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**Original Research Article**

# Cimiracemate A confers protection on arthritic neonatal rats via regulation of iNOS/NF-κB/TLR-4 pathway

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## Abstract

***Purpose:*** *To investigate the protective effect of cimiracemate A on Freund's adjuvant-induced rheumatoid arthritis (RA) in neonatal rats, and the underlying mechanism.*
***Methods:*** *Rheumatoid arthritis was induced in rat pups using Complete Freund's adjuvant (100 µg/100 µL/body weight) which was intra-dermally injected at the tail region. After 21 days of establishment of RA, the rats were randomly assigned to four groups of ten rats each: control group, RA group, 5 mg/kg cimiracemate A group, and 10 mg/kg cimiracemate A group. Cimiracemate A was orally administered for 45 days. The effect of cimiracemate A on oxidative stress biomarkers, superoxide dismutase (SOD), malondialdehyde (MDA) and reduced glutathione (GSH) were determined using standard methods. Plasma levels of the inflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α), and prostaglandin E2 (PGE-2) and matrix metalloproteinase-3 (MMP-3) were determined using enzyme-linked immunosorbent assay (ELISA). Western blotting was used to determine the levels of protein expressions of iNOS, NF-κB and TLR-4.*
***Results:*** *The level of MDA significantly increased and the level of GSH significantly decreased in RA group relative to control group ($p < 0.05$) following treatment with cimiracemate A. SOD activity was significantly reduced in RA group, when compared with control group ($p < 0.05$). However, treatment with cimiracemate A significantly and dose-dependently reversed the altered levels of MDA and GSH and SOD activity, when compared with RA group ($p < 0.05$). Plasma levels of IL-1β, TNF-α, PGE-2 and MMP-3 were significantly higher in RA group than in control group, but were significantly and dose-dependently reduced after treatment with cimiracemate A ($p < 0.05$). There were significant increases in the levels of expression of iNOS, NF-κB and TLR-4 proteins in the chondrocytes of RA group, relative to control group ($p < 0.05$). However, treatment with cimiracemate A significantly and dose-dependently down-regulated the expressions of these proteins, when compared with RA group ($p < 0.05$).*
***Conclusion:*** *The results of this study indicate that cimiracemate A confers some degree of protection on arthritic neonatal rats via a mechanism that involves regulation of iNOS/NF-κB/TLR-4 pathway.*

***Keywords:*** *Rheumatoid arthritis, Cimiracemate A, Chondrocytes, Oxidative stress, Inflammatory cytokines*



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## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by impairment of articular cartilage and inflammation of joints [1]. About 1 % of the world's population suffer from this disease [2]. Environmental, genetic and immunologic factors contribute to the development of RA. Vascular endothelial factors, monocytes, neutrophils, and B and T lymphocytes also contribute to the pathogenesis of RA [3]. Inflammatory cytokines such as IL-1 and TNF-α have been reported to play key roles in the onset of this disease [4]. Toll-like receptor-4 (TLR-4) activates nuclear factor-κB (NF-κB) signaling pathway and innate immunity by binding to lipopolysaccharides (LPS), and the TLR-4/NF-κB pathway has been shown to be an important mechanism for the progression of RA [5].

The use of alternative medicine has shown potential over conventional therapies in the treatment of RA. Cimiracemate A is isolated from *Cimicifuga racemosa* L. (*Ranunculaceae*) and it is used in Traditional Chinese Medicine (TCM) for the treatment of sore throat, pain, headache, fever and inflammation [6]. *Cimicifuga racemosa* L. extract has been reported to possess anti-inflammatory and anti-allergic activities [7]. Cimiracemate A reduces the activity of mitogen-activated protein (MAP) kinase and concentration of TNF-α [8]. Its antioxidant activity has also been reported [9]. The present study investigated the protective effect of cimiracemate A in Freund's adjuvant-induced RA in neonatal rats, and the underlying mechanism.

## EXPERIMENTAL

### Experimental rats

Neonatal rats were obtained from Peking University Health Science Center, China. Rat pups weighing 6 - 7 g (mean weight = 6.5 ± 0.5 g) were used for this study. The rats were housed in plastic cages under optimum conditions: 12 h day/12 h night cycle at 25 °C and relative humidity of 60 - 65 %. They were allowed free access to standard rat feed and clean water. The study protocol was approved by the Institutional Ethics Committee of The First Affiliated Hospital of Nanchang University (approval no. IACUC/FAH/NU/2017/19). Guidelines for the proper use and care of animals as prepared by the National Academy of Sciences, National Institute of Health were followed to provide humane care for all the rats [10].

### Induction of RA

Type II bovine collagen mixed in equal ratio with Complete Freund's adjuvant (*Mycobacterium tuberculosis*) was used for the preparation of emulsion. Complete Freund's adjuvant (100 µg/100 µL) was injected intra-dermally at the tail region for induction of RA, and the rats were observed for 21 days. Booster dose of incomplete Freund's adjuvant was subsequently given to all the rats. The rats were then randomly assigned to four groups of ten rats each: control group, RA group, 5 mg/kg bwt cimiracemate A group, and 10 mg/kg cimiracemate A group. Cimiracemate A was orally administered for 45 days.

### Blood sample collection

At the end of the treatment period, the rats were anesthetized and blood collected from the retro-orbital plexuses. The blood was centrifuged at 2000 rpm for 10 min to obtain plasma which was used for biochemical analyses.

### Determination of markers of oxidative stress

The activity of SOD and plasma levels of MDA and GSH were determined using standard methods.

### Evaluation of levels of inflammatory markers

Plasma levels of PGE-2, IL-1β, TNF-α and MMP-3 were determined using their respective ELISA kits.

### Isolation of chondrocytes

Chondrocytes were isolated using standard methods [11]. Chondrocyte digestion was carried out using trypsin, hyaluronidase and collagenase. The cells were then cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum until they attained 80 % confluency.

### Western blotting

The cells were washed with PBS and ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease inhibitor. The resultant lysate was centrifuged at 12, 000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (20 - 30 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and

90 ° C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies for NF-κB, iNOS, TLR-4 and β-actin at a dilution of 1 to 500. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blot was developed using an x-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software (4.6.2). The respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

### Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using Graph Pad Prism (version 6.1). Groups were compared using Dunnett's post hoc test. Values of $p < 0.05$ were considered statistically significant.

## RESULTS

### Effect of cimiracemate A on oxidative status in RA rats

As shown in Figure 1, the level of MDA was significantly increased and the level of GSH significantly decreased in RA group, relative to control group ($p < 0.05$). The activity of SOD was significantly reduced in RA group, when compared with control group ($p < 0.05$). However, treatment with cimiracemate A significantly and dose-dependently reversed the altered levels of MDA and GSH and activity of SOD, when compared with RA group ($p < 0.05$).

### Levels of inflammatory markers in RA rats

Plasma levels of IL-1β, TNF-α, PGE-2 and MMP-3 were significantly higher in RA group than in control group, but were significantly and dose-dependently reduced after treatment with cimiracemate A ($p < 0.05$; Figure 2).

### Levels of expression of iNOS, NF-κB and TLR-4 proteins in the chondrocytes of RA rats

There were significant increases in the levels of expression of iNOS, NF-κB and TLR-4 proteins in the chondrocytes of RA group relative to control group ($p < 0.05$). However, treatment with cimiracemate A significantly and dose-dependently down-regulated the expressions of these proteins, when compared with RA group ($p < 0.05$). These results are shown in Figure 3.

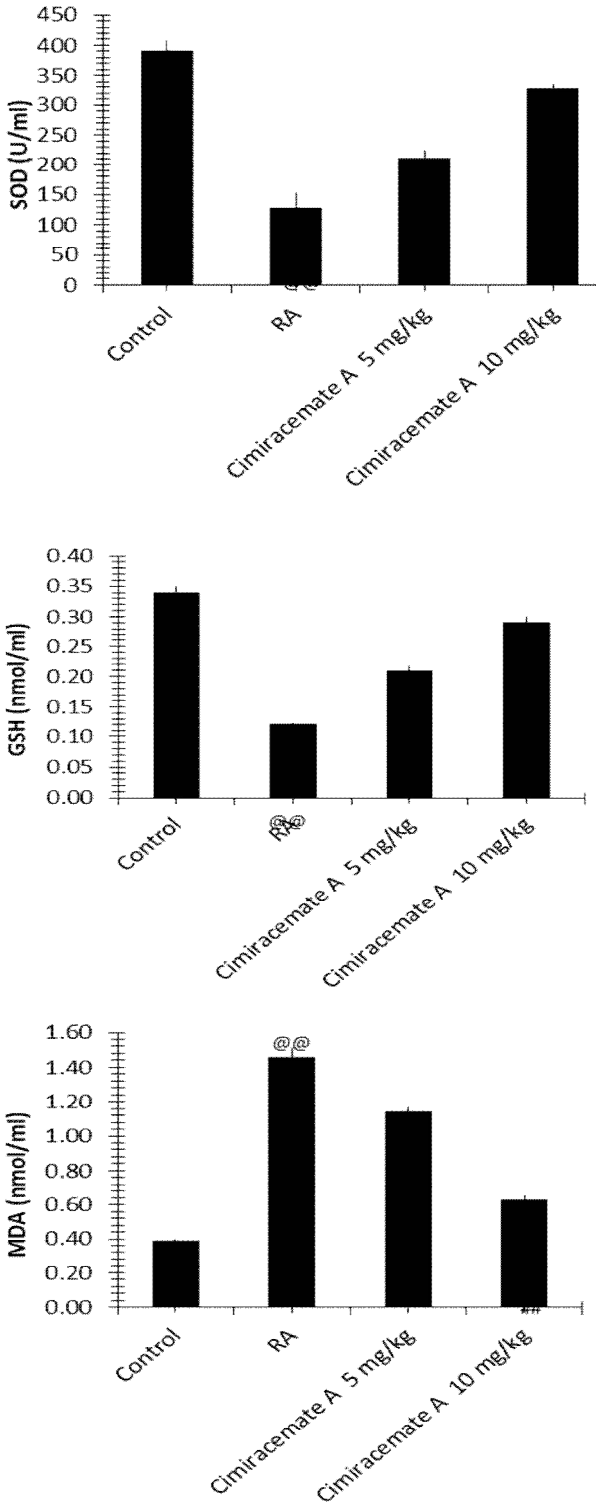


**Figure 1:** Effect of cimiracemate A on markers of oxidative stress in the plasma of RA rats; $^{@@}p < 0.05$, when compared with control group; $^{**}p < 0.05$, when compared with RA group

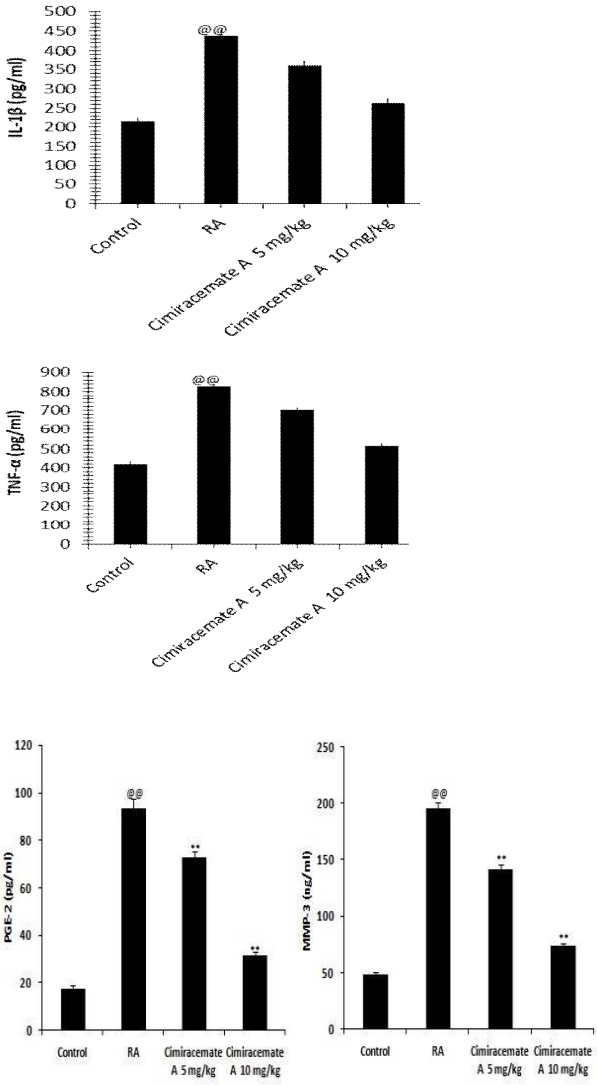


**Figure 2:** Effect of cimiracemate A on plasma levels of inflammatory markers in RA rats; $^{@@}p < 0.05$, when compared with control group; $^{**}p < 0.05$, when compared with RA group

## DISCUSSION

Rheumatoid arthritis is a chronic inflammatory disease of joints characterized by infiltration of activated macrophages and T cells [12]. In the past few decades, alternative medicine has shown potential over conventional therapies in the treatment of RA. The present study investigated the protective effect of cimiracemate A in Freund's adjuvant-induced RA in neonatal rats, and the underlying mechanism. Studies have shown that the severity of RA is significantly elevated by imbalance in the production of free radicals and decreased antioxidant levels at the site of inflammation [13]. Free radical-induced cell membrane damage is due to peroxidation of membrane lipids. In RA, the levels of prostaglandins are significantly increased as a result of oxidative stress and inflammatory injuries [14].

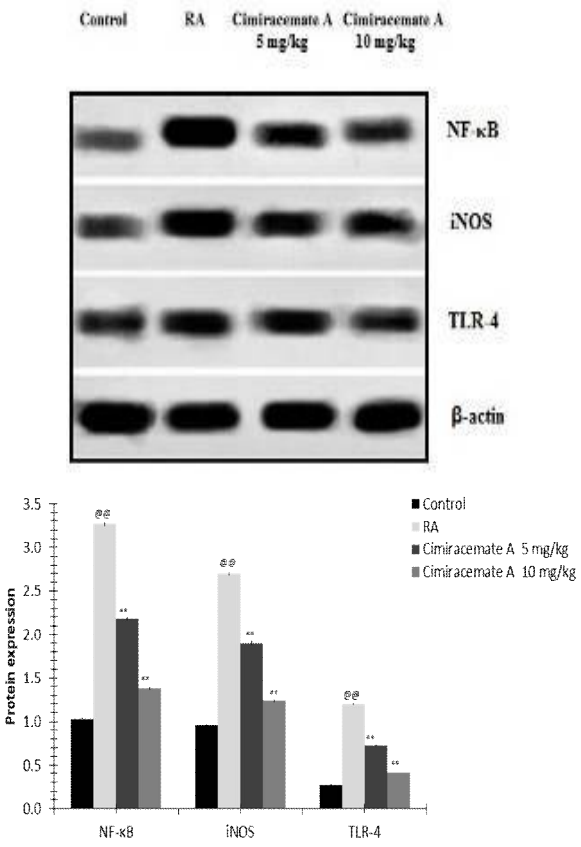


**Figure 3:** Effect of cimiracemate A on the levels of expression of iNOS, NF-κB and TLR-4 proteins; $^{@@}p < 0.05$, when compared with control group; $^{**}p < 0.05$, when compared with RA group

In this study, treatment with cimiracemate A significantly and dose-dependently reversed the altered levels of MDA and GSH, and activity of SOD, when compared with RA group. Plasma levels of IL-1β, TNF-α, PGE-2 and MMP-3 were significantly higher in RA group than in control group, but were significantly and dose-dependently reduced after treatment with cimiracemate A. The inflammatory mediators IL-1, PGE-2 and TNF-α are reported to be significantly increased in RA patients, and anti-inflammatory drugs exert their effects by reducing their levels [15]. The production of MMP-3 is enhanced in RA, and results in the activation of pro-collagenases which catalyze the hydrolysis of proteoglycans and type IX collagen in cartilages [16]. These events lead to destruction of cartilages in RA patients.

It has been reported that inhibition of iNOS/NF-κB/TLR-4 pathway could be used as a strategy for the treatment of RA, and that cimiracemate A inhibits the NF-κB pathway [17]. The results of this study showed that there were significant

increases in the protein expression levels of iNOS, NF-κB and TLR-4 in the chondrocytes of RA group, relative to control group. However, treatment with cimiracemate A significantly and dose-dependently down-regulated the expressions of these proteins, when compared with RA group.

## CONCLUSION

The results of this study have shown that cimiracemate A confers some degree of protection on arthritic neonatal rats, and the underlying mechanism involves regulation of the iNOS/NF-κB/TLR-4 pathway.

## DECLARATIONS

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### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We 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 the authors.

### *Open Access*



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