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Original Research Article

Perfluorocarbon restrains inflammation and cell apoptosis in rats with lung ischemia-reperfusion injury via down-regulation of TLR4/NF-κB signaling pathway

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Abstract

Purpose: *To exaluate the effect of perfluorocarbon on lung ischemia-reperfusion injury in rats, and to unravel the potential underlying mechanism.*
Methods: *A total of 36 Sprague-Dawley (SD) rats were randomly assigned to sham group, model group, and perfluorocarbon group (12 rats per group). The levels of inflammatory factors (TNF-α and IL-1β) were determined using enzyme-linked immunosorbent assay (ELISA). Cell apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, while Western blotting was conducted to determine the protein expressions of TLR4 and NF-κB.*
Results: *The levels of inflammatory factors in the model and perfluorocarbon groups were significantly higher than those in operation group ($p < 0.05$), while their levels in perfluorocarbon group were significantly lower than in model group ($p < 0.05$). The mRNA expression levels of TNF-α and IL-1β in lung tissues rose significantly in both model and perfluorocarbon groups when compared with those in sham group ($p < 0.05$), but declined significantly in the perfluorocarbon group in comparison with those in model group ($p < 0.05$). Furthermore, the perfluorocarbon group exhibited a significantly lower cell apoptosis than model group ($p < 0.05$). The relative protein expression levels of TLR4 and NF-κB declined significantly in perfluorocarbon group than in model group.*
Conclusions: *Perfluorocarbon down-regulates the TLR4/NF-κB signaling pathway, and inhibits inflammation and cell apoptosis after lung ischemia-reperfusion injury in rats, thereby improving their lung function.*

Keywords: *Lung ischemia-reperfusion, Perfluorocarbon, TLR4/NF-κB signaling pathway, Inflammation, Apoptosis*

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INTRODUCTION

Lung ischemia-reperfusion injury is not only a common pathological response process in lung injury, but it is also a prevalent and inevitable complication in clinical lung surgeries. A plethora of inflammatory factors, cytokines, and cytotoxic substances are produced in ischemia and hypoxia, as well as in blood reperfusion, and is caused by the sudden restoration of blood

supply. This triggers multiple pathological responses, thus aggravating lung tissue injury and further affecting lung function [1,2].

Studies have demonstrated that lung ischemia-reperfusion injury, one of the important pathological lung injuries, induces a series of pathological responses such as inflammation, cell apoptosis, and autophagy [3-5]. In the case of inflammation, which is the most common pathological response in lung ischemia-reperfusion injury, massive inflammatory factors such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 and IL-1 beta (IL-1β) may infiltrate the lung tissues, thus exacerbating lung injury [3-5]. As an important inflammation-associated signaling pathway in the body, the Toll-like receptor 4 (TLR4)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, once aberrantly activated, can induce the release of large numbers of inflammatory factors to mediate inflammation [6,7]. Hence, the TLR4/ARE signaling pathway has been confirmed to play a pivotal role in regulating inflammation in lung ischemia-reperfusion injury.

Perfluorocarbon is a fluorocarbon that has been proven to be able to protect tissues and organs in the body well and inhibit inflammation [8], but its mechanisms remain elusive. The present study, therefore, aims to verify whether perfluorocarbon affects lung ischemia-reperfusion injury in rats through down-regulation the of TLR4/NF-κB signaling pathway.

EXPERIMENTAL

Laboratory animals

Thirty-six Sprague-Dawley (SD) rats weighing 200 ± 10 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and fed in the Laboratory Animal Center of Ningxia Medical University. All the rats had free access to purified water and adequate diets and were housed under the 12 h/12 h light/dark cycle. Besides, they were adaptively fed in the Laboratory Animal Center for 7 days before experiments. This study was approved by the Animal Ethics Committee of Ningxia Medical University Animal Center (approval no. 18-NX-no. 011). All procedures were conducted in accordance with the 'Animal Research: Reporting *in vivo* Experiments guidelines 2.0' [9].

Reagents

The reagents used in this study included: Perfluorocarbon (Sigma, St. Louis, MO, USA), anti-TLR4 and anti-NF-κB primary antibodies and secondary antibodies (Abcam, Cambridge, MA, USA), and kits for hematoxylin and eosin (H & E) staining, enzyme-linked immunosorbent assay (ELISA) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Maxim, Fuzhou, China), and quantitative polymerase chain reaction (qPCR) (Vazyme, Nanjing, China).

Grouping of rats and initial treatment

The 36 Sprague-Dawley (SD) rats were divided into sham operation group (n = 12), model group (n = 12), and perfluorocarbon group (n = 12), using a random number table. In the perfluorocarbon group, the rats first underwent thoracotomy to expose pulmonary vessels. Then, perfluorocarbon was injected *via* the femoral vein at a dose of 5 mg/kg, and the lung ischemia-reperfusion injury model was established 30 min later, after which 5 mg/kg perfluorocarbon was injected *via* the femoral vein daily. In the model group, the thoracic cavity of rats was first opened to expose the pulmonary vessels. Then, the rats were injected with an equal amount of normal saline *via* the femoral vein and prepared into the model of lung ischemia-reperfusion injury 30 min later. Subsequently, an equal amount of normal saline was injected *via* the femoral vein daily. In the sham operation group, the rats underwent thoracotomy, and the pulmonary vessels were then exposed, but not clamped. After the operation, the rats were injected with an equal amount of normal saline *via* the femoral vein daily. After 7 consecutive days of intervention, the rats were subjected to sampling.

Preparation of lung ischemia-reperfusion injury model

After being anesthetized successfully *via* intraperitoneal injection of 3 % pentobarbital sodium at 30 mg/kg, the rats were subjected to tracheotomy and tracheal intubation. Then, their respiration was controlled using a small animal ventilator, with the parameters set as follows: oxygen concentration at 21 %, respiratory rate at 50 breaths/min, tidal volume at 10 mL/kg, and inspiratory/expiratory ratio (1:1). Subsequently, 1000 U/kg heparin sodium was infused to a unilateral femoral vein using a micropump. The left 4^{th} - 5^{th} intercostal space was pinpointed, and a thoracotomy was performed to expose and disassociate the left pulmonary hilum which was then clamped using vascular clips for 45 min. Finally, the clips were released for ischemia-reperfusion, followed by 2 h of mechanical ventilation.

Sample collection

The rats were first anesthetized by intraperitoneal injection of 3 % pentobarbital sodium at a dose of 30 mg/kg. Following successful anesthesia, abdominal arterial blood was drawn from each rat to measure the partial pressure of oxygen (PaO_2) therein. Then, 6 rats were randomly selected from each group, and fixed by perfusion of paraformaldehyde. Subsequently, the lung tissues were taken, soaked in paraformaldehyde, and fixed for another 48 h for H & E staining and TUNEL assay. In addition, lung tissues were directly sampled from the remaining 6 rats in each group for Western blotting (WB).

Determination of PaO_2 in arterial blood

The collected abdominal arterial blood was placed in an automatic blood gas analyzer, and the level of PaO_2 in the arterial blood of each rat was determined as an index of the lung function of each rat.

Western blot assay

The lung tissues were added with lysis buffer (Qincheng Biotech, Shanghai, China; Cat no: QC25-05099), bathed on ice for 1 h, and centrifuged at 14,000*g* for 10 min, followed by protein quantification by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The proteins were then determined using a microplate reader, and the protein concentration in the tissues was calculated based on the absorbance value. The extracted proteins were separated using a 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were then incubated successively with the following primary antibodies: anti-TLR4 (1:1,000), anti-NF-κB (1:1,000), and GAPDH (dilution: 1:500), and secondary antibodies (1:1,000). Finally, the rinsed proteins were reacted with a chemiluminescent reagent for 1 min in a dark place to fully develop images.

Determination of TNF-α and IL-1β contents

The fresh lung tissues were first minced, then the inflammatory factors TNF-α and IL-1β in lung tissues were detected by ELISA kit, and the operation was performed according to the instructions of the kit as follows: The samples were loaded into an ELISA plate, then the plate was then added with the standard, biotinylated antibody working solution and enzyme conjugate working solution separately, and then washed. Finally, the samples were detected at 450 nm on the microplate reader. The standard product concentration was taken as the horizontal coordinate and the absorbance value as the vertical coordinate, and the sample concentration was calculated after drawing the standard curve.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The extracted total RNAs were then reversely transcribed into complementary deoxyribose nucleic acids (cDNAs). Subsequently, qRT-PCR was completed in the designed reaction system (20 μL) through the following steps: reaction at 53 °C for 5 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 10 sec and annealing at 62 °C for 30 sec for 35 cycles. The GAPDH served as the internal control. Finally, the relative expression level of the mRNAs was calculated by the $2^{-\Delta\Delta Cq}$ method, and the differences in the expressions of target genes were analyzed. Table 1 shows the specific primer sequences.

TUNEL assay

The paraffin-embedded tissues were first made into 5 μm-thick sections, extended in warm water at 42 °C, mounted, baked, and prepared into paraffin-embedded tissue sections. Then, these sections were routinely de-paraffinized in xylene solution and hydrated in graded ethanol successively. Subsequently, the resulting sections were added dropwise with TdT reaction solution, reacted in the dark for 1 h, and incubated with the deionized water added in drops for 15 min to terminate the reaction. Afterward, hydrogen peroxide was added dropwise to block the activity of endogenous peroxidase, and the sections were added with a working solution in drops, reacted for 1 h, rinsed, and added dropwise with diaminobenzidine (DAB) solution (Solarbio, Beijing, China) for color development. Finally, the rinsed sections were sealed and observed. Then the number of apoptotic positive cells was calculated under the microscope.

Statistical analysis

The SPSS analysis software (version 26.0) was employed for statistical analysis and *t*-test, corrected *t*-test,

Table 1: Primer sequences

Name	Forward (5'-3')	Reverse (5'-3')
TNF-α	ATGAGCACTGAAAGCATGATC	TCACAGGGCAATGATCCCAAAGTAGACCTGCCC
IL-1β	ATGGCAGAAGTACCTAAGCTC	TTAGGAAGACACAAATTGCATGGTGAACTCAGT
GAPDH	ACGGCAAGTTCAACGGCACAG	GAAGACGCCAGTAGACTCCACGAC

and non-parametric test were adopted for the comparisons of data conforming to normal distribution and homogeneity of variance. Those meeting normal distribution and heterogeneity of variance, and those not fulfilling normal distribution or homogeneity of variance, respectively were also computed. Ranked data were analyzed using the rank sum test, while enumeration data were compared *via* the chi-square test.

RESULTS

Level of PaO_2

Both the model group and perfluorocarbon group had a significantly lower level of PaO_2 than the sham group ($p < 0.05$), whereas the level of PaO_2 in the perfluorocarbon group was significantly higher than that in the model group ($p < 0.05$). The above results indicate that perfluorocarbon can considerably improve the lung function of rats with lung ischemia-reperfusion injury (Figure 1).

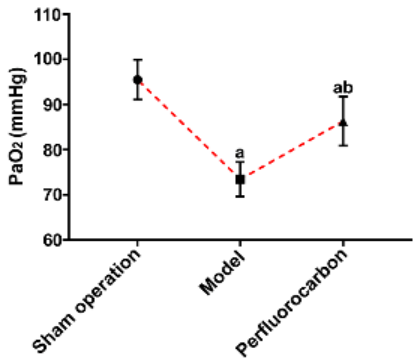

Figure 1: Comparison of PaO_2 among all groups. Note: $^{a}p < 0.05$ *vs.* sham operation group, and $^{b}p < 0.05$ *vs.* model group

Expression of inflammatory factors

Compared with the levels in the sham group, the inflammatory factor levels of TNF-α and IL-1β in the lung tissues increased significantly in the model and perfluorocarbon groups ($p < 0.05$). The TNF-α and IL-1β levels in lung tissues in the perfluorocarbon group were significantly lower than that in the model group ($p < 0.05$). These results suggest that perfluorocarbon can significantly decrease the expressions of the inflammatory factors in rats with lung ischemia-reperfusion injury (Figure 2).

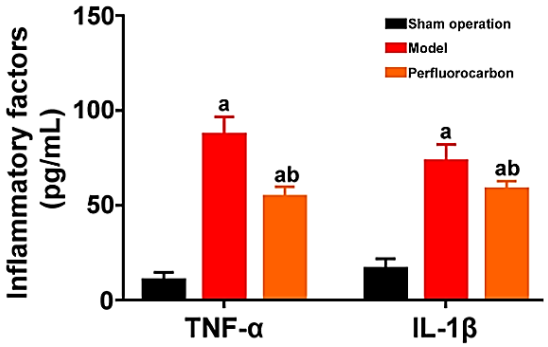

Figure 2: Comparison of levels of inflammatory factors among all groups. $^{a}p < 0.05$ *vs.* sham group, and $^{b}p < 0.05$ *vs.* model group

qPCR results in RNA levels

The TNF-α and IL-1β expression levels of messenger RNA (mRNA) in the lung tissues in both the model group and the perfluorocarbon group were significantly higher than those in the sham operation group ($p < 0.05$), while perfluorocarbon group had a significantly lower TNF-α and IL-1β mRNA expression levels in the lung tissues than the model group ($p < 0.05$). These results suggest that perfluorocarbon can significantly decrease the mRNA expressions of the inflammatory factors in rats with lung ischemia-reperfusion injury (Figure 3).

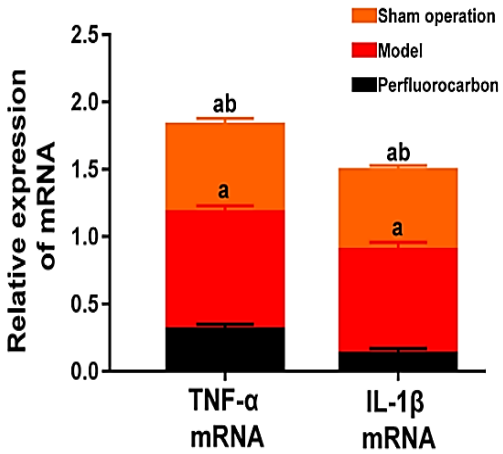

Figure 3: mRNA expressions of inflammatory factors in each group. Note: $^{a}p < 0.05$ *vs.* sham group, and $^{b}p < 0.05$ *vs.* model group

Cell apoptosis

There were fewer apoptotic cells in the sham operation group, but more in the model and perfluorocarbon groups. The apoptosis levels in cells in both model and perfluorocarbon groups were significantly higher than that in the sham operation group ($p < 0.05$), whereas the perfluorocarbon group exhibited a significantly lower cell apoptosis level than the model group ($p < 0.05$). It can be inferred from these results that perfluorocarbon significantly inhibited the apoptosis of cells in the lung tissues of rats with lung ischemia-reperfusion injury (Figure 4).

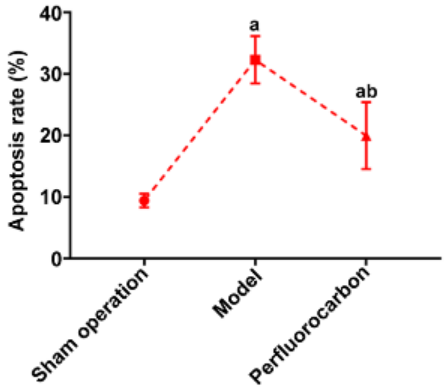

Figure 4: TUNEL apoptosis assay results indicated that perfluorocarbon significantly relieved the apoptosis of cells in the lung tissues of rats with lung ischemia-reperfusion injury

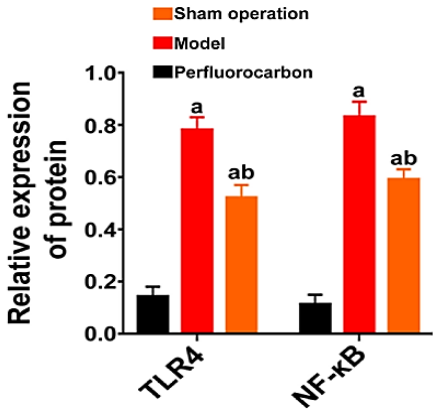

Figure 5: Effect of perfluorocarbon on the relative protein expression levels of TLR4 and NF-κB

Western blotting results

The sham operation group showed protein expansion levels expressed as TLR4 and NF-κB proteins, while the model groups expressed more TLR4 and NF-κB proteins. The relative protein expression levels of TLR4 and NF-κB in the model and perfluorocarbon groups were significantly higher than those in the sham operation group ($p < 0.05$), whereas the perfluorocarbon group had significantly lower TLR4 and NF-κB protein expression levels than the model group ($p < 0.05$) (Figure 5).

DISCUSSION

Ischemia-reperfusion injury is an important pathological injury response in the process of lung injury, which is often composed of lung tissue injury caused by ischemia and hypoxia in the early stage and blood reperfusion injury caused by sudden recovery of lung blood supply in the late stage. Moreover, lung ischemia-reperfusion injury remains one of the crucial pathological factors for multiple pathological responses in lung injury.

Studies have demonstrated that lung ischemia-reperfusion injury increases the synthesis and release of a large number of inflammatory factors, cytotoxic factors, and other substances in lung tissues to induce excessive inflammation, oxidative stress responses, and lipid peroxidation as well as pathological injuries such as apoptosis and necrosis in large numbers of cells in lung tissues. This causes the aggravation of lung tissue injuries, affecting the lung function and hindering the tissue repair and functional recovery after lung injury [10-12]. Therefore, lung ischemia-reperfusion injury is an important process for treating lung injuries and improving lung surgery-induced lung tissue injuries, which has become the difficult point and hotspot of clinical and basic research. The TLR4/NF-κB signaling pathway serves as a pivotal player in regulating many pathological responses such as inflammation and apoptosis. Once injuries get worse, large numbers of inflammatory factors and cytokines are released to abnormally activate the TLR4/NF-κB signaling pathway. As a result, the protein expressions of the key molecules TLR4 and NF-κB in the pathway are aberrantly elevated, helping NF-κB enter the nucleus to bind to its binding site to activate the translation and expression of the downstream proteins.

According to some studies, after the TLR4/NF-κB signaling pathway is activated, on the one hand, the highly expressed NF-κB is transferred into the nucleus to up-regulate the expressions of the inflammatory factors (TNF-α and IL-1β), thereby worsening inflammation. On the other hand, this results in more severe inflammation and leads to massive apoptosis and aggravates tissue damage [13-15]. The results of this study further corroborated that TLR4 and NF-κB were abnormally and highly expressed in the lung tissues of rats with lung ischemia-reperfusion injury, and activated the TLR4/NF-κB signaling

pathway, which may be one reason for the increased expressions of the inflammatory factors (TNF-α and IL-1β) and aggravated cell apoptosis. Several studies have found that the TLR4/NF-κB signaling pathway plays an important role in ischemia-reperfusion injury by regulating inflammation and apoptosis, so it is possible to inhibit ischemia-reperfusion injury by interfering with this pathway [16,17]. Perfluorocarbon is a fluorocarbon characterized by colorlessness, odorlessness, fast volatilization, and stable properties, so it is considered an ideal liquid breathing medium. Besides, perfluorocarbon has now been confirmed to be able to relieve lung tissue injury well, inhibit inflammation and improve lung compliance and function [18-20].

This present study reveals that perfluorocarbon caused an improvement on the lung function of rats with lung ischemia-reperfusion injury and significantly decreased inflammatory factors and cell apoptosis in the lung tissues of rats with lung ischemia-reperfusion injury. Furthermore, perfluorocarbon considerably suppressed the expressions of TLR4, NF-κB, and the key molecules in the TLR4/NF-κB signaling pathway, to repress this pathway, which is probably the reason for perfluorocarbon inhibition of inflammation, and cell apoptosis.

CONCLUSION

Perfluorocarbon down-regulates TLR4/NF-κB signaling pathway, thus inhibiting inflammation and cell apoptosis after lung ischemia-reperfusion injury in rats, thereby improving their lung function. Thus, this compound has potential for use use in the management of lung ischemia-reperfusion injury in clinical practice but clinical studies are required to ascertain this.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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