

Taurochenodeoxycholic Acid Suppresses NF- κ B Activation and Related Cytokines Expression in Peritoneal Macrophages from Adjuvant Arthritis Rat

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Abstract: Taurochenodeoxycholic acid (TCDCA) is one of main bioactive substances of animals' bile acid and confers good anti-inflammatory activity. This study aimed to investigate the mechanism of the anti-adjuvant arthritis activity of TCDCA, the actions were observed in vitro on the protein and mRNA expression of cytokines, the activity of nuclear factor-kappa B (NF- κ B) and its inhibitory I κ B α protein level of peritoneal macrophages (PMs) in adjuvant arthritis (AA) rats. In a definite concentration ranging from 150 μ g/mL to 200 μ g/mL, the over protein and mRNA expression of TNF- α , IL-1 β and IL-6 were remarkably suppressed in the supernatant of PMs treatment with TCDCA. Positive correlations were found between changes of NF- κ B activity and expression of TNF- α , IL-1 β and IL-6 influenced by TCDCA. The activity of NF- κ B was markedly inhibited and I κ B α protein level was increased by TCDCA (150 μ g/mL, 180 μ g/mL and 200 μ g/mL). TCDCA suppresses the protein and mRNA expression of TNF- α , IL-1 β , and IL-6 by inhibiting the NF- κ B binding activity, which is mediated through up-regulating the I κ B α expression of PMs in AA rats.

Keywords: Taurochenodeoxycholic acid; adjuvant arthritis; peritoneal macrophages; nuclear factor-kappa B; cytokines. © 2018 ACG Publications. All rights reserved.

1. Introduction

Bile acids are synthesized in the liver from oxidation of cholesterol and stored in the gallbladder as the main constituent of bile. Chenodeoxycholic acid (CDCA) and cholic acid (CA) are the two primary bile acids and are conjugated mainly to glycine (G) and taurine (T), which are taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA) and taurocholic acid (TCA) [1]. It is clear that bile acids not only facilitate digestion, absorption, and excretion of dietary lipids, but also bile acids interact with numerous cellular signaling pathways [2-5]. TCDCA is synthesized with taurine and chenodeoxycholic acid in organism (Figure 1), which is one of main bioactive substances of bile from animal bile. In our previous study, TCDCA conferred remarkably inhibition on both acute and chronic inflammation, especially it favorably ameliorate the progression development and bone destruction of AA in rat [6].

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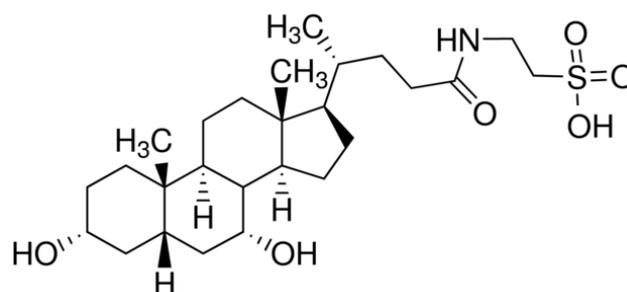


Figure 1. Chemical structure of TCDCA.

NF- κ B is one of most important transcription factors that plays pivotal role and was activated in regulation of inflammation in Rheumatoid arthritis (RA). The activation of NF- κ B is required to induce expression of diverse inflammatory and immune response mediators. Once NF- κ B was activated by a variety of pathogenic stimuli, comprehensive responses were induced, including overproduction of cytokines, such as TNF- α , IL-1 β and IL-6 [7], synoviocytes proliferation, activation of cyclooxygenase 2 and metallo-protease in RA [8,9].

Based on the good anti-adjuvant arthritis activity of TCDCA, we investigated whether TCDCA had directed pharmacologic action on the NF- κ B activity and related cytokines expression in peritoneal macrophages from AA rats. The present study is undertaken to determine the NF- κ B activity, I κ B α protein level and the protein and mRNA expression of TNF- α , IL-1 β and IL-6 in peritoneal macrophages.

2. Materials and Methods

2.1. Reagents

Freund's complete adjuvant (FCA) (Shanghai institute of biological products, China), ELISA kits of TNF- α , IL-1 β and IL-6 (R&D systems, USA), TripureTMRNA reagent, M-MuLV Reverse Transcripts and SYBR[®]PremixEx TagTM (TaKaRa Biotechnology, China), The antibodies against phosphorylated and non-phosphorylated I κ B α (Cell Signaling Technology, USA), Nuclear Extract Kit and TransAM[®]NF- κ Bp65 kit (Active Motif, USA).

2.2. TCDCA Dissociated and Depurated

Fresh chicken gall was collected from chicken slaughterhouse, filtered by filter paper, deproteinated by alcohol, depigmented by activated carbon, condensed by rotary evaporator, salted out, extracted, dewatered, after that crude bile acid was obtained. TCDCA (m/z : 499.69; $\alpha,7\text{-}\alpha$ -dihydroxy-5- β -24-alkylacyl-N-taurine, C₂₃(OH)₂H₃₇CONHCH₂CH₂SO₃H) was dissociated and depurated from crude bile acid by chromatography techniques and the purity was detected by high performance liquid chromatography and its purity was > 99.5%.

2.3. Animals and Induction of AA Rat

The study was approved by Institute's Animal Ethical Committee (Approval ID: 20101027-3) and confirmed to national guidelines on the care and use of laboratory animals. Male wistar rats, 11~13 weeks old, weighing 160~180g, were obtained from experimental animal center, academy of military medical sciences in China. All animals were maintained at a controlled temperature (22 \pm 2 $^{\circ}$ C), and a regular light/dark cycle (7:00~19:00 h, light), and all animals had free access to food and water. AA rat was induced as previously described [10]. Briefly, rats were immunized on day 0 by intradermal injection of FCA into the foot pad, containing 10 mg heat-inactive Bacillus Calmette-Guerin in 1ml paraffin oil, into the left hind paw in 0.1mL for each rat.

2.4. Isolation of Peritoneal Macrophages

The peptone-elicited peritoneal-exudate cells were obtained from AA rats after they were given an intra-peritoneal injection of 1ml 5% peptone solution. 5% Peptone solution followed by a lavage of the peritoneal cavity with 20 mL of medium 2 days later. The cells were washed twice and re-suspended in RPMI-1640 (GIBCO, Grand Island) containing 10% heat-inactivated fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL). The macrophages were isolated from the peritoneal exudate cells using the method described by Um et al [11]. The peritoneal exudate cells were seeded on teflon-coated petri dishes and the macrophages were allowed to adhere for 4 h at 37 °C in a 5% CO₂ humidified atmosphere. Non-adherent cells were removed by washing the dishes twice with prewarmed medium and new medium was added into the plate containing TCDCA at different concentrations.

2.5. Peritoneal Macrophages Proliferation Assay

Peritoneal macrophages were seeded at a concentration of 1×10^5 cells/well in 96-well culture plates and incubated at 37 °C, 5% CO₂ for 4 h. After the cells adhere, the cultures were replaced by new RPMI-1640 with different concentrations of TCDCA (concentration ranging from 0µg/mL to 300 µg/mL). The absorbance (OD value) was measured at 24 h by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)) method after incubation [12].

2.6. Preparation of Peritoneal Macrophages Supernatants and Total RNA

Obtained peritoneal macrophages were resuspended in RPMI-1640 medium at a concentration of 5×10^6 cells/mL and were added to six-well culture plate (final volume was 3 mL/well). After the cells adhere for 4h, new RPMI-1640 medium with different concentration of TCDCA and LPS (5 µg/mL) were added to culture plate. Cells were randomly divided into five groups: Normal group (normal rat cells), AA group (AA rat cells without TCDCA), the remaining three TCDCA groups were treated with different concentrations of TCDCA (150 µg/mL, 180 µg/mL, 200 µg/mL). Culture supernatants were collected and total RNA were isolated using Trizol from peritoneal macrophages after incubation with TCDCA for 24 h.

2.7. Measurement of Related Cytokines Concentration in the Supernatant of Peritoneal Macrophages

The concentrations of TNF- α , IL-1 β and IL-6 in the supernatant of peritoneal macrophages were measured by ELISA according to the manufacturer instructions at 24 h post treatment. The analytic sensitivity for these assays were 5 pg/mL (TNF- α), 5 pg/mL (IL-1 β) and 6 pg/mL (IL-6).

2.8. Relative mRNA Expression of Related Cytokines in Peritoneal Macrophages

Peritoneal macrophages were collected and kept at -80 °C until RNA extraction. Total cellular RNA was extracted using TripureTM RNA reagent. Synthesis of cDNA was performed using an M-MuLV Reverse Transcriptase with random 9-mers according to the manufacturer's protocol. cDNA was amplified using SYBR[®]Premix Ex TagTM (Perfect Real time) kit with specific oligonucleotide primers for target sequences of and in a total of 25 µL reaction mixture (2 µL of cDNA, 12.5 µL of 2 \times SYBR[®]Premix Ex TagTM, 2 µL of each 10µM forward and reverse primers and 8.5 µL of H₂O). The real-time quantitative PCR thermal cycling conditions were 95 °C for 30 s, followed by 95 °C for 5 s and T_m for 30 s for 35 cycles. The primer sequences and T_m were shown in Table1. All primers were synthesized by TaKaRa Biotechnology (China). Data were analyzed according to the comparative Ct method [9] and were normalized by β -actin expression in each sample. Relative mRNA levels were calculated based on the Ct values, corrected for β -actin expression, according to the equation: $2^{-\Delta Ct}$ [$\Delta Ct = Ct(\text{target}) - Ct(\beta\text{-actin})$]. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

Table 1. Primer, temperature and amplified products size and reference in this study

Primer	Temperature	Amplified products size	Sequence
β -Actin	60.0 °C	150 bp	Sense: 5'-CCC ATC TAT GAG GGT TAC GC-3' Antisense: 5'-TTT AAT GTC ACG C AC GAT TTC-3'
TNF- α	58.2 °C	360 bp	Sense: 5'-ACC CCC AAC CTA TGA AGA AA-3' Antisense: 5'-TCC ACG CAAAAC GGA ATG AA-3'
IL-1 β	56.5 °C	377 bp	Sense: 5'TTG TGG CTG TGG AGA AGC TG-3' Antisense: 5'-GCC GTC TTT CAT ACA CAG G'
IL-6	59.8 °C	210 bp	Sense: 5' -TAG AGT CAC AGA AGG AGT GG-3' Antisense: 5' -GCC AGT TCT TCG TAG AGA -3'

2.9. DNA-binding Activity of NF- κ Bp65

Specific binding of NF- κ Bp65 subunit was measured using a TransAM NF- κ Bp65 kit according to the manufacturer's instructions. After treatment with TCDCA, cells were collected by scraping and centrifugation. Cell nuclear extracts were then added into a 96-well plate immobilized with oligonucleotide containing the NF- κ B consensus site (5'-GGG ACT TTC C-3'). Using a primary antibody directed against the NF- κ Bp65 subunit and a second antibody conjugated to horseradish peroxidase, the optical density was qualified by spectrophotometry using Bio-Tek ELX-800 ELIASA (USA).

2.10. Western Blot Analysis

After treatment with TCDCA for 24 h, PMs were washed twice with ice-cold PBS. The cells were lysed with lysis buffer (50mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% NaN₃, and 1% NP40). The cytosolic fraction was obtained from the supernatant after 12,000 rpm centrifugation at 4 °C for 15 min. Samples (20 mg of protein) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS-Tween (0.1%, PBST) for 1 h, and then incubated with monoclonal antibodies against rat I κ B α , and phosphorylated-I κ B α (p-I κ B α) in PBST containing 1% nonfat milk for 1 h. After washing three times with PBST, the membrane was hybridized with secondary antibody conjugated to horseradish peroxidase for 1 h and washed three times with PBST. The membrane was incubated with ECL reagent for 2 min and exposed to X-ray film [13].

2.11. Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software. Significance of the differences between controls and experimental groups was determined by one-way ANOVA analysis. $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Induction of AA rat

All rats developed severe inflammation, and typically non-injected hind limb became severely red and edematous on days 13 after were injected with FCA. Involvement was predominantly distal with ankle, tarsal and inter phalangeal inflammation and few nodules in the tail. Peak severity occurred within 13~15 days after the onset with each involved limb usually reaching a maximum score by the arthritic index (Figure 2).



Figure 2. The changes of right paw and tail of one rat within 15 days after immunization. Compared to the normal rat, there were severely red and edematous swelling of paw and apparent nodules in the tail on days 15 after immunization.

3.2. Effects of TCDCA on Peritoneal Macrophages Proliferation

With the concentration ranging from 0 $\mu\text{g/mL}$ to 200 $\mu\text{g/mL}$, TCDCA showed no toxicity on the peritoneal macrophages proliferation at 24 h post treatment. From 200 $\mu\text{g/mL}$ to 300 $\mu\text{g/mL}$, TCDCA appeared markedly progressing inhibition, respectively (Figure 3). Based on this results, the concentration of TCDCA 150 $\mu\text{g/mL}$, 180 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$ was used in our further experiments.

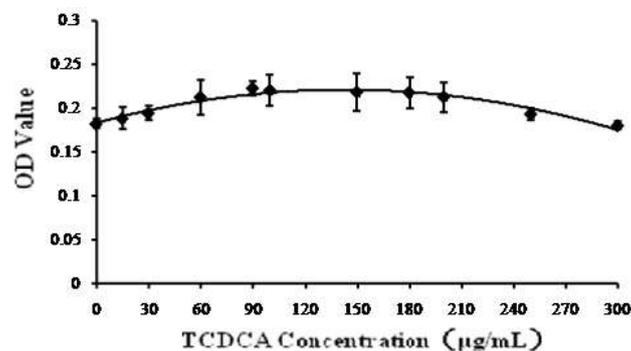


Figure 3. Effects of TCDCA on peritoneal macrophages proliferation of AA rats. In a define concentration ranging from 0 $\mu\text{g/mL}$ to 300 $\mu\text{g/mL}$, effects of TCDCA on peritoneal macrophages proliferation were detected by MTT method. With the concentration ranging from 0 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$, TCDCA showed promoting action, and from 100 $\mu\text{g/mL}$ to 300 $\mu\text{g/mL}$, TCDCA appeared markedly progressing inhibition. Data are expressed as mean \pm S.D (n=5). * $P < 0.05$, ** $P < 0.01$ vs. the OD value of peritoneal macrophages without TCDCA.

3.3. Effects of TCDCA on Related Cytokines Production

The concentration of TNF- α , IL-1 β and IL-6 were markedly increased in the peritoneal macrophages from AA rats compare to normal rats. TNF- α , IL-1 β and IL-6 production were markedly decreased by TCDCA (150 $\mu\text{g/mL}$, 180 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$) on a concentration-dependent manner (Figure 4).

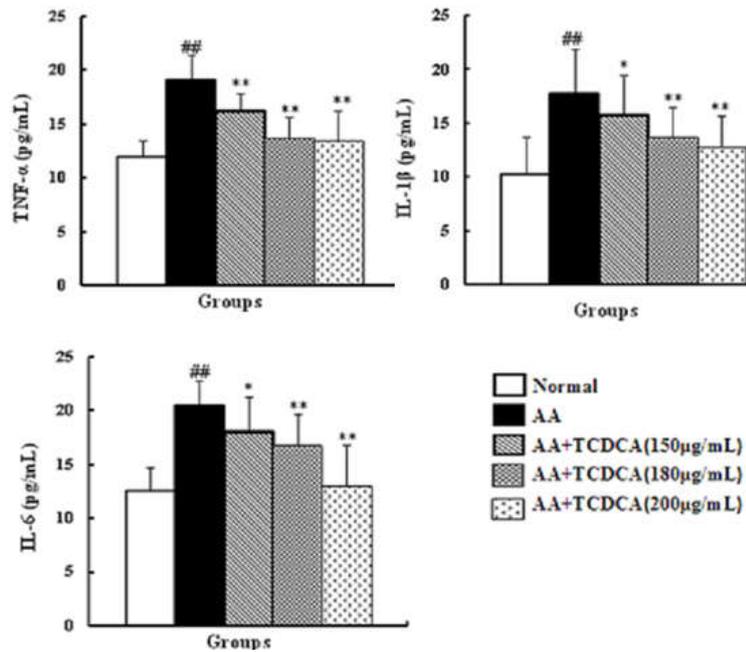


Figure 4. Effects of TCDCA on cytokines production in the supernatant of peritoneal macrophages. TNF-α, IL-1β, IL-6 and IL-10 content were detected by ELISA in the supernatant. The concentration of TNF-α, IL-1β and IL-6 were significant higher in peritoneal macrophages of AA rat than of normal rat. Overproduction of TNF-α, IL-1β and IL-6 were inhibited by TCDCA (from 100 μg/mL to 300 μg/mL). Data are expressed as mean ± S.D (n = 8). [#]*p* < 0.05, ^{##}*p* < 0.01 vs normal peritoneal macrophages; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs. AA peritoneal macrophages.

3.4. Effects of TCDCA on Related Cytokines mRNA Expression

mRNA expression levels of TNF-α, IL-1β and IL-6 were markedly increased in the peritoneal macrophages was seen from AA rats compare to normal rats. mRNA expression levels of TNF-α, IL-1β and IL-6 were markedly inhibited by TCDCA (150 μg/mL, 180 μg/mL and 200 μg/mL) (Figure 5).

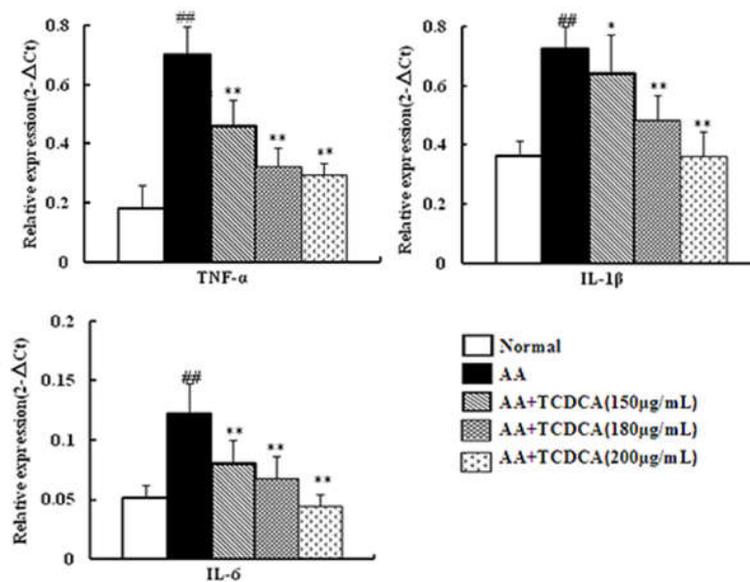


Figure 5. Effects of TCDCA on related cytokines mRNA expression in peritoneal macrophages of AA rats. mRNA expression levels of related cytokines were determined by real time RT-PCR in peritoneal macrophages. mRNA expression levels of TNF-α, IL-1β and IL-6 were markedly inhibited by TCDCA (150 μg/mL, 180 μg/mL and 200 μg/mL). Data are expressed as mean ± S.D (n = 8). [#]*p* < 0.05, ^{##}*p* < 0.01 vs normal synoviocytes; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs AA synoviocytes.

3.5. Effects of TCDCA on DNA-binding Activity of NF- κ Bp65

DNA-binding activity of the NF- κ B p65 subunit was evaluated using a sensitive multi-well colorimetric assay. Cells were pretreated with TCDCA for 24 h. It showed that 2.35-fold-enhanced DNA-binding capacity in the peritoneal macrophages from AA rats compare to normal rats. Cells treated with TCDCA showed weaker NF- κ Bp65 activity with different concentration and statistical significance was detected compare to cells without treatment of TCDCA (Figure 6).

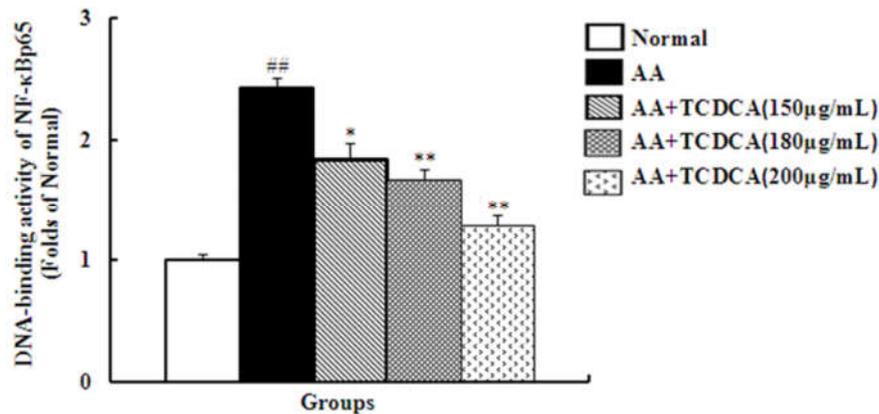


Figure 6. Effects of TCDCA on DNA-binding activity of NF- κ Bp65 in peritoneal macrophages of AA rats. DNA-binding activity of the NF- κ B p65 subunit was evaluated using a sensitive multiwell colorimetric assay. Activity of the NF- κ B p65 was inhibited by treatment with TCDCA (150 μ g/mL, 180 μ g/mL and 200 μ g/mL), and statistical significance was detected compare to cells without TCDCA. Data are expressed as mean \pm S.D (n = 8). #*p* < 0.05, ##*p* < 0.01 vs normal peritoneal macrophages; **p* < 0.05, ***p* < 0.01 vs AA peritoneal macrophages.

3.6. Effects of TCDCA on Expression of I κ B α and Phospho-I κ B α

Since activation of NF- κ B is correlated with I κ B α degradation, the effect of TCDCA on I κ B α and phospho-I κ B α expression was examined to clarify the inhibitory action of TCDCA (Figure 7). It caused a marked reduction in I κ B α protein and augmentation of phospho-I κ B α protein level in peritoneal macrophages from AA rats. Treatment of TCDCA with different concentration resulted in marked augmentation of I κ B α protein and reduction of phospho-I κ B α protein level in the cytoplasmic fractions of peritoneal macrophages.

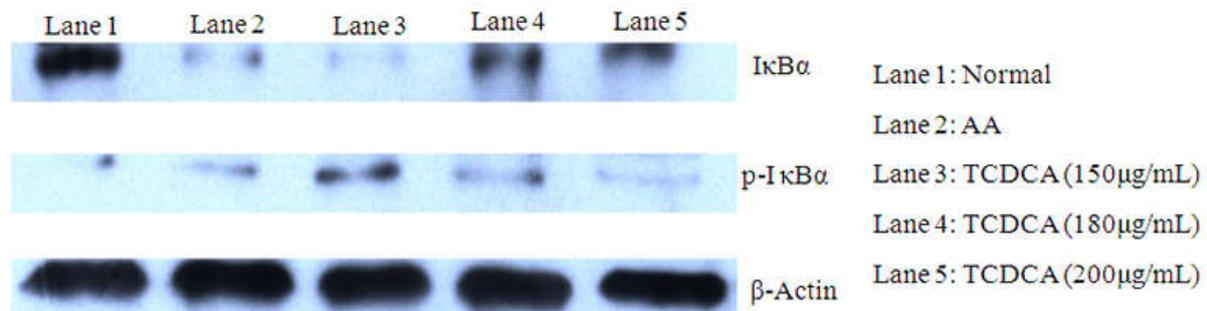


Figure 7. Effects of TCDCA on expression of I κ B α and phospho-I κ B α in peritoneal macrophages of AA rats. I κ B α and phospho-I κ B α expression was analyzed in the cytoplasmic fractions of peritoneal macrophages by western-blot. Treatment with TCDCA caused a marked augmentation in I κ B α protein and reduction of phospho-I κ B α protein level in the peritoneal macrophages from AA rats.

RA is a chronic autoimmune disease associated with multiple inflammatory mediators that lead to joint damage, synovial inflammation and cartilage and bone damage [14,15]. Nowadays, there is dramatically growing interest in herbal medicines among persons with RA and in RA research community. In fact, herbal medicine is being widely used virtually around the world for treatment of rheumatic and arthritic diseases and in recent year, considerable advances have been made in both clinical and basic research on the treatment of RA. As one kind of natural bioactive substance of animal bile acid dissociated and deputed from chicken bile, TCDCA showed noticeable anti-inflammatory and immuno-regulation roles in both acute and chronic inflammation. In our previous study, it was proved that TCDCA conferred a good anti-adjuvant arthritis activity in rats and inhibited the expression of inflammatory cytokines in the synoviocytes [16]. However, the action of TCDCA, on the NF- κ B and related cytokines expression in peritoneal macrophages, was unknown.

One of the most widely used models for studying the anti-inflammatory, anti-rheumatic properties of compounds is adjuvant-induced arthritis in rats. It is an experimental immunopathy and to be considered to share many features with human rheumatoid arthritis. In this model, arthritis develops within 2 weeks and is characterized by developed severe inflammation, and typically non-injected hind limb became severely red and edematous, and few nodules in the tail (Figure 2).

Macrophages and synoviocytes, in particular, are the most vigorous producers of intercellular mediators in RA. Peritoneal macrophages can be stimulated by a variety of agents such as IFN- γ , lipopolysaccharides, or other microbial products [17-19] and some of these have also been shown to trigger the release of TNF- α , IL-1 β and IL-6 by the macrophages [20-23]. Peritoneal macrophages proliferation result showed that from 100 μ g/mL to 300 μ g/mL, TCDCA appeared markedly progressing inhibition, and its inhibited rates reached to 10%, 20% and 30% in the concentration of TCDCA 150 μ g/mL, 180 μ g/mL and 200 μ g/mL, respectively (Figure 3).

Modulation of NF- κ B activation could be an important strategy to reduce cellular injury. There is in fact increasing evidence to suggest that NF- κ B is an important mediator in the pathophysiology of diseases characterized by elevated levels of cytokines such as sepsis and inflammation [24,25]. Our data demonstrate for the first time that TCDCA has the ability to block induced activation of NF- κ B in peritoneal macrophages from AA rats. NF- κ B activity was effectively suppressed during TCDCA incubation on a concentration-dependent manner (from 150 μ g/mL to 200 μ g/mL) (Figure 6).

Accordingly, an NF- κ B inhibitory protein known as I κ B α , its protein expression was marked increased and phospho-I κ B α protein expression was marked decreased by TCDCA (150 μ g/mL, 180 μ g/mL and 200 μ g/mL) as shown in Figure 7. The present data demonstrate that it induced a significant decrease in I κ B α and increase of phospho-I κ B α in peritoneal macrophages from AA rats. TCDCA inhibited I κ B α degradation and decreased the expression levels of phospho-I κ B α with different concentration on a concentration-dependent manner, and thereby blocked NF- κ B translocation to the nucleus. Thus, this mechanism appears to be involved in the action of anti-adjuvant arthritis activity.

NF- κ B is a ubiquitous transcription factor that plays a key role in the expression of many genes central to RA, including IL-1 β in monocytes, as well as intercellular adhesion molecule-1 (ICAM-1), TNF- α and IL-6 [26-28]. The present study indicated that additional treatment with TCDCA reduced the elevated binding activity on a concentration-dependent manner (Figure 6). Furthermore, positive correlations were found between changes of NF- κ B and the mRNA and secretion of TNF- α , IL-1 β and IL-6 treatment with TCDCA. Over production of TNF- α , IL-1 β and IL-6 was markedly decreased by treatment with TCDCA (150 μ g/mL, 180 μ g/mL and 200 μ g/mL) on a concentration-dependent manner, in contrast (Figure 4). mRNA expression levels of TNF- α , IL-1 β and IL-6 were markedly inhibited by TCDCA (150 μ g/mL, 180 μ g/mL and 200 μ g/mL) (Figure 5).

These results demonstrated that TCDCA decreases the mRNA expression and secretion of TNF- α , IL-1 β and IL-6 by inhibiting the NF- κ B binding activity. Accordingly, NF- κ B might be the upstream mechanism of the inhibitory effect of TCDCA on AA-induced TNF- α , IL-1 β and IL-6 expressions. This could also explain the anti-RA role of TCDCA, because NF- κ B acts as an important transcriptional factor in the pathogenesis of RA

4. Conclusion

In this study, TCDCa reduces the pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6 production in PMs from AA rats at transcriptional level by inhibiting NF- κ B activity, and the inhibitory effect of NF- κ B activity is mediated through up-regulating the I κ B α expression, which might be one of the anti-adjuvant arthritis activity mechanisms of TCDCa.

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Competing interests: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. The authors declare that they have no competing interests.

Ethics approval and consent to participate: The study was approved by the farm owner and all animal experiments were conducted according to the International Guiding Principles for Biomedical Research. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Inner Mongolia Agricultural University (Approval ID: 20101027-3).

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