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Research Article

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CPG-52364 targeted TLR9 regulate lung injury induced by inflammation reaction resulting from elderly hip fracture caused mtDNA release

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Abstract

Background: *Acute lung injury (ALI) after elderly hip fracture is the serious and fatal complication, it is associated with inflammation induced by mtDNA release activates TLR9/NF-KB pathway after trauma. Inhibiting TLR9 pathway activation can alleviate the systemic inflammation and ALI after elderly hip fracture. In the first part of our study, we investigate the effect of the TLR9 inhibitor (CPG-52364) in the pathological process of inflammation and ALI after elderly hip fracture. In the second part of our study, we investigate the effect of CPG-52364 in the pathological process of inflammation and ALI induced by mtDNA. Material and Methods- In the first part of our study, the elderly rats received hip fracture operations and CPG-52364 managed. The degree of lung injury and inflammation were evaluated, TLR9/NF-KB were determined using Western blot, and mtDNA were analyzed by fluorescent quantitative polymerase chain reaction. In the second part of our study, the elderly rats received mtDNA injection and CPG-52364 managed, the specimens were collected and detected as the first part.*

Results: *Both hip fracture and mtDNA injection can cause significant cytokines (IL-6 and IL-10) release, TLR9 and NF-KBp65 expression, and lung injury in the elderly rats. CPG-52364 can effectively down-regulate the expression of the above pathological indicators.*

Conclusion: *These results suggest that the CPG-52364 can down-regulate the TLR9/NF-KB pathway to control the inflammation and ALI induced by mtDNA release after elderly hip fracture.*

Keywords: Elderly Hip Fracture, mtDNA, ALI, CPG-52364.

Introduction

The mortality rate of elderly hip fracture remains unacceptably high that is associated with acute lung injury (ALI) and subsequent lung infection after trauma [1-4]. Pulmonary infection is a common complication of the elderly hip fracture that is related to mitochondrial DNA (mtDNA) release after fracture [5-7]. MtDNA a newly identified damage-associated molecular pattern, is positively correlated with the risk of systemic inflammatory response syndrome (SIRS) in the pathologic process of acute trauma [8,9]. MtDNA derived from the circular genomes of bacteria that evolved into the mitochondria of eukaryotic cells. Similar to bacterial DNA, mtDNA contains a higher frequency of unmethylated cytosine–phosphate–guanine (CpG) dinucleotides. Unmethylated CpG motifs can be recognized by the innate immune system and exerts powerful immunostimulatory effects. During stress and injury, certain molecules released from impaired mitochondria function as danger-associated molecular patterns (DAMPs), including mtDNA, which activates neutrophil through TLR9/p38 MAPK, leading to inflammatory cytokines (such as IL-1β, IL-6, TNF-α, etc) release. If inflammation is not effectively controlled, it potentially contributes to the development of post traumatic SIRS [10,11]. Our previous research also showed that in an elderly hip fracture model of acute lung injury (ALI) that mitochondrial DNA (mtDNA) release induce inflammatory mediator production, it leads to systemic inflammation and lung injury which is correlated with activation of TLR9/NF-ΚB pathway [5,6]. Hence, Inhibiting TLR9 pathway activation induced by post-trauma mtDNA release is the key of alleviating the systemic inflammation and lung injury after elderly hip fracture. Among the antagonists of TLR9, CPG-52364 deserves attention. CPG-52364 is endosomal TLR9 inhibitor that blocks ligand-induced activation of TLR9[12]. Since CPG-52364 was tested in some autoimmune diseases. The results were positive and indicated that CPG-52364 suppressed autoimmune antibody production and reduced inflammation by inhibiting TLR9 activation [13]. CPG-52364 is being considered for early stage clin-

ical development. The purpose of our study was to investigate the effect of CPG-52364 in inhibiting inflammatory response and ALI induced by mtDNA releasing after elderly hip fracture.

Materials and Methods

This paper consisted of two parts. In the first part, we studied the influence of CPG-52364 on inflammatory response and ALI in the elderly hip fracture rats. In the second part, we investigated the effects of CPG-52364 in inflammatory response and ALI induced by mtDNA in the elderly rats.

Animals

Rats of 22 to 23 months old are considered elderly [4-6]. A total of 80 elderly male Sprague-Dawley (SD) rats (age:22-23months; weight:450– 550g Animal Experiment Center of Southern Medical University, China) were allowed to acclimate for 1 week prior to the experimental procedures. Experiments were performed according to the guidelines for experimental animal care and use approved by the Experimental Animal Ethics Committee of Zhujiang Hospital, Southern Medical University.

First part

Grouping of animals- 40 elderly rats were divided into 4 groups randomly (sham group, fracture group, control group, CPG-52364 group). The sham group (n=10) only received anesthesia, cannulation, and observation. In addition to these, the other three groups also received hip fracture operations, the models are made by referring to our previous articles [4-6]. Moreover, the control group (n=10) immediately received intravenous injections with 1 ml saline after hip fracture, and the CPG-52364 group (n=10) received intravenous injections with 1 ml CPG-52364 solution (1.25mg/ml).

Collection of samples-The rats were sacrificed by cervical dislocation at 24 h after treatment, samples were collected by referring to our previous articles [5].

Lung tissue W/D ratio-The right lower lung lobe specimens were collected and weighed to measure the wet weight; subsequently, the samples were placed in an -80°Cdrying oven for 48h until the weights unchanged. The dried lung tissue was weighed again for dry weight, and then W/D ratio of lung tissue was calculated [5].

Histological analysis-Lung specimens were embedded in paraffin. Tissue sections (5-8μm) were prepared and stained with hematoxylin and eosin. All slides were examined and scored as our previous study [5].

Protein, cytokine, MPO and NE assay-The protein, cytokine, MPO and NE assay were performed following the manufacturer's instructions [5].

Western blot- After the protein was extracted and determined, it was separated, transferred and blocked, then incubated with the corresponding primary and second antibodies for TLR9, phosphorylated(p)-NF-κBp65 and GAPDH. An imaging densitometer (LI-COR Bioscience, Lincoln, Neb) was used to analyze the relative density of each band [5].

Serum mtDNA isolation- Serum DNA was extracted from serum using a QIAamp Blood Mini Kit based on affinity columns (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Real-time fluorescent quantitative polymerase chain reaction-Mitochondrial DNA gene primers were designed according to a previous study [5] as follows: forward: CAGCCGCTATTAAAGGTTCG, reverse: CCTGGATTACTCCGGTCTGA. The product size was 79 base pairs. The mtDNA plasmid was constructed, the purified polymerase chain reaction (PCR) products were linked into the pMD18-T vector(TaKaRa, Japan), and the connective product was transformed to DH5 α competent

Escherichia coli. The positive clone E.coli was screened and enriched, then mtDNA was extracted from the plasmid and measured. A standard curve was generated. Fluorescent quantitative PCR (FQ-PCR) reactions were conducted. Each sample and DNA standard were analyzed in duplicate, and the mean value was used for quantification as our previous articles [5].

Second part

Rat femur mtDNA preparation-Ten rats were sacrificed by cervical dislocation, and then femurs were collected; the femurs' mtDNA were extracted using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. The purity and concentrations of the mtDNA were determined by FQ-PCR and spectrophotometry, respectively [5].

Mitochondrial DNA inoculation of animals-40 elderly rats were divided into 4 groups randomly (sham group, mtDNA group, CPG-52364 group and control group). Then, the control group(n=10) received intravenous injections with 1mL phosphate-buffered saline and 1mL of 10μg/ml mtDNA, the mtDNA group(n=10) received injections with 1mL of 10μ g/ml mtDNA, and the CPG-52364 group(n=10) received injections with 1mL of 10μg/ml mtDNA and 1 ml CPG-52364 solution (1.25mg/ml) .

The mtDNA concentration was selected according to our previous experiments [5]. After 24h, the animals were sacrificed by cervical dislocation, and then specimens were collected and detected as in the first part.

Statistical Analysis-Data were presented as means ± standard deviation. All data were analyzed by SPSS software (version13.0). One-way ANOVA with Bonferroni post hoc tests were performed to compare the data of all groups at each monitoring point. Quantitative data were compared between two groups using t-test. A P value of less than 0.05 was considered to be statistically significant.

Results

First part

As shown in Fig.1A, the lung tissue structure was clear, the alveolar walls were normal without significant inflammatory corpuscle infiltration after treatment. However, the pulmonary tissue slices of the fracture group (Fig.1B) and the control group (Fig.1D) exhibited increased congestion, pulmonary edema, polymorphonuclear and mononuclear cell infiltrates, and dam-

aged alveolar architecture. The above pathological changes were significantly alleviated in the CPG-52364 group (Fig.1C).

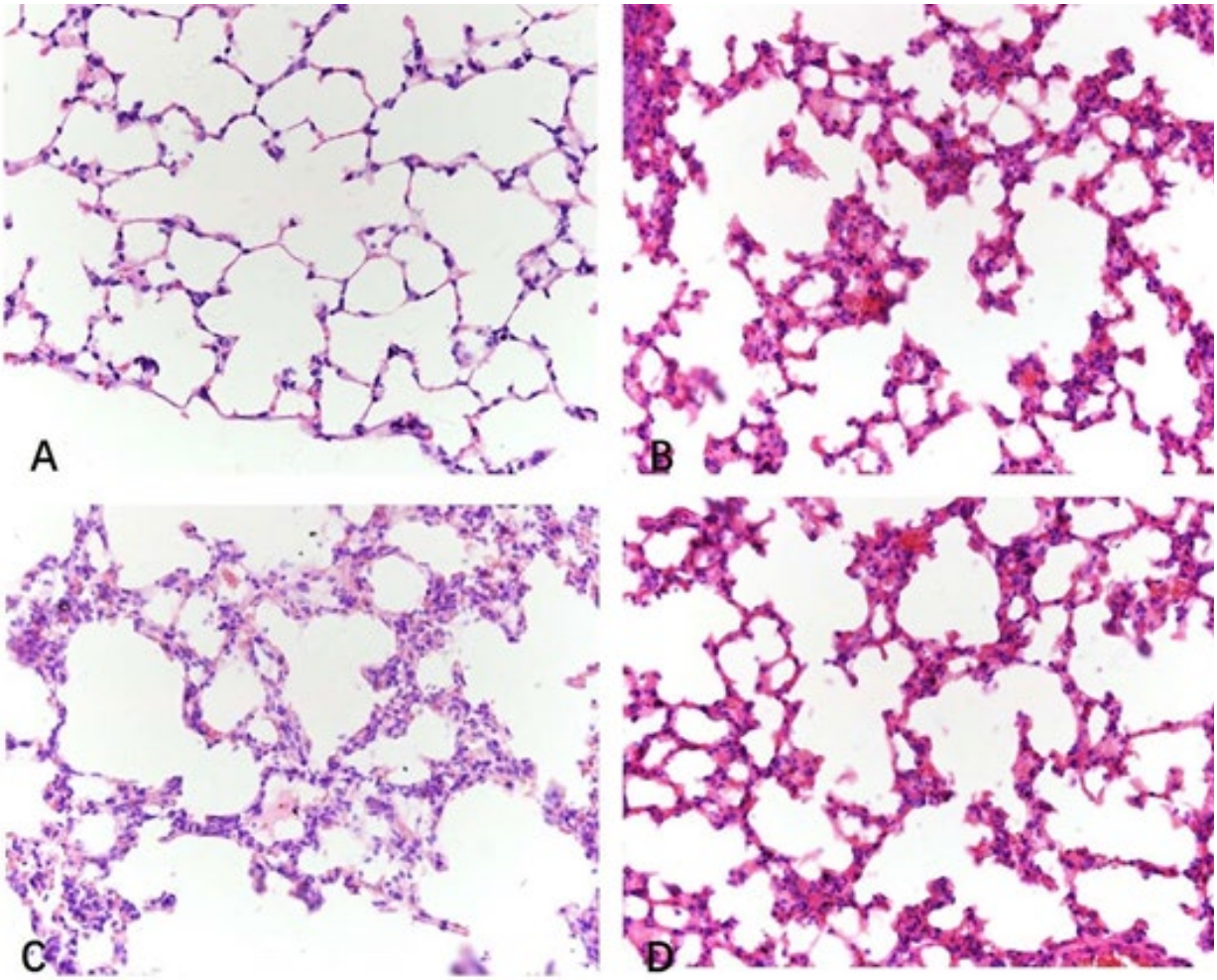

Figure 1: Representative H&E sections of pulmonary tissues (magnification: 200×). At 24 h after treatment, the sham group (A) exhibited no obvious inflammation. The fracture group (B) and the control group (D) showed typical manifestations of acute lung injury. The lung inflammatory manifestations of CPG-52364 group (C) had relieved.

Compared to the sham group (Fig.2 and Fig.3), the serum mtDNA, IL-6, and IL-10 levels, the pulmonary histological score, the cytokines (IL-6 and IL-10) and protein concentrations in the BALF, the lung tissue W/D ratio, MPO and NE activities, and the TLR9 and NF- ΚBp65 protein expressions increased significantly in the fracture group and the control group (all $P < 0.05$). These indicators decreased significantly in the CPG-52364 group (all $P < 0.05$).

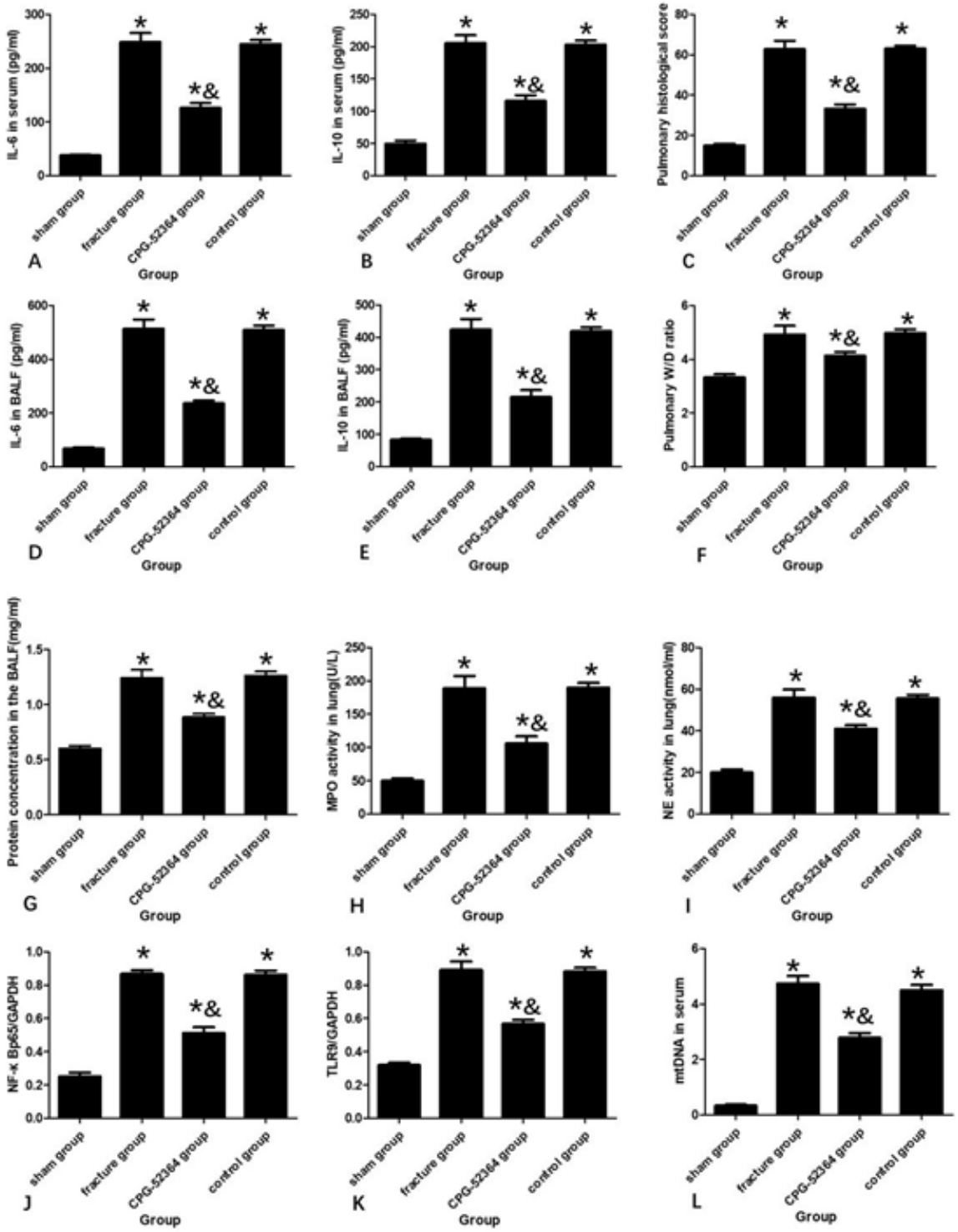

Figure 2: The concentrations of IL-6(A and D), and IL-10 (B and E) in the serum and BALF, pulmonary histological score (C), the lung tissue W/D ratio (F), the BALF protein concentration (G), the MPO (H) and NE (I) activity, the TLR9 (J) and NF-κB p65(K) protein expression, and serum mtDNA levels(L). Results are expressed as mean ±SD, *P<0.05 for the sham group versus the fracture group, CPG-52364 group and control group. &P<0.05 for the CPG-52364 group versus the fracture group and the control group.a

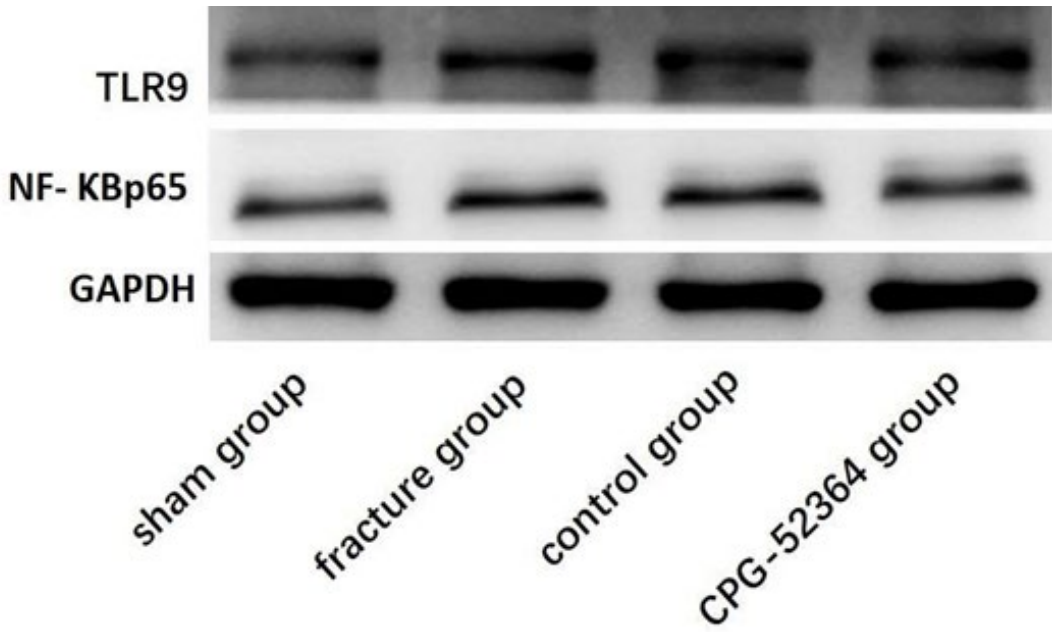

Figure 3: Western blot analysis of the TLR9 and NF-κBp65 protein levels. After treatment, the TLR9 and NF-κBp65 protein levels were significantly increased in the fracture group and control group, and relatively reduced in the CPG-52364 group.

Second part

As shown in Fig.4, the pulmonary tissue slices of the mtDNA group (Fig.4B) show typical manifestations of acute lung injury. The pathological changes were significantly alleviated in the CPG-52364 group (Fig.4C).

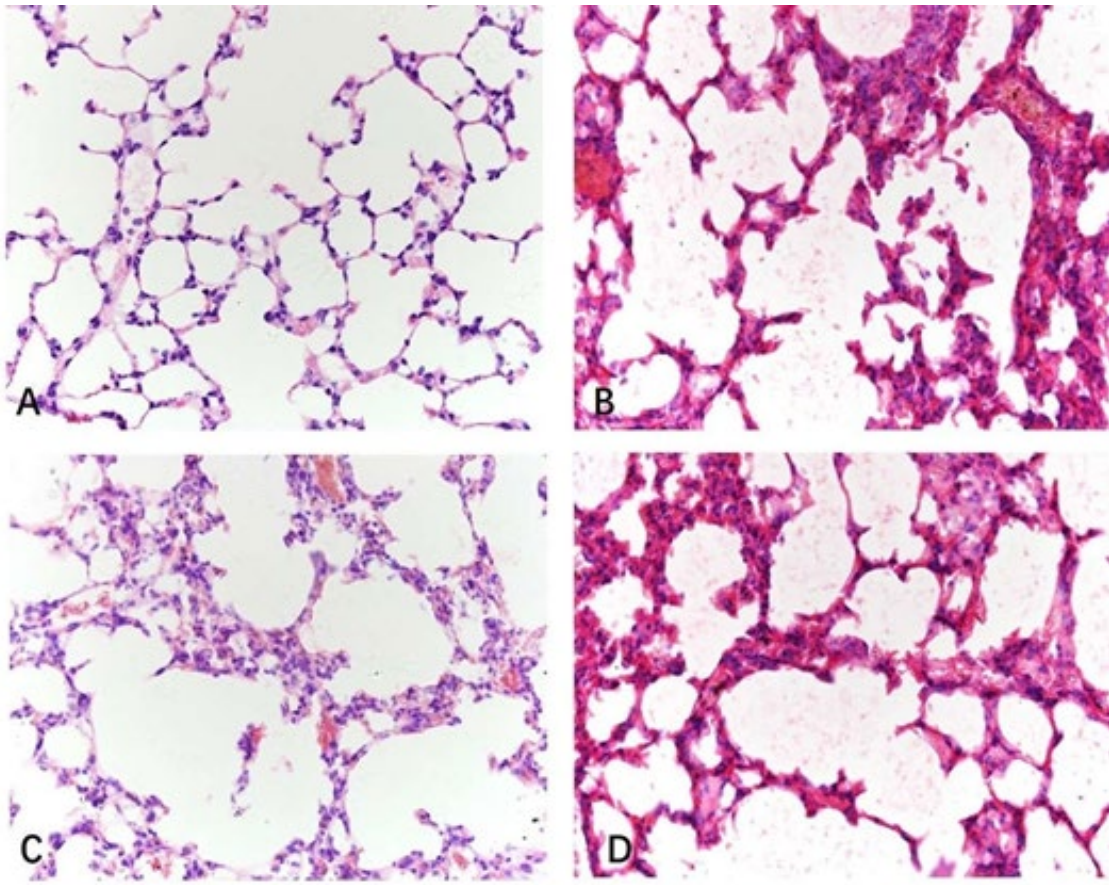

Figure 4: Representative H&E sections of pulmonary tissues (magnification: 200×). At 24 h after treatment, the sham group (A) exhibited no obvious inflammation. The mtDNA group (B) and the control group (D) showed typical manifestations of acute lung injury. The lung inflammatory manifestations of CPG-52364 group (C) had relieved.

Compared to the sham group(Fig.5and Fig.6), the serum IL-6 and IL-10 levels, the pulmonary histological score, the cytokines (IL-6 and IL-10) and protein concentrations in the BALF, the lung tissue W/D ratio, MPO and NE activities, and the TLR9 and NF- ΚBp65 protein expressions increased significantly in the mtDNA group (all $P < 0.05$). These indicators decreased significantly in the CPG-52364 group (all $P < 0.05$).

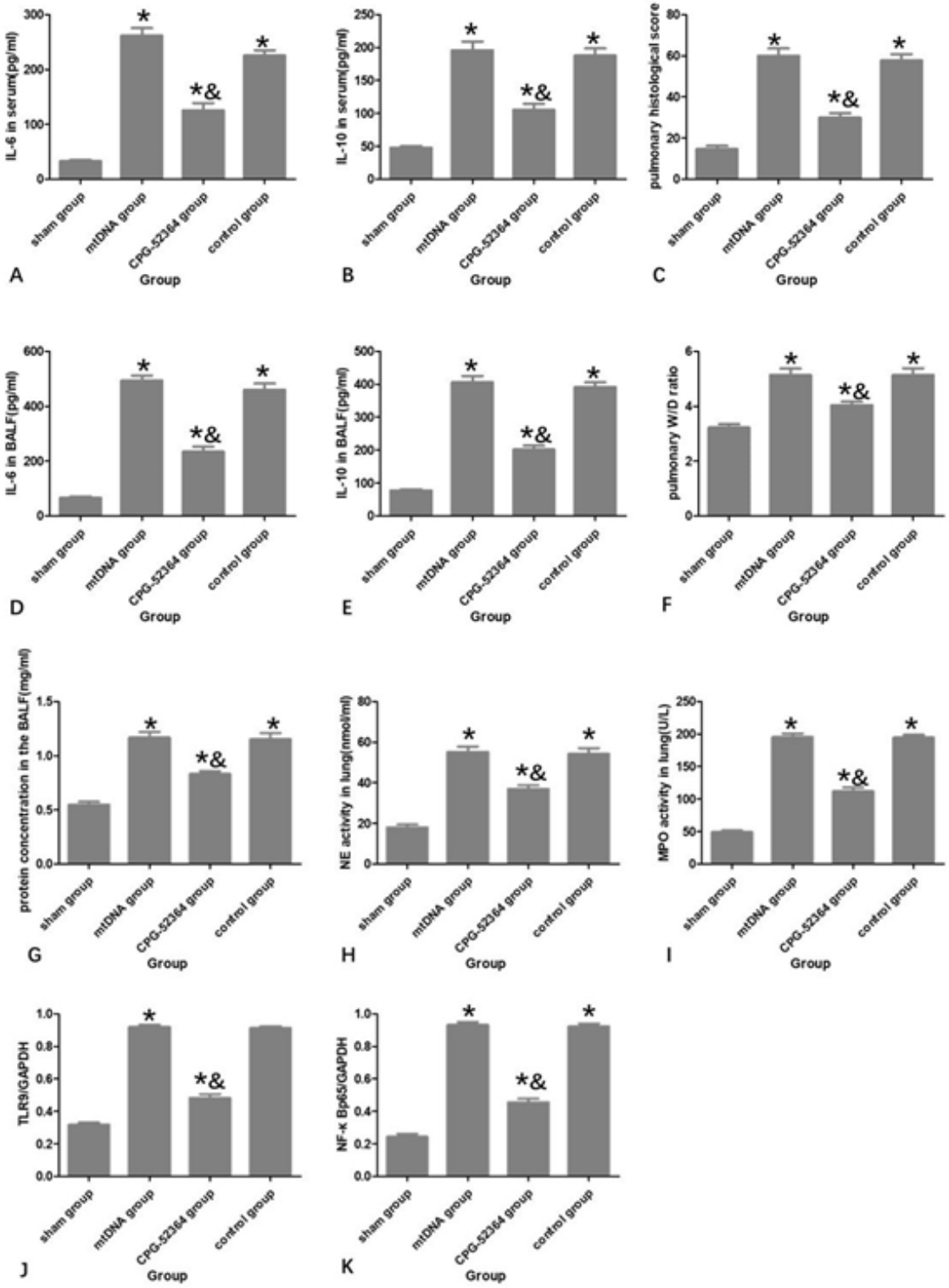

Figure 5: The concentrations of IL-6(A and D), and IL-10 (B and E) in the serum and BALF, pulmonary histological score (C), the lung tissue W/D ratio (F), the BALF protein concentration (G), the NE (H) and MPO (I) activity, the TLR9 (J) and NF-κB p65(K) protein expression. Results are expressed as mean ±SD, *P<0.05 for the sham group versus the mtDNA group, CPG-52364 group and control group. &P<0.05 for the CPG-52364 group versus the mtDNA group and the control group.

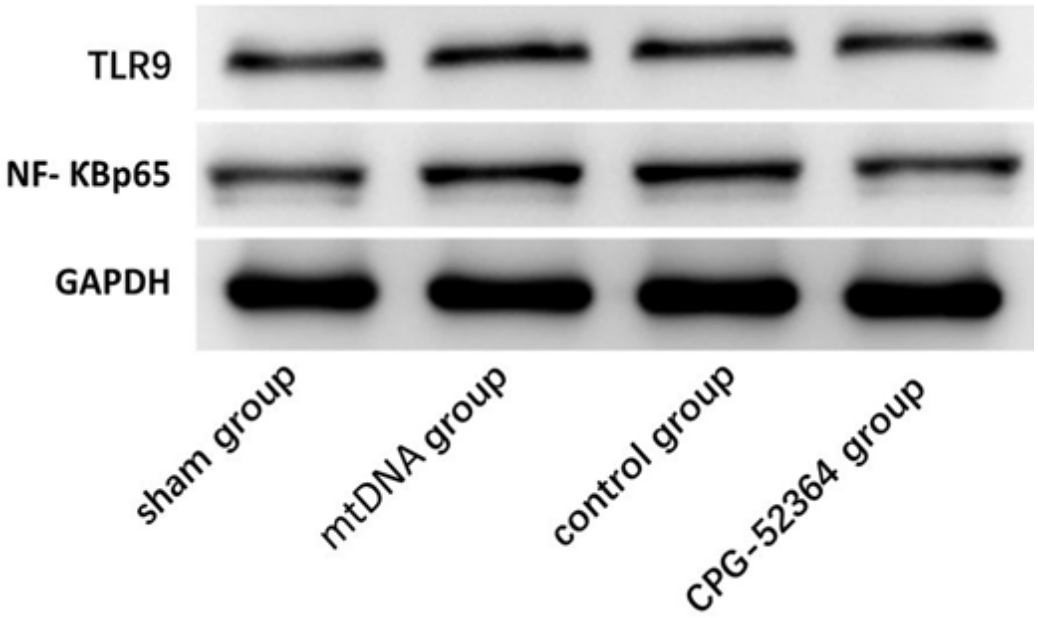

Figure 6: Western blot analysis of the TLR9 and NF-κBp65 protein levels. After treatment, the TLR9 and NF-κBp65 protein levels were significantly increased in the mtDNA group and control group, and relatively reduced in the CPG-52364 group.

Discussion

Recognition of pathogen-associated molecular patterns (PAMPs) or endogenous DAMPs by innate immune cells as well as non-immune cells is the first line of response to pathogen or sterile tissue injury. Innate immune signaling triggered by DAMPs (including mtDNA) during sterile inflammation or the persistence of pathogens [15-17]. TLR9 play an important role in the recognition of exogenous and endogenous danger signals. TLR9 gives rise to rapid activation of signaling pathways, such as those involving NF-Ƙβ responsive factors, thereby leading to the secretion of pro-inflammatory cytokines, reactive oxygen species, antimicrobial peptides and acute-phase proteins. The signaling through TLR9 is an important element of host defense-induced by mtDNA [18-20].

MtDNA damage and exposure, it is an early event in damage exposed cells that provokes the immune response via the activation of TLR9, can trigger a systemic inflammatory response and result in ALI [21]. Thus, inhibition of TLR9 activation induced by post-traumatic mtDNA release indeed has the potential to inhibit systemic inflammation and lung injury after trauma.

In this study, we have characterized CPG-52364, a new TLR9 antagonist by animal studies, as a potential promising therapeutic for the treatment of systemic inflammation and subsequent ALI, which are induced by mtDNA release after elderly hip fracture. In the first part of experiment, the inflammatory chain reaction caused by TLR9 activation-induced mtDNA release after elderly hip fracture is effectively controlled by the CPG-52364. In the CPG-52364 group, the TLR9 pathway activating protein (TLR9 and NF-ƘBp65) expressions decreased significantly; the serum mtDNA levels, the indicators of systemic inflammation (serum IL-6 and IL-10 levels) and ALI (pulmonary histological score, cytokine and protein concentrations in the BALF, lung tissue W/D ratio, MPO and NE activities) were effectively controlled.

In order to further verify the role of CPG-52364 in the pathological process of mtDNA-induced inflammatory response, we carried out the second part of this experiment. In the CPG-52364 group, the TLR9 pathway activating protein (TLR9 and NF-ƘBp65) expressions decreased significantly; the indicators of systemic inflammation and ALI also were effectively controlled. The CPG-52364 can indeed inhibit mtDNA-induced activation of the TLR9 pathway, thus inhibiting the downstream inflammatory responses.

A growing body of evidence indicates that dysregulation of the receptors play a role in the pathogenesis of sepsis. The massive release of inflammatory mediators into the bloodstream following TLR activation is associated with sepsis, culminating in multiple organ failure. TLR9 play an important role in the recognition of exogenous and endogenous danger signals. TLR9 engagement gives rise to rapid activation of signaling pathways, such as it involving NF-Ƙβ responsive factors, thereby leading to the secretion of pro-inflammatory cytokines, reactive oxygen species, antimicrobial peptides and acute-phase proteins [21].

Although some novel therapeutic agents (the TLR9 blockader) have recently been introduced in the field of sepsis, their clinical efficacy remain controversial, as the mortality rate remains unacceptably high and they are associated with significant side effects [22,23]. Hence, further investigation into the side effects of the CPG-52364 are required in controlling lung injury induced by inflammation reaction resulting from elderly hip fracture caused mtDNA release.

Authors' contributions

Xiao Yang, Guangpeng Ou and Bei Li contributed equally to this paper.

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