Effects of *L*-arginine on lipopolysaccharides-induced acute lung injury by inhibiting apoptotic pathway

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Abstract：AIM　To investigate the effect and mechanism of *L*-arginine (*L*-Arg) on lipopolysaccharides (LPS)-induced acute lung injury (ALI). **METHODS** Models of ALI were established by injection (iv) with LPS 5 mg·kg^{-1} in male SD rats. The rats were randomly divided into 3 groups：① saline group；② LPS group；③ *L*-Arg group. The rats in each group were further divided into 2 subgroups according to *L*-Arg-supplemented time：1 h + 3 h group and 6 h + 3 h group. *L*-Arg 500 mg·kg^{-1} or saline (saline and LPS groups) was administrated at 1 or 6 h after LPS injection, respectively. The treatment lasted for 3 h, and the rats were sacrificed at 4 or 9 h after LPS injection. Apoptotic rate, caspase 3, and Bcl-2 and Bax were evaluated by flow cytometry, Western blot analysis and immunohistochemistry, respectively；meanwhile, the pathological changes of lung tissue were observed by electron microscope. **RESULTS**　Compared with saline group, apoptosis of pulmonary cells and caspase 3 expression were significantly increased, Bcl-2 was decreased, while Bax was elevated in alveolar and airway epithelial cells in LPS group. Compared with LPS 1 h + 3 h group, *L*-Arg 1 h + 3 h decreased apoptotic pulmonary cells[(23.8 ± 2.8)% *vs* (15.4 ± 2.3)%]； moreover, expressions of caspase 3 (0.80 ± 0.06 *vs* 0.67 ± 0.10) and Bax (0.115 ± 0.012 *vs* 0.091 ± 0.014) were significantly decreased, while expression of Bcl-2 (0.067 ± 0.011 *vs* 0.075 ± 0.009) and Bcl-2/Bax ratio (0.586 ± 0.114 *vs* 0.833 ± 0.142) in alveolar and airway epithelial cells were markedly increased, and lung damage was alleviated. *L*-Arg 6 h + 3 h also reduced apoptotic pulmonary cells and caspase 3 expression compared with LPS group, but the lung injury relieved slightly. **CONCLUSION**　Relatively early administration of *L*-Arg can protect lungs from LPS-induced injury through inhibiting cell apoptosis, as well as increasing the expression of anti-apoptotic protein Bcl-2 and decreasing the expression of proapoptotic protein Bax and caspase 3.

Key words：arginine；apoptosis；respiration disorders

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　　Lipopolysaccharides (LPS)-induced acute lung injury (ALI) is characterized by polymorphonuclear neutrophil accumulation, pulmonary hypertension, pulmonary endothelial cell damage, and increased capillary permeability[1]. The importance of pulmonary cell apoptosis in the pathogenesis of ALI has recently been demonstrated[2], but the underlying mechanisms of their involvement in the development of ALI remain unknown. Apoptosis, which is observed in the pulmonary cells in ALI, might affect the integrity of the bronchial wall by increasing its permeability, and further disturbing the main barrier between the environment and respiratory system[3].

　　Previous studies had shown that nitric oxide (NO) was an important apoptotic modulator in pulmonary cells. *L*-Arginine (*L*-Arg) is the critical substrate for NO production through enzymatic oxidation catalyzed NO synthase (NOS)[4]. The inducible NOS (iNOS) was induced when cells were exposed to LPS and interferon-γ(IFN-γ). Increased rate of Arg catabolism was observed in patients with septic shock or with a lack of arginine associated with

a slower production[5]. The rate of Arg catabolism in lungs was detected to be more-than-3-fold higher after burn and smoke inhalation injury. The concentration of Arg in plasma was decreased to a great extent in the nonsurviving sheep after endotoxin challenge, and administration of Arg could improve the function of the lung after endotoxin insult[6]. Administration of L-Arg could also improve gas exchange and pulmonary function, indicating it as a potential treatment for the patients with ALI[7]. Supplementation with L-Arg had been shown beneficially by restoring vascular function and improving the clinical symptoms of various diseases associated with vascular dysfunction[8].

So far, there has been no report on the mechanism of L-Arg which involved in ALI process by inhibiting apoptotic pathway. In this study, the effect of L-Arg on LPS-induced lung injury was investigated, and tried to explore the possible mechanism of lung injury in rats.

1 MATERIALS AND METHODS

1. 1 Drugs, reagents and instruments

LPS and L-Arg were purchased from Sigma Chemicals (St Louis, MO, USA). Rat caspase 3 monoclonal antibody, rabbit Bcl-2 and Bax polyclonal antibodies, streptavidin-perosidase and diaminobenzidine (DAB) were purchased from Santa Cruz Biotechnologies (Santa Cruz, USA). Epics-XL flow cytometer (Beckmen Coulter, USA), microscope (Olympus BX40, Japan) and electronic microscope (H-7500, Hitachi, Japan) were used.

1. 2 Animal treatment and administration

A total of 48 male SD rats, weighing 250 – 290 g, were obtained from Experimental Animal Center of Hebei Province. The rats were in thermoregulated environment, under a 12 h light/12 h dark cycle. All animals were treated according to the Chinese Council on Animal Care Guidelines. SD rats were randomly divided into 3 groups：① saline group；② LPS group；③ L-Arg group. LPS 5 mg·kg^{-1} was iv administrated in L-Arg group

and LPS group, saline was administrated in saline group. The rats in each group were further divided into 2 subgroups according to L-Arg supplemented time：1 h + 3 h group and 6 h + 3 h group. L-Arg or saline was administrated at 1 or 6 h after LPS injection. L-Arg 500 mg·kg^{-1} was ip administrated in L-Arg group, saline was administrated in saline and LPS groups. The rats were sacrificed at 4 or 9 h after administration of saline or LPS. The dosage of L-Arg was used according to the previous work[9].

1. 3 Determination of apoptotic rate by flow cytometry

The lung tissue was minced and fixed overnight in 70% ethanol at 4℃. The sample staining was performed using immunofluorescence labeling method. Stained samples were analyzed by Epics-XL flow cytometer. The analytic data were processed with Expo32 v 1. 2 software. The quantitative assessment of sub-G$_1$ cells by flow cytometry was used to estimate the number of apoptotic cells.

1. 4 Western blot analysis for caspase 3 protein expression

Firstly, total protein was extracted from 100 mg lung tissue using 1 mL radioimmunoprecipitation assay buffer, the insoluble materials were removed by centrifugation at 12 000 × g for 10 min at 4℃. Proteins were resolved by 12% SDS-polyacrylamide and transferred onto nitrocellulose membranes. The membrane was blocked with 5% bovine serum albumin (BSA) for 2 h followed by incubation with primary antibodies (1∶200) overnight at 4℃. After washing with Tween/Tris buffered salt solution, the membranes were incubated with horseradish peroxidase-conjugated antibodies for 1 h. Finally, the proteins were visualized using DAB. The intensity of protein bands was measured with the analysis software (BIO-PROFIF, France).

1. 5 Measurement of expressions of Bcl-2 and Bax with immunohistochemistry

Lung tissue samples were fixed by 10% Formalin. After processing, samples were embedded in paraffin. 5 μm-Thick tissue sections were cut

using a rotary microtome and mounted onto histological slides. The expressions of Bcl-2 and Bax proteins were determined by immunhistochemistry S-P methods. The positive cells showed brownyellow grains in the cytoplasm. The average optical densities of positive cells at 5 different high fields of vision (×400) were calculated with image analyzer(Motic6. 0) in order to quantify the expression in each slide(6 slides per rat).

1.6　Detection of ultrastructure of lung tissue

The part of lung tissues was cut down and immediately cut into 1. 0 mm^3 pieces on ice,and fixed with 4% glutaraldehyde fixation solution for 24 h. Then, the tissue sections were anhydrated, embedded, chipped and stained with uranyl acetate-lead citrate, and observed via transmission electron microscope.

1.7　Data were shown as $\bar{x} \pm s$. The statistical analysis was preformed with one-way ANOVA and Dunnett t test by SPSS 11. 0 software.

2　RESULTS

2.1　Effect of *L*-Arg on apoptosis of lung cells in ALI rats

The results in Tab 1 and Fig 1 showed that the apoptosis of lung cells was significantly increased in LPS 1 h + 3 h and LPS 6 h + 3 h groups compared with that of the saline group, respectively. But after the administration of *L*-Arg, the apoptotic cells were decreased compared with that of the corresponding LPS group, respectively. These results suggested that *L*-Arg inhibit lung cell apoptosis in rats with ALI.

2.2　Effects of *L*-Arg on caspase 3 protein expression in ALI rats

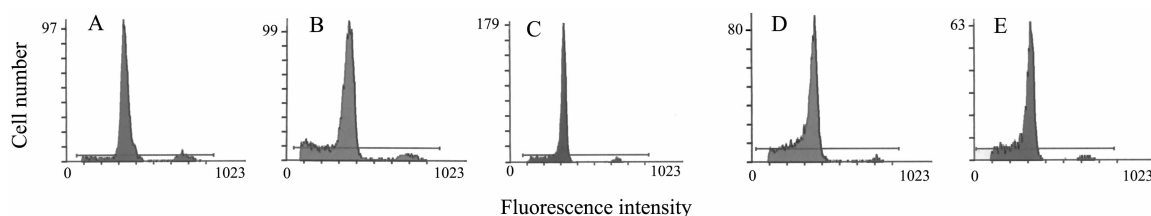The results from Western blot showed that

Tab 1.　Effect of *L*-arginine (*L*-Arg) on apoptotic rate of lung cells in rats with acute lung injury (ALI) induced by lipopolysaccharides (LPS)
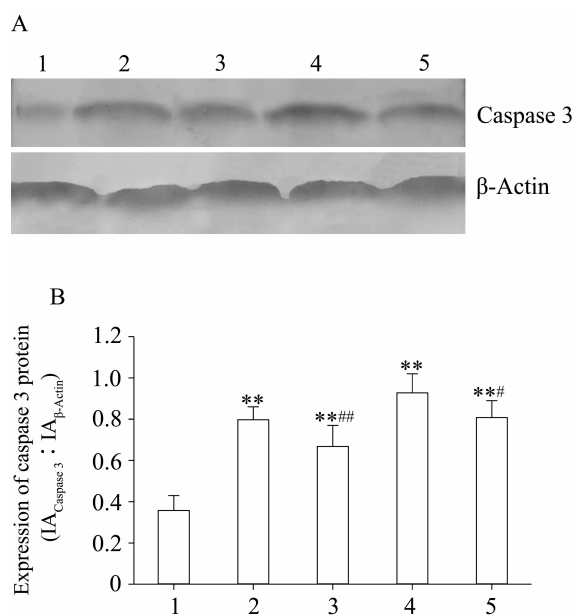
Group	Apoptotic rate/%
Saline 1 h + 3 h	11. 4 ± 3. 1
Saline 6 h + 3 h	12. 0 ± 3. 1
LPS 1 h + 3 h	23. 8 ± 2. 8 **
LPS 6 h + 3 h	27. 2 ± 4. 3 **
L-Arg 1 h + 3 h	15. 4 ± 2. 3 ##
L-Arg 6 h + 3 h	22. 8 ± 2. 4 # **

The rats in control group were given saline. The rats in model group were given iv LPS 5 mg·kg^{-1}. The rats in *L*-Arg 1 h + 3 h group and *L*-Arg 6 h + 3 h group were given ip *L*-Arg 500 mg·kg^{-1} 1 or 6 h after LPS injection. Then, 3 h after *L*-Arg treatment, the rats were sacrificed and apoptotic rate of lung cells was measured by flow cytometry. $\bar{x} \pm s$, $n = 8$. ** $P < 0.01$, compared with saline group; # $P < 0.05$, ## $P < 0.01$, compared with LPS group.

the expressions of caspase 3 were remarkably increased in LPS 1 h + 3 h group and in LPS 6 h + 3 h group compared with saline group. Compared with LPS group, expression of caspase 3 was significantly decreased in *L*-Arg group (Fig 2).

2.3　Effect of *L*-Arg on Bcl-2 and Bax protein expressions in ALI rats

The results in Tab 2 showed that Bcl-2 and Bax positive cells could be found at endochylema of alveolar and airway epithelial cells in saline group. In LPS group, a few positive expression of Bcl-2 and considerable positive expression of Bax were observed. The ratios of Bcl-2/Bax were lower after LPS injection at 4 and 9 h group than that in saline group. Compared with that of LPS group, the expression of Bax was markedly decreased, while the expression of Bcl-2 and the ratios of Bcl-2 to Bax were significantly increased in *L*-Arg 1 h + 3 h group. However, in the lungs,

Fig 1.　Effect of *L*-Arg on apoptotic rate in ALI rats. See legend of Tab 1 for treatments. A：saline；B：LPS 1 h + 3 h；C：*L*-Arg 1 h + 3 h；D：LPS 6 h + 3 h；E：*L*-Arg 6 h + 3 h.

A

B

Fig 2. Effect of *L*-Arg on caspase 3 protein expression in ALI rats by Western blot analysis. See legend of Tab 1 for treatments. 1：saline；2：LPS 1 h + 3 h；3：*L*-Arg 1 h + 3 h；4：LPS 6 h + 3 h；5：*L*-Arg 6 h + 3 h. $\bar{x} \pm s$, $n = 8$. $^{**}P < 0.01$, compared with saline group；$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with LPS group.

Tab 2. Effects of *L*-Arg on Bcl-2 and Bax expressions of lung tissue in ALI rats

Group	Bcl-2	Bax	Bcl-2: Bax
Saline 1 h + 3 h	0.073 ± 0.009	0.076 ± 0.010	0.976 ± 0.157
Saline 6 h + 3 h	0.072 ± 0.010	0.077 ± 0.007	0.929 ± 0.118
LPS 1 h + 3 h	$0.067 \pm 0.011^{*}$	$0.115 \pm 0.012^{**}$	$0.586 \pm 0.114^{**}$
LPS 6 h + 3 h	$0.056 \pm 0.010^{**}$	$0.172 \pm 0.012^{**}$	$0.327 \pm 0.067^{**}$
L-Arg 1 h + 3 h	$0.075 \pm 0.009^{\#\#}$	$0.091 \pm 0.014^{**}_{\#\#}$	$0.833 \pm 0.142^{**}_{\#\#}$
L-Arg 6 h + 3 h	$0.062 \pm 0.011^{**}$	$0.168 \pm 0.012^{**}$	$0.371 \pm 0.071^{**}$

See Tab 1 for the legend. The expressions of Bcl-2 and Bax proteins were determined by immunohistochemistry S-P mehtod. $\bar{x} \pm s$, $n = 240$ fields (5 fields per slide, 6 slides per rat, 8 rats in each subgroup). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with saline group；$^{\#\#}P < 0.01$, compared with LPS group.

the expression of Bcl-2, Bax and the ratio of Bcl-2 to Bax showed no significant difference between LPS 6 h + 3 h and *L*-Arg 6 h + 3 h groups. These results suggested that administration of *L*-Arg at 1 h after LPS affected apoptosis-regulation protein induced by LPS：anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, through which inhibiting pulmonary apoptosis.

2. 4 Effect of *L*-Arg on pathological changes in lung tissue of ALI rats

The result in Fig 3 showed the number of microvillus and osmiophilic multilamellar body of alveolar type II epithelial cells decreased after administration of LPS；the mitochondria swelled and the cristae disrupted, dissolved even disappeared in LPS group (Fig 3B and 3D), but these changes were not observed in saline group. The degree of ALI became worse gradually after administration of LPS, while the administration of *L*-Arg could ameliorate the pathological alterations.

3 DISCUSSION

The importance of pulmonary cell apoptosis in the pathogenesis of ALI has recently been underscored. The results of current study showed that LPS 5 mg·kg^{-1} caused an increase in lung apoptosis as early as 4 and 9 h after LPS injection. The findings from the electron microscopy demonstrated morphological characteristics of apoptosis on the pulmonary endothelial and epithelial cells. The results were partly consistent with other studies[10]. Lung injury could cause the losing of normal function cells. *L*-Arg could
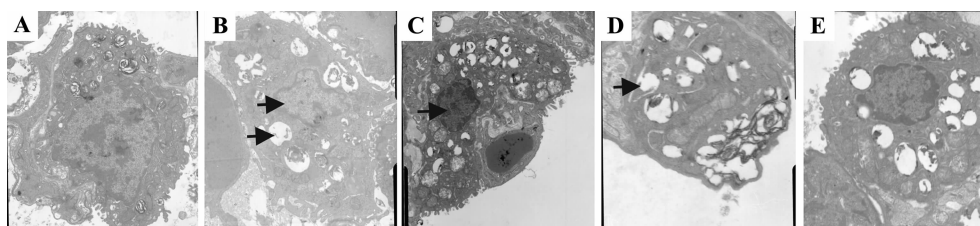
Fig 3. Ultrastructural pathological changes in lung tissue of ALI rats under transmission electron microscope. See legend of Tab 1 for experiment procedure. A：saline(×7000)；B：LPS 1 h + 3 h(×7000)；C：*L*-Arg 1 h + 3 h (×6000), only few evacuated lamellar body and slight mitochondrial cristae damage；D：LPS 6 h + 3 h(×12 000), lamellar bodies are evacuated；mitochondria appear swelling and mitochondrial cristae are damaged in B and D；E：*L*-Arg 6 h + 3 h (×8000).

significantly ameliorate pathological alterations and attenuated pulmonary injury induced by LPS, and apoptotic cells were significantly decreased in the lungs of SD rats after *L*-Arg injection.

　　Previous studies had showed that iNOS and NO end products increased in the lungs of patients with ARDS and induced ALI[11]. However, NO had been shown to inhibit alveolar epithelial cell apoptosis which induced by either hyperoxia, stretch, or ischemia-reperfusion injury[12]. The results confirmed that the expression of caspase 3 protein was associated with LPS-induced apoptosis, meanwhile, the decrease of Bcl-2, and increase of Bax were also detected during the process of LPS-induced apoptosis. But, *L*-Arg increased Bcl-2 expression and induced activation of caspase 3, while inhibited induction of Bax. These data suggested that *L*-Arg could inhibit LPS-induced pulmonary cell apoptosis and exert its anti-apoptotic function by blocking intrinsic (mitochondria-dependent) pathway, one of apoptosis signal pathways, which involves cytochrome c release from the mitochondria and the activation of caspase 9, which then cleaves and activates caspase 3, caspase 7, and caspase 6. The cytochrome c release is determined by the ratio of anti-apoptotic proteins (Bcl-2 and Bcl-xL) and proapoptotic proteins (Bax, Bad, Bik and Bid)[13], caspase 3 is a downstream member of the caspase cascade and acts as a central effector in the execution phase. Its activation leads to cell apoptosis, DNA fragmentation and nuclear morphologic changes, and the inhibition of its activity retards or even prevents apoptosis and attenuates the degree of ALI and animal mortality[14-15]. Bcl-2 family proteins are a critical regulatory factor in cellular responce to apoptosis through mitochondrial pathways. In the present, it was shown that *L*-Arg exerts its anti-apoptotic at least in part through increasing expression of Bcl-2.

　　Our previous study showed that *L*-Arg treatment decreased malondialdehyde and increased superoxide dismutase levels[9]. The results suggested that *L*-Arg could protect lungs

from peroxidation injury through reducing peroxidation during ALI. After endotoxin challenge, the concentration of Arg in plasma was decreased[6]. In response to the lack of substrate, iNOS began to oxidize oxygen molecules, thereby formed superoxide species, so the depletion of *L*-Arg resulted in the production of both and NO by iNOS, which led to peroxynitrite (ONOO⁻) mediated oxidative damage[16], and ONOO⁻ induced apoptosis had been demonstrated in multiple cell types such as type Ⅱ epithelial cells[17], and oxidative stress often caused cell apoptosis[18]. So the antiapoptotic effect of *L*-Arg was associated with inhibition peroxidation in this rat ALI model.

　　In conclusion, early administration of *L*-Arg can protects lungs during ALI through inhibiting oxidative stress and apoptosis. These findings provided a novel insight into the role of *L*-Arg on ALI treatment. However, relevance of oxidative stress to apoptosis needs further clarification, and further studies will help to determine the detail pathways and make possibility of *L*-Arg in the treatment to LPS-induced ALI.

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L-精氨酸通过抑制凋亡途径对脂多糖导致的急性肺损伤的作用

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摘要：**目的**　探讨一氧化氮供体 *L*-精氨酸（*L*-Arg）对大鼠不同病程阶段的急性肺损伤（ALI）的影响及机制。**方法**　采用注射内毒素脂多糖（LPS）的方法制备大鼠肺损伤模型。健康雄性 SD 大鼠，随机分为：① 对照组；② LPS 组；③ *L*-Arg 治疗组。各组按治疗时间又分为给 LPS 1 h 后治疗 3 h（1 h + 3 h）组和给 LPS 6 h 后治疗 3 h（6 h + 3 h）组，并在给予 LPS 1 和 6 h 后再分别 ip 给予生理盐水（对照组及 LPS 组）和 *L*-Arg 500 mg·kg^{-1}（*L*-Arg 治疗组）治疗 3 h。采用流式细胞术检测肺细胞凋亡率；Western 蛋白印迹法检测胱天蛋白酶 3（caspase 3）蛋白的表达；免疫组化法测定 Bcl-2 和 Bax 蛋白的表达；电镜观察肺组织病理变化。**结果**　与对照组比较，LPS 1 h + 3 h 及 LPS 6 h + 3 h 组细胞凋亡率和 caspase 3 蛋白表达明显升高，Bcl-2 蛋白表达下降，Bax 表达增加，Bcl-2/Bax 比值降低，肺组织出现明显的病理变化。与 LPS 1 h + 3 h 组相比，*L*-Arg 1 h + 3 h 组细胞凋亡率〔（23.8 ± 2.8）% *vs*（15.4 ± 2.3）%〕，caspase 3（0.80 ± 0.06 *vs* 0.67 ± 0.10）和 Bax 蛋白表达（0.115 ± 0.012 *vs* 0.091 ± 0.014）显著降低，Bcl-2 蛋白表达（0.067 ± 0.011 *vs* 0.075 ± 0.009）和 Bcl-2/Bax 比值（0.586 ± 0.114 *vs* 0.833 ± 0.142）显著升高；肺组织病理改变明显减轻。*L*-Arg 6 h + 3 h 组细胞凋亡率和 caspase 3 蛋白表达低于 LPS 6 h + 3 h 组，肺组织病理改变稍有减轻。**结论**　较早给予 *L*-Arg 可减轻 ALI，其机制可能与降低 caspase 3 和 Bax 蛋白表达、增强 Bcl-2 蛋白表达有关。

关键词：精氨酸；细胞凋亡；呼吸障碍

（本文编辑　乔　虹）