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## SCIENTIFIC ARTICLE

# Dexmedetomidine preconditioning protects against lipopolysaccharides-induced injury in the human alveolar epithelial cells



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### KEYWORDS

Dexmedetomidine;  
Lipopolysaccharides;  
Preconditioning;  
Acute lung injury;  
Alveolar epithelial  
cell

### Abstract

**Background and objectives:** Dexmedetomidine (DEX) has demonstrated the preconditioning effect and shown protective effects against organize injury. In this study, using A549 (human alveolar epithelial cell) cell lines, we investigated whether DEX preconditioning protected against acute lung injury (ALI) in vitro.

**Methods:** A549 were randomly divided into four groups ( $n=5$ ): control group, DEX group, lipopolysaccharides (LPS) group, and D-LPS (DEX + LPS) group. Phosphate buffer saline (PBS) or DEX were administered. After 2 h preconditioning, the medium was refreshed and the cells were challenged with LPS for 24 h on the LPS and D-LPS group. Then the malondialdehyde (MDA), superoxide dismutase (SOD), Bcl-2, Bax, caspase-3 and the cytochrome c in the A549 were tested. The apoptosis was also evaluated in the cells.

**Results:** Compare with LPS group, DEX preconditioning reduced the apoptosis ( $26.43\% \pm 1.05\%$  vs.  $33.58\% \pm 1.16\%$ ,  $p < 0.05$ ) in the A549, which is correlated with decreased MDA ( $12.84 \pm 1.05$  vs.  $19.16 \pm 1.89 \text{ nmoL} \cdot \text{mg}^{-1}$  protein,  $p < 0.05$ ) and increased SOD activity ( $30.28 \pm 2.38$  vs.  $20.86 \pm 2.19 \text{ U} \cdot \text{mg}^{-1}$  protein,  $p < 0.05$ ). DEX preconditioning also increased the Bcl-2 level ( $0.53 \pm 0.03$  vs.  $0.32 \pm 0.04$ ,  $p < 0.05$ ) and decreased the level of Bax ( $0.49 \pm 0.04$  vs.  $0.65 \pm 0.04$ ,  $p < 0.05$ ), caspase-3 ( $0.54 \pm 0.04$  vs.  $0.76 \pm 0.04$ ,  $p < 0.05$ ) and cytochrome c.

**Conclusion:** DEX preconditioning has a protective effect against ALI in vitro. The potential mechanisms involved are the inhibition of cell death and improvement of antioxidation.

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**PALAVRAS-CHAVE**

Dexmedetomidina;  
Lipopolissacarídeos;  
Pré-  
condicionamento;  
Lesão pulmonar  
aguda;  
Células epiteliais  
alveolares

## Pré-condicionamento com dexmedetomidina protege contra lesões induzidas por lipopolissacarídeos em células epiteliais alveolares humanas

**Resumo**

**Justificativa e objetivos:** Dexmedetomidina (DEX) demonstrou possuir efeito pré-condicionante e também efeitos protetores contra lesão organizada. Neste estudo, usando células A549 (células epiteliais alveolares humanas), investigamos se o pré-condicionamento com DEX proporcionaria proteção contra lesão pulmonar aguda (LPA) *in vitro*.

**Métodos:** Células A549 foram aleatoriamente distribuídas em quatro grupos (n=5): grupo controle, grupo DEX, grupo lipopolissacarídeos (LPS) e grupo D-LPS (DEX+LPS). Administramos solução de PBS (tampão fosfato-alcalino) ou DEX. Após 2 h de pré-condicionamento, o meio foi renovado e as células desafiadas com LPS por 24 h nos grupos LPS e D-LPS. Em seguida, malondialdeído (MDA), superóxido dismutase (SOD), Bcl-2, Bax, caspase-3 e citocromo c em A549 foram testados. Apoptose também foi avaliada nas células.

**Resultados:** Em comparação com o grupo LPS, o pré-condicionamento com DEX reduziu a apoptose ( $26,43\% \pm 1,05\%$  vs.  $33,58\% \pm 1,16\%$ ,  $p < 0,05$ ) em células A549, o que está correlacionado com a diminuição de MDA ( $12,84 \pm 1,05$  vs.  $19,16 \pm 1,89$  nmol.mg<sup>-1</sup> de proteína,  $p < 0,05$ ) e aumento da atividade de SOD ( $30,28 \pm 2,38$  vs.  $20,86 \pm 2,19$  U.mg<sup>-1</sup> de proteína,  $p < 0,05$ ). O pré-condicionamento com DEX também aumentou o nível de Bcl-2 ( $0,53 \pm 0,03$  vs.  $0,32 \pm 0,04$ ,  $p < 0,05$ ) e diminuiu o nível de Bax ( $0,49 \pm 0,04$  vs.  $0,65 \pm 0,04$ ,  $p < 0,05$ ), caspase-3 ( $0,54 \pm 0,04$  vs.  $0,76 \pm 0,04$ ,  $p < 0,05$ ) e citocromo c.

**Conclusão:** O pré-condicionamento com DEX tem efeito protetor contra LPA *in vitro*. Os potenciais mecanismos envolvidos são inibição da morte celular e melhora da antioxidação.

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**Introduction**

Acute Lung Injury (ALI) and its most severe form, Acute Respiratory Distress Syndrome (ARDS), is a relatively common syndrome in critically ill patients associated with high morbidity and mortality. Which are characterized by high-protein pulmonary edema and severe hypoxemic respiratory failure and may result from many clinical insults, including sepsis and pneumonia.<sup>1-3</sup> Despite intense research and an improved understanding of the pathophysiology of ALI/ARDS, there are no specific pharmacological treatments of proven benefit for them. So the morbidity and mortality remains significant at 35%–40%.<sup>3-5</sup>

Preconditioning has been reported to offer an effective protection against ALI by a previous stimulus.<sup>6,7</sup> To date, it has been extensively studied for its potential to offer a unique opportunity to exert protective effects in clinical practice. Today, we know that the protection can be elicited more safely by many drugs such as dexmedetomidine (DEX).<sup>8,9</sup>

DEX has been commonly used as a sedative in clinical settings. Recently, investigators found that DEX was capable of mimicking the preconditioning effect and shown protective effects against organize injury caused by lipopolysaccharides (LPS).<sup>10,11</sup> That evidence indicates that DEX may have protective effect against ALI. Here, we used the LPS-induced A549 injury model to simulate ALI *in vitro*, investigating whether DEX preconditioning would produce protection against ALI. We have also investigated the effects of DEX preconditioning on cell death and antioxidation function to gain a better insight into the mechanism(s).

**Materials and methods****Cell culture and treatments**

Human alveolar epithelial cell line A549 was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan University, Wuhan, China). A549 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol.L<sup>-1</sup> glutamine, 100 U.mL<sup>-1</sup> penicillin and 100 mg.mL<sup>-1</sup> streptomycin, and maintained in a humid environment at 37 °C and 5% CO<sub>2</sub>.

The A549 at the log phase of growth were seeded into a 96 well plate, 2 × 10<sup>3</sup> well. After overnight culture, these cells were randomly allocated to four different treatment groups: the DEX (Jiangsu Hengrui Medicine Co.; Ltd., Jiangsu, China) and D-LPS group received DEX (10 μg.mL<sup>-1</sup>); the control and LPS (Sigma-Aldrich, San Luis, MO, USA) group received the same volume of phosphate buffer saline (PBS). After 2 h exposure to the pharmacological agent each well plate washed and the cells were challenged with LPS (50 μg.mL<sup>-1</sup>) for 24 h on the LPS and D-LPS group. Then the cells were collected for further analysis.

**Measurement of malondialdehyde (MDA) formation and superoxide dismutase (SOD) activity**

The level of MDA formation and SOD activity were determined as markers of oxidative stress using commercially available kits (Jiancheng Biologic Project Co.; Nanjing, China). The protein concentration was determined by the

BCA protein assay kit (BestBio Co.; Shanghai, China) according to the manufacturer's protocol.

## Western blotting

Cells were homogenized and the protein concentration of its supernatant was determined by the BCA method. The supernatant containing 50  $\mu\text{g}$  of protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Primary antibodies included caspase-3, Bcl-2, Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and  $\beta$ -actin (Abcam, Cambridge, MA, USA). The membrane was then incubated with an HRP-conjugated anti-rabbit secondary antibody (1:20,000; Pierce) for 1 h and the blot was developed with a Supersignal chemiluminescence detection kit (Pierce). The immunoblotting was visualized with a Kodak X-ray Processor 102 (Eastman Kodak, Rochester, NY, USA) and analyzed with Quantity One-4.2.3 software (Bio-Rad, Hercules, CA).

## Cytochrome c release assay

As previously described,<sup>12</sup> the A549 cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked using 3% serum dissolved in PBS for 30 min at room temperature. The cells were then probed with anti-cytochrome c antibody (1:100; Santa Cruz Biotechnology) overnight at 4 °C. The cells were washed with PBS twice and incubated with FITC conjugated secondary antibody (1:200; Biovision, China) for 2 h in the dark at 37 °C. After washing, images of stained cells were obtained using a fluorescence microscope.

## Flow cytometric detection of apoptosis

Using an Annexin V-PE/7-AAD Apoptosis Detection Kit (Bio-Vision, MA, USA), the apoptotic cells was evaluated by flow cytometry analysis (FACScan, BD Biosciences, San Jose, CA, USA).<sup>13</sup> The data were analyzed using the CellQuest software (BD Biosciences).

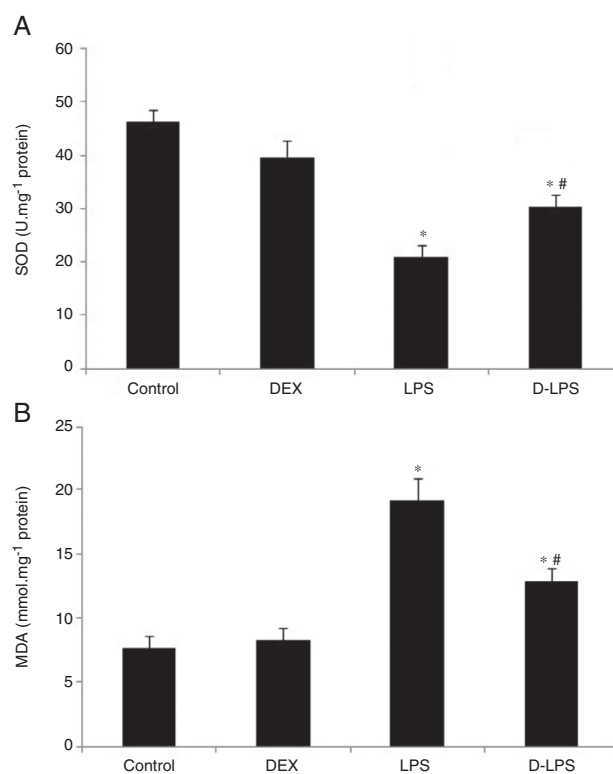
## Statistical analysis

The data are reported as the mean  $\pm$  SEM of five independent experiments. ANOVA and Student-Newman-Keuls (SNK) test were performed to determine statistical significance. Significant differences were established at  $p < 0.05$ .

## Results

### Effects of DEX on oxidative stress in the A549

Compared with the control group, LPS decreased SOD activity in the A549 ( $46.34 \pm 2.24 \text{ U} \cdot \text{mg}^{-1} \text{ protein}$  vs.  $20.86 \pm 2.19 \text{ U} \cdot \text{mg}^{-1} \text{ protein}$ ,  $p < 0.05$ ) (Fig. 1A) and increased the formation of MDA ( $7.68 \pm 0.92 \text{ nmol} \cdot \text{mg}^{-1} \text{ protein}$  vs.  $19.16 \pm 1.89 \text{ nmol} \cdot \text{mg}^{-1} \text{ protein}$ ,  $p < 0.05$ ) (Fig. 1B). Such an effect was significantly attenuated



**Figure 1** (A) SOD activity in the A549. (B) MDA levels in the A549. Control, without injury; DEX, dexmedetomidine; LPS, lipopolysaccharide; D-LPS, DEX + LPS. Values were presented as mean  $\pm$  SEM,  $n = 5$  for each group. \* $p < 0.05$  vs. Control group and # $p < 0.05$  vs. LPS group.

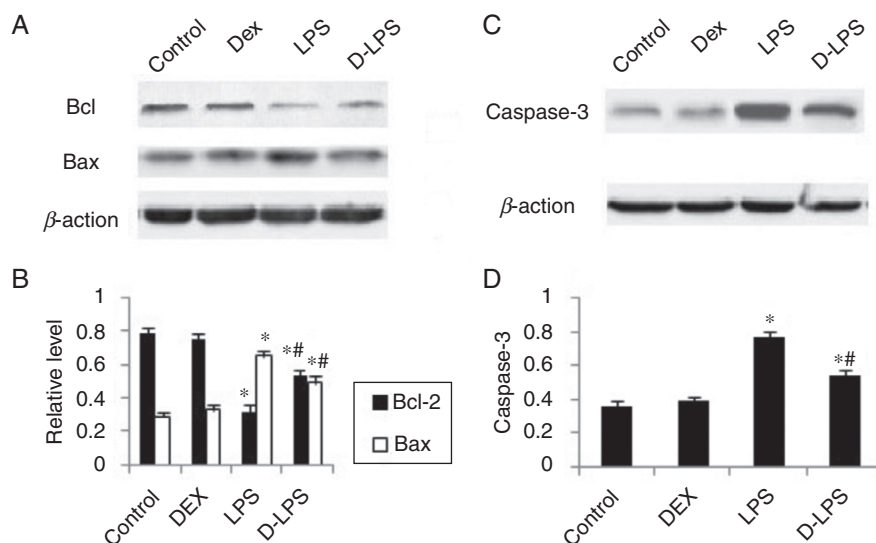
by DEX preconditioning ( $30.28 \pm 2.38 \text{ U} \cdot \text{mg}^{-1} \text{ protein}$  and  $12.84 \pm 1.05 \text{ nmol} \cdot \text{mg}^{-1} \text{ protein}$ ,  $p < 0.05$  vs. LPS group).

### Effects of DEX on the expression of Bcl-2, Bax and caspase-3

Compared with the control group, LPS decreased Bcl-2 level ( $0.79 \pm 0.03$  vs.  $0.32 \pm 0.04$ ,  $p < 0.05$ ) and increased the expression of Bax ( $0.29 \pm 0.03$  vs.  $0.65 \pm 0.04$ ,  $p < 0.05$ ) in the A549 (Fig. 2A and B). Such an effect was significantly attenuated by DEX preconditioning ( $0.53 \pm 0.03$  and  $0.49 \pm 0.04$ ,  $p < 0.05$  vs. LPS group). Compared with the control group, LPS increased the expression of caspase-3 ( $0.36 \pm 0.03$  vs.  $0.76 \pm 0.04$ ,  $p < 0.05$ ) in the A549 (Fig. 2C and D). Such an effect was significantly attenuated by DEX preconditioning ( $0.54 \pm 0.04$ ,  $p < 0.05$  vs. LPS group).

### Effects of DEX on cytochrome c release

Cytochrome c from the mitochondrial intermembrane space into the cytoplasm is a critical step in the progression of the intrinsic apoptotic pathway. In the control group cytochrome c had barely detectable level, whereas LPS resulted in significant rises. Such an effect of LPS was attenuated by DEX preconditioning (Fig. 3).



**Figure 2** (A) Representative picture of Bcl-2 and Bax. (B) The level of Bcl-2 and Bax. (C) Representative picture of caspase-3. (D) The level of caspase-3. Control, without injury; DEX, dexmedetomidine; LPS, lipopolysaccharide; D-LPS, DEX + LPS. Values were presented as mean  $\pm$  SEM,  $n=5$  for each group. \* $p < 0.05$  vs. Control group and # $p < 0.05$  vs. LPS group.

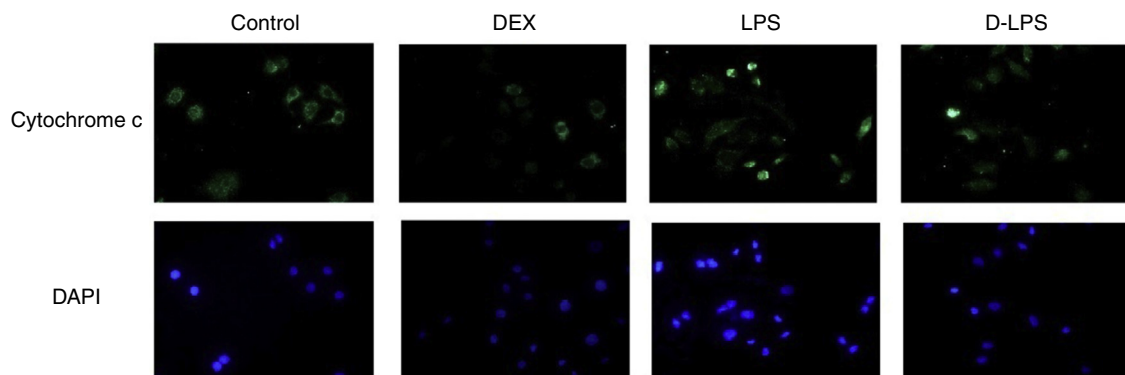
### Effects of DEX on apoptosis

Compared with the control group, LPS increased the percentage of apoptotic cells in the A549 ( $1.57\% \pm 0.52\%$  vs.  $33.58\% \pm 1.16\%$ ,  $p < 0.05$ ) (Fig. 4A and B). Such an effect was significantly attenuated by DEX preconditioning ( $26.43\% \pm 1.05\%$ ,  $p < 0.05$  vs. LPS group).

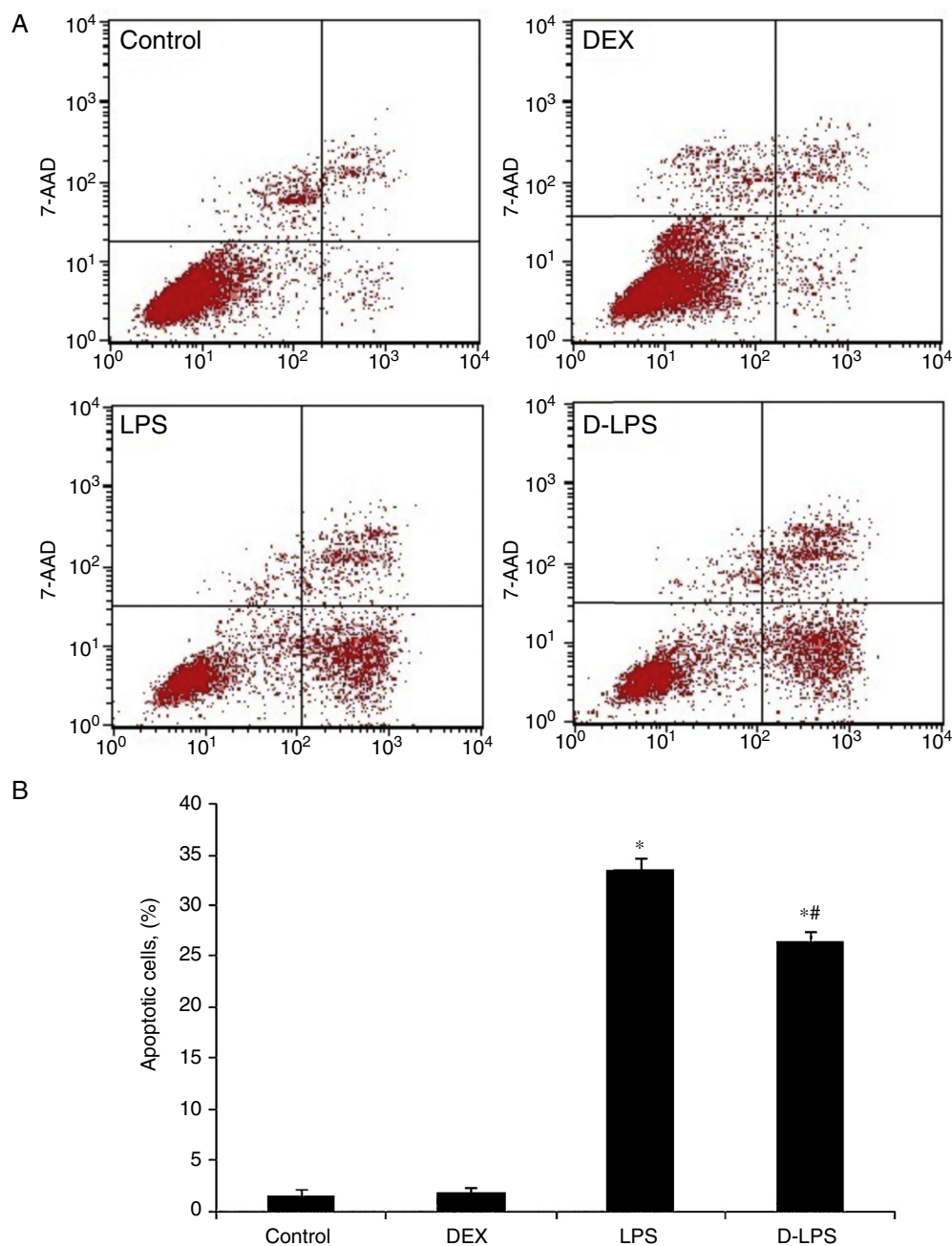
### Discussion

This study demonstrates that DEX preconditioning significantly decreased LPS-induced injury in the A549. The main findings are as follows: (1) DEX preconditioning reduced MDA and increased SOD activity in the A549; (2) DEX preconditioning increased the Bcl-2/Bax ratio and decreased the caspase-3 and cytochrome c level; (3) DEX preconditioning attenuated apoptosis in the A549. This data provides the first evidence that DEX preconditioning attenuates the A549 injury caused by LPS.

Studies have been reported that oxidative stress plays an important role on LPS-induced injury.<sup>14,15</sup> Consistent with the previous study, we also found the LPS-induced oxidative stress by increased MDA but decreased SOD in the A549. MDA is the degradation products of the oxygen-derived free radicals and lipid peroxidation. Therefore, the increase of MDA implies impairment of the normal membrane structure and oxidative tissue damage.<sup>16</sup> In contrast, SOD is thought to be an important intracellular antioxidant enzyme with multiple biological functions. With an increase in the antioxidant enzyme SOD, it can indicate the cellular capability of scavenging and quenching free radicals.<sup>17,18</sup> Previous studies also reported that DEX prevented peroxidation reactions by increased SOD but decreased MDA in tissues.<sup>19,20</sup> In the experiment, our results obviously demonstrated that DEX preconditioning resulted in an increase of the SOD activity and a decrease of the MDA in the A549. These findings both suggest that DEX preconditioning could protect against oxidative damage by LPS-induced in the A549, thereby modulating the cellular injury and dysfunction.



**Figure 3** Representative cytochrome c sections. Control, without injury; DEX, dexmedetomidine; LPS, lipopolysaccharide; D-LPS, DEX + LPS. The green fluorescence shown cytochrome c and the blue fluorescence (DAPI) shown DNA. Original magnification  $400\times$ .



**Figure 4** (A) Representative picture of flow cytometer. (B) The grade of cell apoptosis. Control, without injury; DEX, dexmedetomidine; LPS, lipopolysaccharide; D-LPS, DEX + LPS. Values were presented as mean  $\pm$  SEM,  $n = 5$  for each group. \* $p < 0.05$  vs. Control group and # $p < 0.05$  vs. LPS group.

Several evidences have indicated that oxidative stress was involved in underlying pathological mechanisms of LPS-induced apoptosis in A549.<sup>21,22</sup> As suggested from our results, we also found that LPS had significantly reduced ratio of Bcl-2/Bax associated with the increase of MDA and the decrease of SOD activity in A549. Both Bcl-2 and Bax belong to Bcl-2 family. However, Bcl-2 may be regarded as an important cellular component that not only guards against apoptotic cell death but also influences multiple cellular events. In recent studies, Bcl-2 was found to protect against LPS-induced injury in some organs including A549.<sup>23,24</sup> In contrast, Bax exhibits proapoptotic actions. When Bax was

over expressed, it may form channels or pores allowing for the release of factors such as cytochrome c from the mitochondria to propagate apoptosis.<sup>25</sup> Thus the Bcl-2/Bax protein ratio is likely critical for cell survival after injury.<sup>24</sup> In the present study, our results indicated that DEX increased the expression of Bcl-2 and decreased the expression of Bax, leading to an increase of the Bcl-2/Bax ratio.

Cytochrome c release and caspase-3 activation is pivotal point in the apoptotic cascade and can be regulated by the Bcl-2/Bax ratio.<sup>26</sup> Previous studies showed that excessive generation of oxidation might cause cytochrome c release and caspase-3 activation.<sup>25</sup> And the process can be



depressed by Bcl-2. The released cytochrome c and activated caspase-3 cleaves downstream critical cellular targets involved in chromatin condensation, DNA fragmentation, and cytoskeletal destruction, thereby expressing the dramatic morphological changes of apoptosis.<sup>25–27</sup> LPS has been demonstrated to be one of the ways of inducing cytochrome c release and caspase-3 activation in A549.<sup>28</sup> In the present study, our data also shows that cytochrome c release and caspase-3 activation significantly increased after LPS administration in the A549, consistent with the previous studies. Furthermore, we observed that the cytochrome c release and caspase-3 activation was depressed by DEX preconditioning.

Apoptosis is a fundamental process of cell death that occurs via activation of distinct signaling pathways involving down-regulation of Bcl-2/Bax ratio and release of cytochrome c and activation of caspase-3 in vitro.<sup>28,29</sup> Ultimately, cells undergo destruction and formation of apoptotic bodies.<sup>30</sup> LPS have been shown to initiate this apoptotic cascade in A549.<sup>24</sup> Using an in vitro model of ALI, our data demonstrate significant reduction of apoptosis after DEX preconditioning, which correlated with up-regulation of Bcl-2/Bax ratio and depression of caspase-3 activation and cytochrome c release.

However, the descriptive study has some obvious limitations that need to be addressed. Which included one sampling time point, the brief period of observation, and lack of correlation with clinical measurements of ALI. Moreover, we have only investigated single DEX concentration, which is very large to the clinical practice. Compared with clinical, a large dose is usually used in laboratory.<sup>31,32</sup> Some studies even reported a heavy dose in experiment. These may be related with different species.<sup>31,32</sup> The relation between dose and effect remains unclear. Therefore, studies related to dose–effect relationship need further exploration in the model.

## Summary

Our data show that DEX preconditioning can effectively attenuate the LPS-induced injury in A549. The protective effects may involve a reduction in oxidative stress and cell death induced by LPS. These experimental results suggested that DEX may be efficacious in the treatment of LPS-induced ALI.

## Conflict of interest

The authors declare no conflicts of interest.

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