

Original Article

Remifentanil ameliorates lung injury in neonate rats with acute respiratory distress by down-regulating TIMP1 expression

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Abstract: Acute respiratory distress syndrome (ARDS) is a critical clinical disease characterized by diffuse inflammation of lung parenchyma and refractory hypoxemia. Remifentanil has been reported to act as an anti-inflammatory antioxidant in a variety of diseases. However, whether Remifentanil has a protective effect in ARDS and its mechanism remains to be further studied. This study was designed to investigate the effects of Remifentanil on ARDS in neonate rats. In this study, we established the model of acute respiratory distress in neonate rats. To study the effects of Remifentanil on ARDS through a series of *in vitro* and *in vivo* experiments. Furthermore, the overexpression vector of recombinant tissue inhibitors of metalloproteinase 1 (TIMP1) was injected into the neonate rat before the operation to explore the effect of TIMP-1 overexpression on acute respiratory distress rats through the above experiments. Remifentanil reduced lung injury in rats with acute respiratory distress, reduced inflammation, oxidative stress and tissue cell apoptosis in rats with acute respiratory distress. Remifentanil inhibited the expression of TIMP-1 in rats with acute respiratory distress, and TIMP-1 overexpression inhibited the protective effect of Remifentanil on rats with acute respiratory distress. Remifentanil can reduce lung injury and inflammatory response in young mice with acute respiratory distress and play a protective role by down-regulating the expression of TIMP-1.

Keywords: Remifentanil, acute respiratory distress syndrome, TIMP-1, neonate rat

Introduction

Acute respiratory distress syndrome (ARDS) is a critical clinical illness characterized by diffuse pulmonary inflammation, non-cardiogenic pulmonary edema and respiratory failure [1, 2]. The role of oxidative stress and inflammatory response in its pathogenesis is critical and has complex interactions [3, 4]. Oxidative stress is a state of tissue and organ damage caused by the imbalance between the body's oxidation and antioxidant systems, while inflammatory response is a host response to pathogen stimulation or tissue damage, and its overactivation will cause body damage [5]. The two promote and complement each other, playing a key role in the occurrence and development of diseases [6].

Remifentanil is a kind of synthetic opioids for cesarean delivery analgesia, are reported in a variety of diseases in the role of anti-inflamma-

tory antioxidants, in neonate brain injury, Remifentanil significantly reduces the production of ROS, cortex cysteine protease activity, DNA fragmentation, the levels of interleukin 1 beta and reactive stellate [7] and fentanyl may inhibit the Fas/FasL signal transduction pathways to reduce myocardial cell apoptosis and oxidative stress and inflammation induced by IR [8], Remifentanil inhibited the increase of il-6 mRNA level in the brain of mice, and inhibited the plasma il-6, corticosterone and norepinephrine responses in the inflammatory state [9]. However, the effect of Remifentanil on acute respiratory distress in neonate mice has not been studied.

Matrix metalloprotein 9 (MMP9) was found when the target gene of Remifentanil was searched by STITCH website, and recombinant tissue inhibitors of metalloproteinase 1 (TIMP1) was found when the protein interacting with MMP9 was predicted by string website. It was

reported that the expression of TIMP1 was increased in rats with acute respiratory distress [10]. Compared with TIMP1 $-/-$ mice infected with influenza, WT mice lost significantly more weight. Wild-type mice showed more immune cell infiltration and airway inflammation [11]. Similarly, the role of TIMP1 in lung injury in neonate rats with acute respiratory distress has not been studied. Hence, this study was designed to investigate the effects of Remifentanil on ARDS in neonate rats, which will be of critical significance for the clinical treatment of ARDS.

Materials and methods

Animals

Sprague-Dawley (SD) neonate rats, weight 44–61 g, were obtained from the Hangzhou Children's Hospital. All animals received human care and were housed under controlled temperature-controlled condition and were access to food and water. All experimental operations performed on all rats were approved by the Animal Experiment Ethics Committee of Hangzhou Children's Hospital.

The method of ARDS model preparation

The standard establishment step mainly refer to the report of Li et al. [12]. In brief, neonate rats were given intraperitoneal injection of anaesthetic to anesthetize ketamine and toluenthiazide by normal saline lavage and mechanical ventilation. It was then placed in a heated water bath at 37°C for tracheotomy and the blunt cannula was inserted and secured. Ventilate with 100% oxygen at a rate of 30 breaths per minute with a volume (VT) of 8 ml/kg, an inhale/exhale ratio of 1:2, PEEP of 3 cm H₂O. Pampermine is injected into the muscles to relax them and prevent spontaneous breathing. After 15 minutes of ventilation, VT was increased to 16 ml/kg, PEEP was increased to 8 cm H₂O, followed by 15 minutes of ventilation. The lungs were then washed 10 times with preheated saline to deplete the surfactant and ventilate again for 3.5 h. Intraperitoneal administration of ketamine/toluene thiazide and pencuprimo was maintained at half the initial dose every 45 minutes to maintain anaesthesia and muscle relaxation. The oxygenation index was less than 150 mmHg, and the dynamic lung compliance was reduced to 50%.

Dry/wet ratio of lung

Partial resection of the right upper right lung of the rat and placed in an oven at 70°C for 48 h to constant weight to calculate the lung wet/dry weight ratio (W/D).

Histopathological analysis

Appropriate weight lung tissues were conventionally fixed in 10% buffered formalin overnight and the tissues were embedded in paraffin. Then, all the sections were dehydrated with hematoxylin and eosin (HE). The stained slides were observed and evaluated under a light microscope (Olympus Corp., Tokyo, Japan) using 200× magnification (100 fields per section).

Enzyme-linked immunosorbent assay (ELISA)

The expression of TNF- α , IL-1 β , IL-6 ICAM-1 and MIP-2 in serum and the levels of MDA, MPO and NADPH in lung tissues were determined using ELISA assay kits (NeoBioscience Technology Co., Ltd.) with a microplate reader (MULTISKAN MK3, Thermo, San Jose, CA, USA) according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was employed to test apoptosis of alveolar epithelial cells in accordance to the manufacturer's instruction (Millipore, Sigma, Unite States).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA sample was extracted by using Trizol Reagent according to the manufacturer's instructions. The synthesis of cDNA was carried out with the RevertAid™ First Strand cDNA Synthesis Kit. QRT-PCR was performed using the SYBR Green Master Mix I (TaKaRa, Otsu, Shiga, Japan) on the ABI 7900 Fast Real-Time PCR System (ABI, Foster City, CA, USA). The reaction conditions were set as follows: 10 min at 95°C followed by 35 cycles of 15 s at 95°C and 40 s at 55°C. GAPDH was used as the housekeeping gene and relative quantification was performed using the 2^{- $\Delta\Delta C_T$} method [13]. The primers were designed and synthe-

Remifentanil ameliorates lung injury

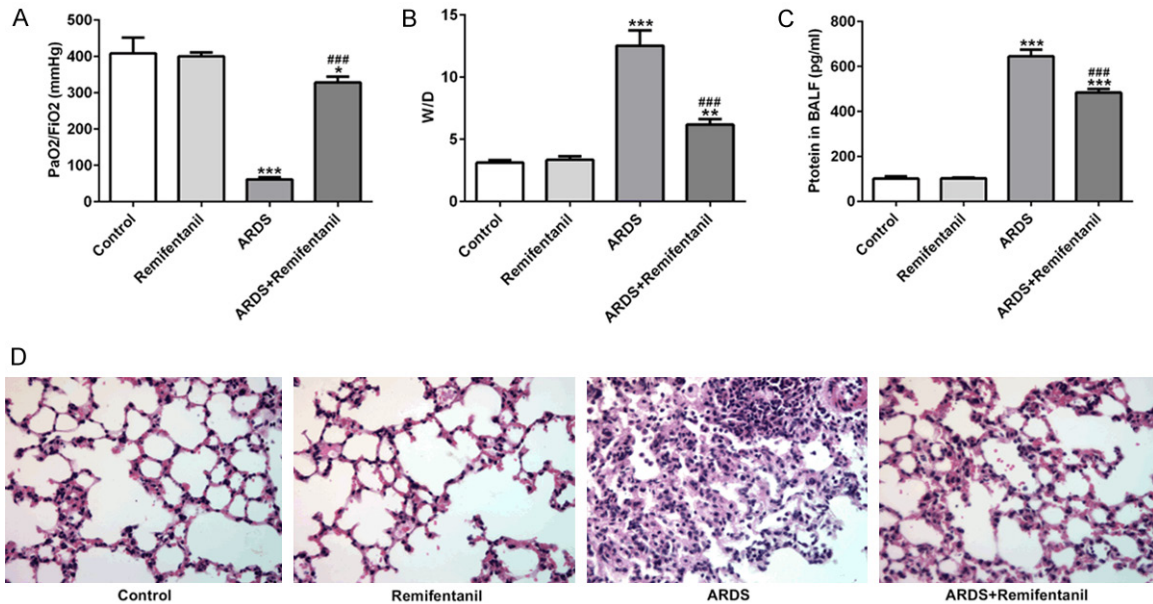


Figure 1. Remifentanil alleviated lung injury in rats with acute respiratory distress. (A) The pulmonary function was tested by $\text{PaO}_2/\text{FiO}_2$ ratio. We measured lung wet-dry ratio (W/D) (B) and total protein level (C) in BALF in control, remifentanil, ARDS and ARDS+remifentanil group. (D) Representative images of H&E staining from each experimental group at 12 h after administration. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. ARDS.

sized by Gemma (Shanghai, China). The primer sequences used were as followed: TIMP-1 forward, 5'-TCTGGCATCCTCTTGTG-3', and reverse 5'-GGTGGTCTCGTTGATTCT-3'; GAPDH forward, 5'-CCAGGGGTGCCTTCTCTT-3', and reverse 5'-CCGTGGGTAGAGTCATACTGG.

Western blot assay

The cells were harvested and total proteins were obtained using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein was separated on 10% SDS-PAGE gel with a 10% gel and subsequently transferred to PVDF membranes (EDM Millipore). The membranes were blocked with 5% non-fat milk for 2 h, followed by incubation overnight for 4°C with the following primary antibodies: anti-B-cell lymphoma (Bcl)-2 (1:1,000, #3498, Cell Signaling Technology, Inc.), anti-BCL2 associated X (Bax) (1:1,000, #5023, Cell Signaling Technology, Inc.), anti-cleaved-caspase-3 (1:500, #9579, Cell Signaling Technology, Inc.) and GAPDH (1:2,000, #MAB374, EDM Millipore). As a secondary antibody, mouse anti-rabbit (1:10000, sc-2357, Santa Cruz), rabbit anti-mouse (1:10000, sc-358914, Santa Cruz), the membrane was incubated for 2 h at room temperature. Proteins were detected by using enhanced

chemiluminescence and imaged. β -actin was used as an internal control.

Statistical analysis

All experiments were repeated at least three times, and data were expressed as the mean $\pm$ standard deviation (SD). SPSS 17.0 software was used to conduct all statistical analyses (SPSS, Inc., USA). A one-way ANOVA followed by a Tukey's or Dunnett's test was performed using GraphPad Prism 5 software (GraphPad Software, Inc.) to determine statistical comparisons between groups. Differences with $P < 0.05$ were considered significant.

Results

Remifentanil alleviated lung injury in rats with acute respiratory distress

We tested the pulmonary function by $\text{PaO}_2/\text{FiO}_2$ ratio, as shown in **Figure 1A**, $\text{PaO}_2/\text{FiO}_2$ ratio was significantly decreased in ARDS group compared with control group, while remifentanil treatment significantly increased $\text{PaO}_2/\text{FiO}_2$ ratio compared with ARDS group. Simultaneously, we measured lung W/D (**Figure 1B**) and total protein level (**Figure 1C**) in BALF in control, remifentanil, ARDS and ARDS+

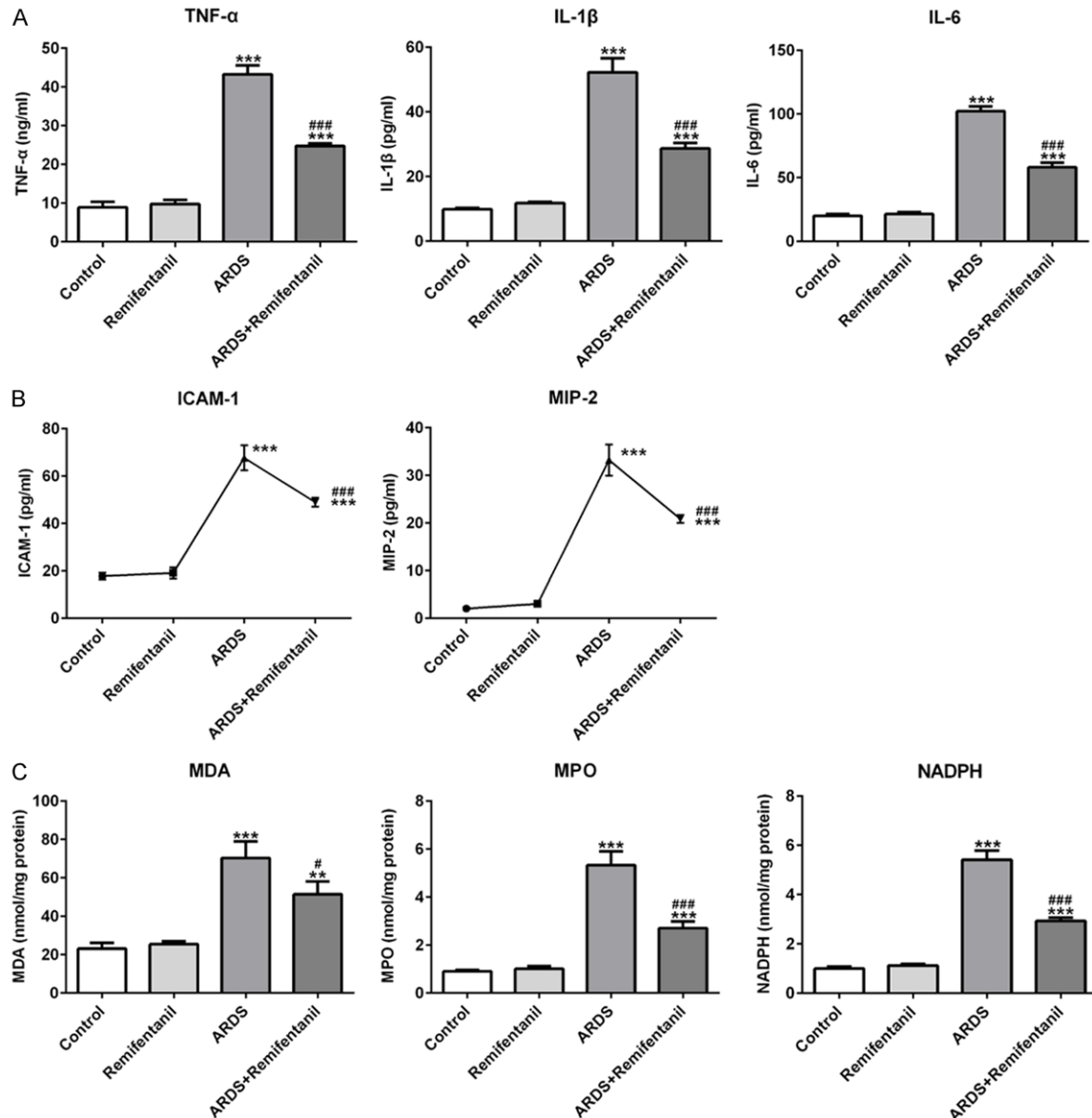


Figure 2. Remifentanyl treatment alleviates inflammation and oxidative stress in rats with acute respiratory distress. A. The levels of TNF- α , IL-1 β and IL-6 were detected by ELISA. B. The expression of ICAM-1 and MIP-2 in serum were detected by ELISA. C. The level of oxidative stress factors (MDA, MPO and NADPH) in lung tissues was detected with commercial kits. ** $P < 0.01$ and *** $P < 0.001$ vs. control; # $P < 0.05$ and ### $P < 0.001$ vs. ARDS.

remifentanyl group. The results showed that remifentanyl treatment significantly reduced the degree of lung edema. Furthermore, we observed pulmonary histopathology by H&E staining to assess the protective effect of remifentanyl, as shown in **Figure 1D**, the lung tissues in control group and remifentanyl group present normal structure. The ARDS group showed neutrophil infiltration, protein edema fluid, intravascular coagulation, alveolar hemorrhage, and alveolar walls disruption. However, these typical features were visibly attenuated in rats treated with remifentanyl. Taken together,

remifentanyl alleviated lung injury in rats with acute respiratory distress.

Remifentanyl treatment alleviates inflammation and oxidative stress in rats with acute respiratory distress

The effect of remifentanyl on the expression of inflammatory cytokines in rats with acute respiratory distress was explored. The levels of TNF- α , IL-1 β and IL-6 were detected by ELISA. As presented in **Figure 2A**, the levels of TNF- α , IL-1 β and IL-6 were increased notably in ARDS

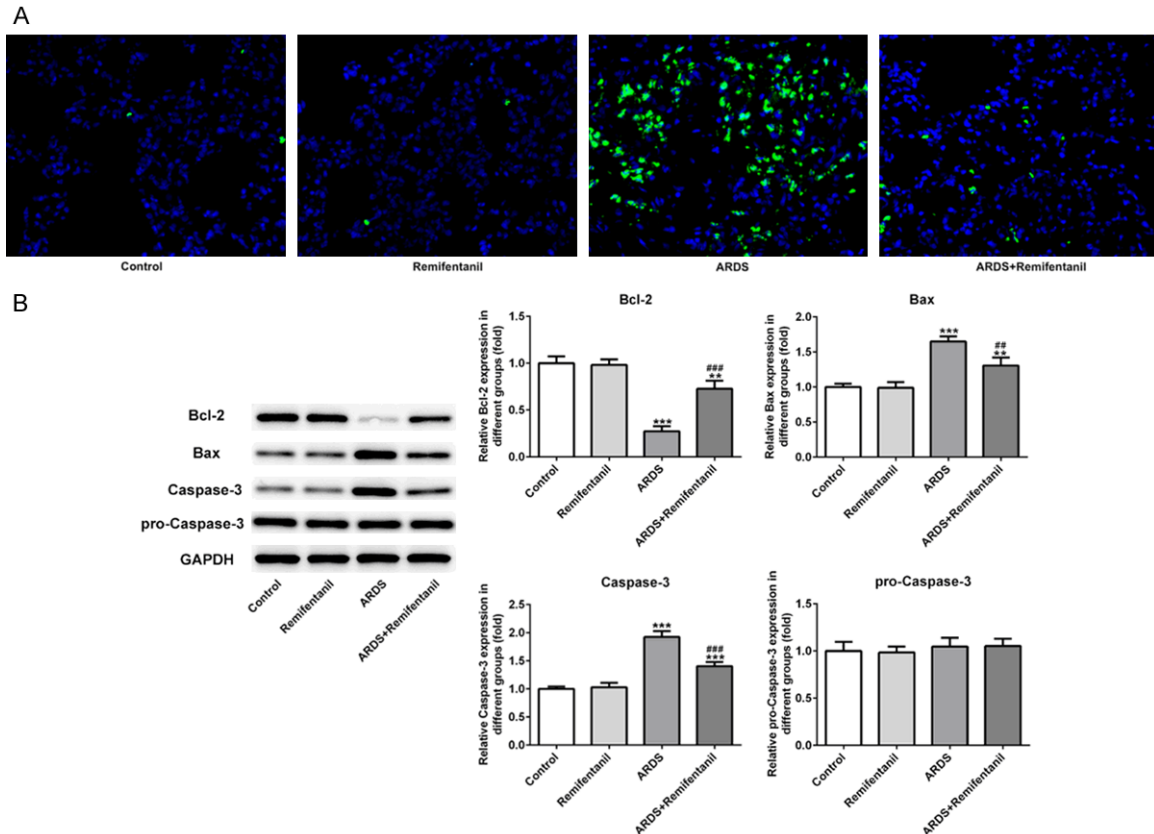


Figure 3. Remifentanyl treatment alleviates cell apoptosis in rats with acute respiratory distress. A. TUNEL assay was applied for the detection of cell apoptosis. B. The protein expression levels of the Bcl-2, Bax and Caspase-3 were detected by Western blot analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. control; ** $P < 0.01$ and *** $P < 0.001$ vs. ARDS.

group. Following treatment with remifentanyl, the levels of TNF- α , IL-1 β and IL-6 were significantly decreased. Above data indicated that remifentanyl treatment decreased the levels of inflammatory factor. Furthermore, the expression of ICAM-1 and MIP-2 in serum were detected by ELISA, the ICAM-1 and MIP-2 level in remifentanyl treatment (ARDS+remifentanyl) group was decreased dramatically compared to ARDS group (**Figure 2B**). The level of oxidative stress factors (MDA, MPO and NADPH) in lung tissues was detected with commercial kits (**Figure 2C**).

Overall, these results indicate that remifentanyl treatment alleviates inflammation and oxidative stress in rats with acute respiratory distress.

Remifentanyl treatment alleviates cell apoptosis in rats with acute respiratory distress

The TUNEL results indicated that ARDS markedly increase the cell apoptosis rate compared with

control group (**Figure 3A**) and remifentanyl treatment could treatment alleviates cell apoptosis compared with ARDS group. At the same time, the protein expression levels of the Bcl-2, Bax and Caspase-3 were detected by Western blot analysis. As the results showed that, the protein expression of Bcl-2 was reduced, while the protein expression of Bax and Caspase-3 was increased after remifentanyl treatment (**Figure 3B**).

Overexpression of TIMP-1 inhibited the protective effect of remifentanyl on rats with acute respiratory distress

MMP9 was found when the target gene of Remifentanyl was searched by STITCH website, and TIMP-1 was found when the protein interacting with MMP9 was predicted by string website. We detected the expression of TIMP-1 in serum by qRT-PCR. TIMP-1 was dramatically higher in ARDS group than that of control group, and dramatically lower in remifentanyl treatment (ARDS+remifentanyl) group than that of

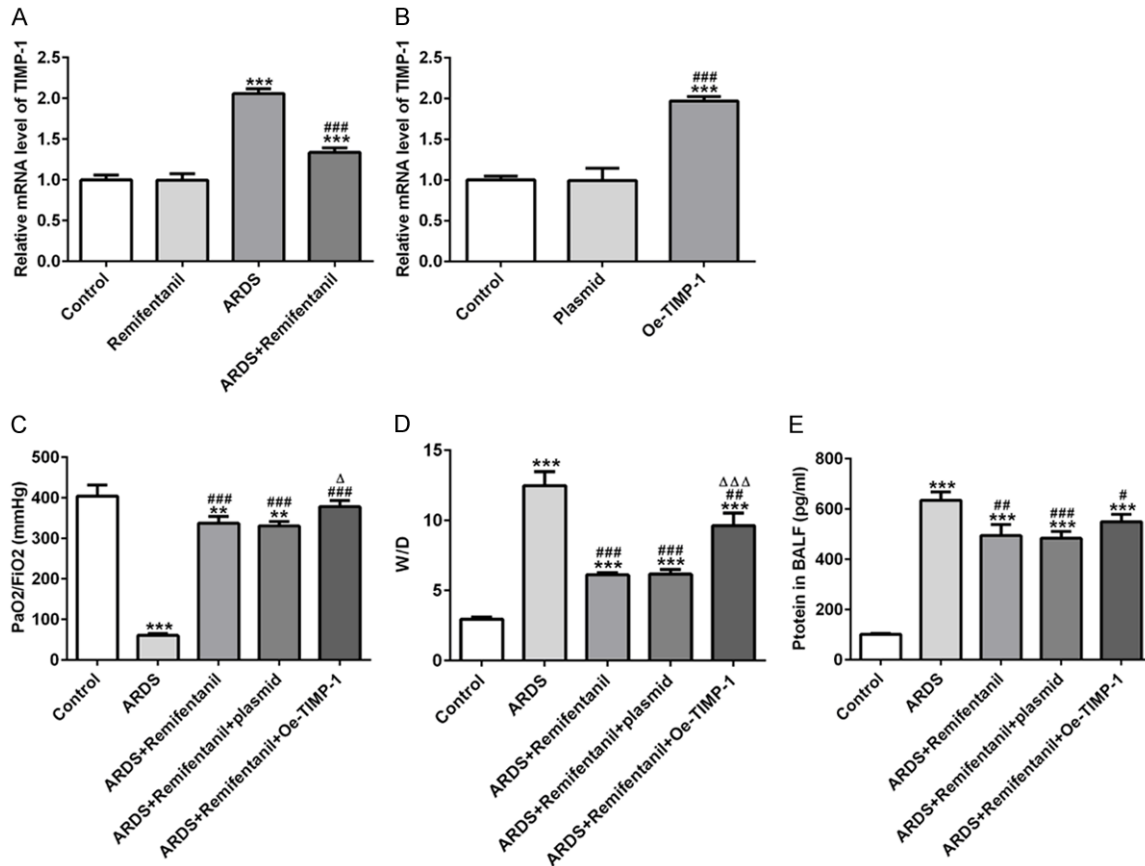


Figure 4. Overexpression of TIMP-1 inhibited the protective effect of remifentanil on rats with acute respiratory distress. (A) The expression of TIMP-1 in serum by qRT-PCR. (B) The transfection efficiency of Overexpression-TIMP-1 was measured by qRT-PCR. The results of PaO₂/FiO₂ ratio (C), (W/D) ratio (D) and total protein level (E) in BALF in ARDS+Remi+Oe-TIMP-1 (ARDS rat injection with Overexpression-TIMP-1) group show that Overexpression of TIMP-1 inhibited the protective effect of remifentanil on lung injury in rats with acute respiratory distress. **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. ARDS; ^ΔP<0.05 and ^{ΔΔΔ}P<0.001 vs. ARDS+remifentanil+plasmid.

ARDS group (Figure 4A). Then, we investigated the role of TIMP1 in lung injury in newborn rats with acute respiratory distress, as shown in Figure 4B, the transfection efficiency of Overexpression-TIMP-1 was measured by qRT-PCR. The results of PaO₂/FiO₂ ratio (Figure 4C), (W/D) ratio (Figure 4D) and total protein level (Figure 4E) in BALF in ARDS+Remi+Oe-TIMP-1 (ARDS rat injection with Overexpression-TIMP-1) group show that Overexpression of TIMP-1 inhibited the protective effect of remifentanil on lung injury in rats with acute respiratory distress. Lung histopathology was observed by H&E staining, which further demonstrated that TIMP-1 overexpression inhibited the protective effect of remifentanil on rats with acute respiratory distress (Figure 5A). Furthermore, the level of inflammation factors

(TNF-α, IL-1β and IL-6) (Figure 5B), ICAM-1, MIP-2 (Figure 6A) and oxidative stress factors (MDA, MPO and NADPH) (Figure 6B) were measured by ELISA. The results demonstrate that Overexpression of TIMP-1 inhibited the effects of remifentanil on inflammation and oxidative stress in rats with acute respiratory distress. Tunnel assay (Figure 7A) and western blot (Figure 7B) results demonstrated that overexpression of TIMP-1 inhibited the cell apoptosis of remifentanil on rats with acute respiratory distress.

Discussion

ARDS is the barrier destruction of alveolar epithelial-mesenchym-endothelial complex caused by pathogenic factors inside and outside the lung, and the accumulation of high protein

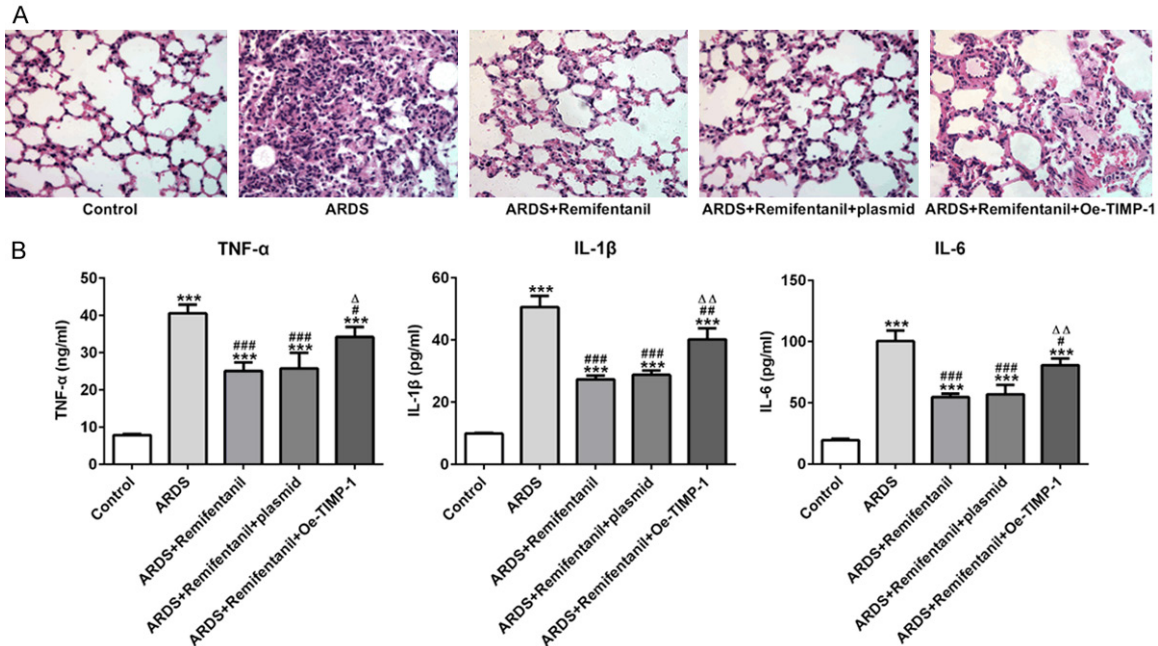


Figure 5. Overexpression of TIMP-1 inhibited the protective effect of remifentanyl on rats with acute respiratory distress. A. Lung histopathology was observed by H&E staining. B. The level of inflammation factors (TNF- α , IL-1 β and IL-6) were measured by ELISA. *** P <0.001 vs. control; # P <0.05, ## P <0.01 and ### P <0.001 vs. ARDS; Δ P <0.05 and $\Delta\Delta$ P <0.01 vs. ARDS+remifentanyl+plasmid.

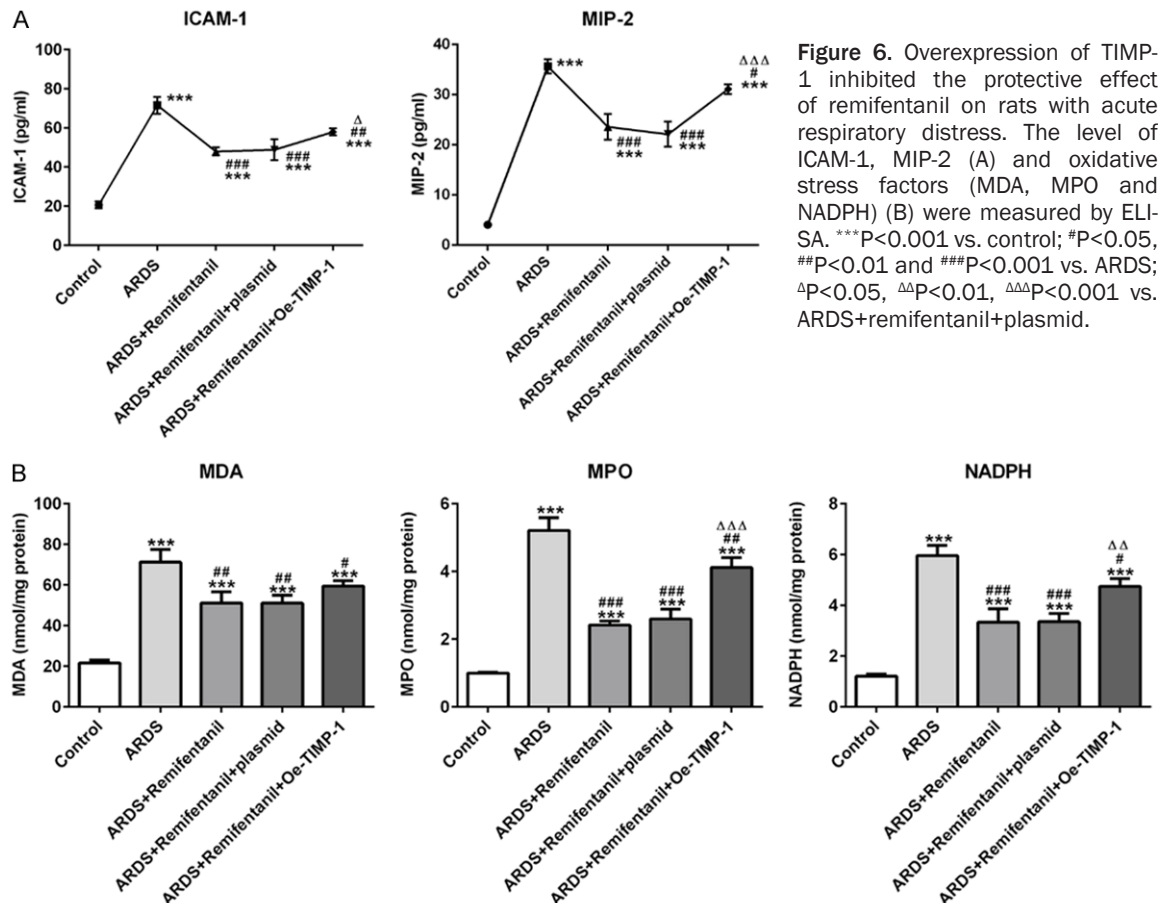


Figure 6. Overexpression of TIMP-1 inhibited the protective effect of remifentanyl on rats with acute respiratory distress. The level of ICAM-1, MIP-2 (A) and oxidative stress factors (MDA, MPO and NADPH) (B) were measured by ELISA. *** P <0.001 vs. control; # P <0.05, ## P <0.01 and ### P <0.001 vs. ARDS; Δ P <0.05, $\Delta\Delta$ P <0.01, $\Delta\Delta\Delta$ P <0.001 vs. ARDS+remifentanyl+plasmid.

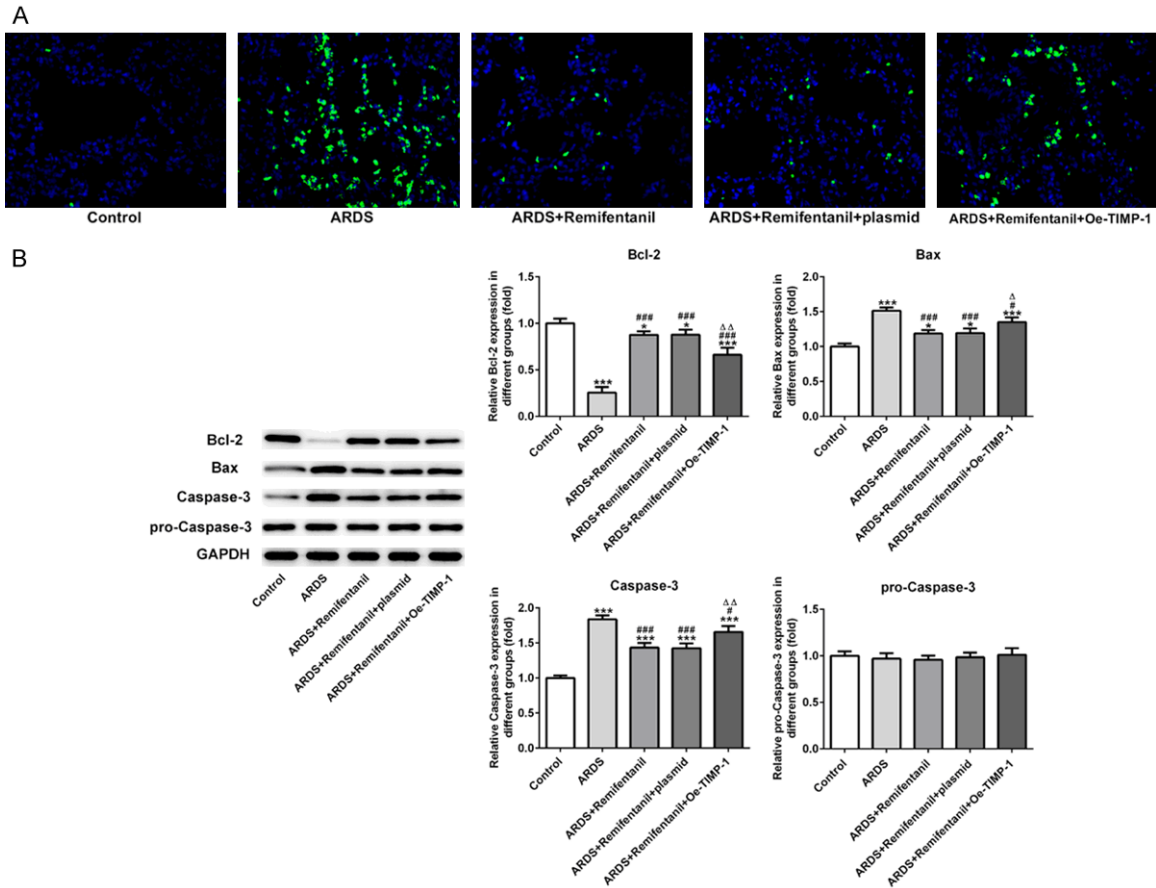


Figure 7. Overexpression of TIMP-1 inhibited the protective effect of remifentanyl on rats with acute respiratory distress. Tunnel assay (A) and western blot (B) results demonstrated that overexpression of TIMP-1 inhibited the cell apoptosis of remifentanyl on rats with acute respiratory distress. *** $P < 0.001$ vs. control; # $P < 0.05$ and ### $P < 0.001$ vs. ARDS; $\Delta P < 0.05$ and $\Delta\Delta P < 0.01$ vs. ARDS+remifentanyl+plasmid.

secretions in the lung stroma and alveoli, which leads to the ventilation and diffusion dysfunction of the lung tissue, and further leads to the clinical syndrome of refractory hypoxemia [14, 15]. Its typical histopathological features are damage of alveolar epithelial cells and pulmonary vascular endothelium, infiltration of inflammatory cells in alveolar septum and alveolar cavity, partial congestion and edema, collapse and fusion of a large number of alveoli, and collagen deposition in alveoli and interstitial lung, and infiltration and fibrosis [16]. In this study, we successfully established a classical animal model of ARDS, the lung histopathological results suggested that the normal structure of the lung tissue was destroyed, edema fluid was filled in the alveoli, diffuse inflammatory cell infiltration in the alveolar cavity and interstitium, and alveolar septum was widened. In addition, the lung tissue W/D value increased

and the arterial blood oxygenation index decreased. There was an increase in inflammatory cells and an increase in inflammatory cytokines in BALF. These are consistent with previous research results [17, 18].

Inflammatory factors can destroy the integrity of pulmonary capillaries, increase vascular permeability, further induce pulmonary edema, leading to respiratory dysfunction [19]. Remifentanyl is a kind of synthetic opioids for cesarean delivery analgesia, are reported in a variety of diseases in the role of anti-inflammatory antioxidants [20]. A large number of studies have shown that the opioid receptor agonist remifentanyl can alleviate ALI by inhibiting the release of inflammatory cytokines and has a pulmonary protective effect on LPS-induced ALI animals [21, 22]. Studies have shown that oxidative stress can also increase the permea-

bility of endothelial and epithelial cells, impair the function of alveolar epithelial cells, and accelerate lung injury [23]. The results showed that SOD content and lung injury were negatively correlated with [24]. Yoon et al. found that remifentanil can exert cellular protection by inhibiting H₂O₂-induced oxidative stress [25, 26]. This study found that remifentanil treatment alleviates inflammation and oxidative stress in rats with acute respiratory distress via down-regulating TIMP1 expression. Studies have shown that apoptosis of endothelial and epithelial cells can accelerate destruction in alveolar capillaries [27].

ICAM-1 is a member of the immunoglobulin superfamily inflammatory factors are released when inflammation occurs and induce endothelial cells and other cells to express ICAM-1 on the cell surface. In the present study, the expression of ICAM-1 was significantly decreased in ARDS+Remi compared with ARDS group, it showed that the administration of remifentanil reduced the release of certain cytokines and proinflammatory factors in ARDS, and protected cells to some extent.

Apoptosis is the result of multiple molecular mechanisms. The release of a large number of inflammatory factors can directly induce cell apoptosis [23]. The Caspase family is the ultimate executor of apoptosis, and inhibition of Caspase activity can alleviate ARDS in mice. We have demonstrated that remifentanil treatment alleviates inflammation and oxidative stress in rats with acute respiratory distress. In addition, remifentanil can significantly inhibit the apoptotic and the expression of caspase-3 in the lung tissues of ARDS rats. These results suggested that remifentanil treatment alleviates cell apoptosis in rats with acute respiratory distress.

Conclusion

In conclusion, we found that remifentanil can reduce lung injury and inflammatory response in young mice with acute respiratory distress and play a protective role by down-regulating the expression of TIMP-1, which will be of critical significance for the clinical treatment of ARDS.

Disclosure of conflict of interest

None.

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