0 | 100% | | |
| | | 專書 | 0 | 0 | 100% | 章/本 | |
| | 專利 | 申請中件數 | 0 | 0 | 100% | 件 | |
| | | 已獲得件數 | 0 | 0 | 100% | | |
| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（外國籍） | 碩士生 | 0 | 0 | 100% | 人次 | |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 0 | 0 | 100% | | |

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|---|---|
| 其他成果<br>(無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。) | 無 |

| | 成果項目 | 量化 | 名稱或內容性質簡述 |
|---|---|---|---|
| 科教處計畫加填項目 | 測驗工具(含質性與量性) | 0 | |
| | 課程/模組 | 0 | |
| | 電腦及網路系統或工具 | 0 | |
| | 教材 | 0 | |
| | 舉辦之活動/競賽 | 0 | |
| | 研討會/工作坊 | 0 | |
| | 電子報、網站 | 0 | |
| | 計畫成果推廣之參與（閱聽）人數 | 0 | |

# 國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

經由第一年的研究發現 luteolin 經由降低脂多醣所引發 Akt, MAPK 與 NF 岂 B 的活化，而降低急性肺損傷的發炎現象。經由第二年度的計畫發現 luteolin 經由降低巨噬細胞活化與內皮細胞滲透度增加，而降低急性肺損傷的發炎現象。經由第三年度的計畫發現木犀草素以濃度依存性的方式降低由 fMLP 或脂多醣所引起嗜中性球趨化或呼吸爆發作用，但並不影響 PMA 對嗜中性白血球活化的作用。而此種抑制作用的機轉主要是經由 MAPK/ERK kinase 1/2 (MEK), extracellular signal-regulated kinase (ERK), 與 Akt 磷酸化所致。綜合上述，木犀草素為一有效預防脂多醣所誘發的急性肺損傷疾病的保健食品物質。