Methotrexate ameliorates pristane-induced arthritis by decreasing IFN-γ and IL-17A expressions[*]

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Abstract:　Objective: This study was carried out to test the effects of methotrexate (MTX) and black seed oil (BSO) on pristane-induced arthritis (PIA) in rats. Methods: Inbred dark agouti (DA) rats were induced by a single subcutaneous injection of pristane, and then treated with MTX or BSO. Arthritis severity was evaluated macroscopically and microscopically. Plasma nitric oxide (NO) concentration was determined by the Griess method and cytokine mRNA expression in the spleen was detected by the real-time reverse transcription-polymerase chain reaction (RT-PCR). Results: The clinical arthritis severity was decreased after MTX treatment, while the BSO groups did not show significant changes compared with the disease group. The plasma NO level of the MTX group was significantly decreased compared with the disease group, but the BSO groups showed no difference from the disease group in plasma NO levels. The interferon-γ (IFN-γ) and interleukin-17A (IL-17A) mRNA expressions in the spleens were significantly decreased in the MTX group, but only showed a declining trend in the BSO groups compared with the disease group. Neither MTX nor BSO had an effect on the mRNA expressions of IL-4, transforming growth factor β (TGF-β), and tumor necrosis factor-α (TNF-α) in the spleen. Conclusions: MTX, but not BSO, can reduce the arthritis severity and decrease the mRNA expressions of IFN-γ and IL-17A in pristane-induced arthritis of rats.

Key words:　Methotrexate (MTX), Black seed oil (BSO), Pristane-induced arthritis (PIA), Interferon-γ (IFN-γ), Interleukin-17A (IL-17A)

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1 Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory disease that principally affects diarthrodial joints, and the central pathology is synovitis (Helliwell *et al.*, 2000; Yamamoto, 2009). The mechanism of RA is complicated and still unclear. The commonly used agents for RA are non-specific and have many adverse effects (Lee and Weinblatt, 2001; Klareskog *et al.*, 2006; Pratt *et al.*, 2009).

Methotrexate (MTX) is the most frequently used disease-modifying anti-rheumatic drug (DMARD). Although MTX can prevent de novo pyrimidine and purine synthesis, the mechanism of MTX in RA remains obscure (Alarcón, 2000; Cronstein, 2005; Wessels *et al.*, 2007; McLean-Tooke *et al.*, 2009). Black seed oil (BSO) obtained from *Nigella sativa* has long been used in many kinds of illnesses, such as

asthma, headache, hypertension, eczema, and rheumatism (Salem, 2005; Shahzad *et al.*, 2009). Tekeoglu *et al.* (2006) reported that thymoquinone (TQ), one of the main constituents of BSO, can suppress the clinical and radiological severities of adjuvant-induced arthritis in Sprague-Dawley and Wistar rats.

In recent years, many researchers have focused on imbalances within the immune system to explain the development of RA and its therapy. T helper cells and their cytokines are important factors in regulating immune balance. Interferon-γ (IFN-γ), interleukin-4 (IL-4), and interleukin-17A (IL-17A) represent the hallmarks for Th1, Th2, and Th17 cells, respectively (Mosmann and Coffman, 1989; Ivanov *et al.*, 2006; Mucida *et al.*, 2007; Bettelli *et al.*, 2008).

In this study, we hypothesized that MTX or BSO can ameliorate pristane-induced arthritis (PIA) by regulating immune balance. Arthritis in dark agouti (DA) rats was induced by pristane and then treated with MTX or BSO. We observed the arthritis severity macroscopically and microscopically, and measured gene expressions of IFN-γ, IL-4, IL-17A, transforming growth factor β (TGF-β), and tumor necrosis factor-α (TNF-α) in the rat spleens.

2 Materials and methods

2.1 Animals

DA rats (originated from Zentralinstitut für Versuchstierzucht, Hannover, Germany) were bred in the animal house under specific pathogen free (SPF) conditions with a 12 h:12 h of light/dark cycle and air-conditioning. The rats were housed in polystyrene cages with standard rodent chow and water ad libitum. Rats at the age of 8–12 weeks were randomly divided into seven groups matched for sex and age: four rats in the control group (NC) and eight rats in each of BSO prevention low dose group (PL), BSO prevention high dose group (PH), BSO treatment low dose group (TL), BSO treatment high dose group (TH), MTX treatment group (MTX), and disease group (D). Rats were anesthetized intraperitoneally by 20 mg/ml pentobarbital sodium (0.15 ml/100 g body weight (BW)) at 26 d post-induction. Blood samples were collected into heparin sodium anticoagulation tubes (Jiangxi Sophisticated Medical Equipment Co., China)

by abdominal aorta punctures, and then were centrifuged at 3 000 r/min for 20 min, and stored at −80 °C. Left posterior paws were removed and fixed in 40 mg/ml paraformaldehyde. Spleens were collected and stored at −80 °C. All experiments had been approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University, China.

2.2 Induction and evaluation of arthritis

Arthritis was induced by a single subcutaneous injection of 150 µl pristane (Sigma-Aldrich, USA) at the base of the rat tail. Rats in the NC group were injected with 150 µl phosphate buffered saline (PBS) instead.

A macroscopic scoring system was used to monitor arthritis development once every two days after pristane injection in all four limbs. The redness and swelling of each joint were observed, and the maximum score was 15 points per limb.

Rat left posterior paws were fixed and decalcified, then stained with hematoxylin and eosin (H&E). A pathological scoring system was used to evaluate the severity of arthritis. Synovitis, inflammation, and repair each were given the maximum of 3 points.

2.3 Treatment with BSO and MTX

The rats were given BSO (Quinessence Aromatherapy Ltd., UK) at different time points by intragastric administration. We began to give the medicine one day before induction in the prevention groups, and started to administrate the medicine at 13 d after induction in the treatment groups, as our previous observations showed that the average onset of PIA was at 14 d after induction (Meng *et al.*, 2010). Both groups were given the medicine continuously for 5 d. Two dose groups of BSO for both prevention and treatment were used, with 2.5 and 5 ml/kg BW for low and high doses, respectively. MTX (Pfizer Pharmaceuticals Ltd., Australia) was given by intramuscular injection at the dose of 1 mg/kg BW once at 13 d after induction. PBS was used in control rats.

2.4 Plasma nitric oxide (NO) detection

Plasma NO level was assayed by the Griess reaction. Briefly, 100 µl blood plasma was mixed with 80 µl of 375 mmol/L ZnSO$_4$ and 120 µl of 275 mmol/L NaOH, and then centrifuged at 13 000 r/min for 20 min.

The supernatant was obtained and added with 400 mg of copper-plated cadmium and 100 µl of 0.2 mol/L glycine buffer (pH=7.5). The mixture was shaken for 2.5 h at room temperature. Then 100 µl of supernatant of each well was added into 96-well plates, and reacted with 100 µl Griess reagent (1 mg/ml *N*-(1-naphthyl) ethylenediamine dihydrochloride:10 mg/ml sulfanilamide) to develop color. The optical density (OD) was measured at 545 nm wavelength by a microplate reader (Thermo Electron Co., Finland). The NO concentration was obtained by calculating OD values against a standard curve from gradient concentrations of sodium nitrite.

2.5 RNA isolation and mRNA expression detection

Total RNA was isolated from the spleens using the TRIzol method (Invitrogen, USA). The complementary DNA (cDNA) was obtained by reverse transcription kits according to the manufacturer's protocol (RevertAid, Fermentas Life Sciences, Canada). The IFN-γ, IL-17A, IL-4, TGF-β, and TNF-α mRNA expression levels in the spleen were determined by the real-time reverse transcription-polymerase chain reaction (RT-PCR), and the primers used are shown in Table 1. Then cDNA expression was determined by SYBR Green II Supermix (Ta-KaRa, China) using the iQ5 real-time PCR detection system (Bio-Rad, USA).

2.6 Statistical analysis

Data were expressed as mean±standard error of mean (SEM). Mann-Whitney test was used to analyze all the experiments. The correlation analysis between two parameters was performed by Pearson method. A *P* value <0.05 was considered statistically significant.

3 Results

3.1 Effects of MTX and BSO on clinical arthritis severity

The clinical arthritis severity was evaluated by a macroscopic scoring system once every two days. The arthritis severity started to decrease after MTX treatment, while that of rats in the BSO groups only had declining trends, but no significant changes compared with the disease group, regardless of whether the BSO group was prevention or treatment, low or high dosage (Fig. 1 a).

3.2 Effects of MTX and BSO on ankle joint pathology

Left posterior paw of each rat was harvested at 26 d after immunization and stained with H&E. The ankle joint was evaluated by a pathological scoring system, which considers synovitis, joint destruction, and repair. The pathological score was correlated with the clinical arthritis score (r=0.573, P=0.007). There was no difference of pathological scores among the D, PL, PH, TL, TH, and MTX groups (Fig. 1b). Thus, both BSO and MTX did not affect ankle joint pathology at 26 d after pristane injection.

3.3 Effects of MTX and BSO on plasma NO levels in PIA rats

Plasma NO levels of the D, PL, PH, and TL groups were significantly increased compared with those of the NC group. Plasma NO levels of the MTX group were significantly decreased compared with those of the D group, and there was no difference in plasma NO levels between the NC and MTX groups (Fig. 1c). We concluded that MTX could decrease plasma NO levels in PIA rats.

Table 1 Primers used for amplification of cytokines by real-time RT-PCR

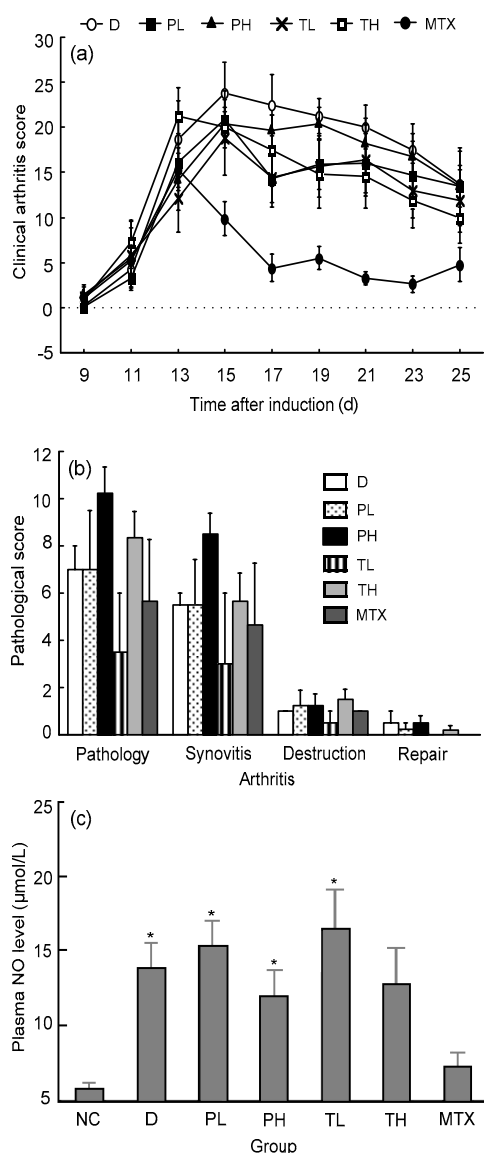| Cytokine	Primer sequence	Amplified length (bp)	Annealing temp. (°C)
IFN-γ	Sense: CCCTCTCTGGCTGTTACTGC	149	65
	Antisense: TTTCGTGTTACCGTCCTTTTG		
IL-17A	Sense: CTACCTCAACCGTTCCACTT	191	65
	Antisense: ACTTCTCAGGCTCCCTCTTC		
IL-4	Sense: TGCACCGAGATGTTTGTACCAGA	92	63
	Antisense: TTGCGAAGCACCCTGGAAG		
TGF-β	Sense: GGACTACTACGCCAAAGAAG	294	64
	Antisense: TCAAAAGACAGCCACTCAGG		
TNF-α	Sense: TCAGCCTCTTCTCATTCCTGC	202	60
	Antisense: TTGGTGGTTTGCTACGACGTG		

Fig. 1 Evaluation of clinical and pathological arthritis scores and plasma NO levels of rats in each group
(a) Clinical arthritis score of each group rats after pristane induction; (b) Pathological evaluation of ankle joints by H&E staining. A pathological scoring system was adopted to evaluate the severity of arthritis. 'Pathology' indicated total pathological arthritis scores of ankle, which include synovitis, destruction, and repair; (c) Plasma NO level of each group rats detected by the Griess reaction method. Mann-Whitney test was used to analyze the data: * $P<0.05$ compared with the NC group. All data are expressed as mean± SEM. NC: normal control group; D: disease group; PL: BSO prevention low dose group; PH: BSO prevention high dose group; TL: BSO treatment low dose group; TH: BSO treatment high dose group; MTX: MTX treatment group

3.4 Effects of MTX and BSO on the mRNA expressions of IFN-γ and IL-17A in rat spleens

Spleen total RNA was isolated and reverse-transcribed into cDNA. The IFN-γ and IL-17A mRNA expressions were significantly decreased in the MTX group, but in the BSO groups, only IL-17A showed declining trends compared with the disease group (Figs. 2a and 2b). The IL-17A mRNA expression was correlated with repair ($r=0.835$, $P=0.001$), while IFN-γ was correlated with plasma NO levels ($r=0.511$, $P=0.002$). Neither MTX nor BSO affected the expressions of IL-4, TGF-β, and TNF-α (Figs. 2c, 2d, and 2e).

4 Discussion

Our results indicate that MTX ameliorates the clinical arthritis severity and decreases the IFN-γ and IL-17A mRNA expressions in PIA rat spleens. The clinical arthritis score and the mRNA expressions of IFN-γ and IL-17A had declining trends ($P>0.05$) in the BSO groups.

RA is a chronic polyarthritis disorder, and PIA is one of its animal models, a T cell-dependent model which can be used for mechanism and therapy research (Lu *et al.*, 2002; Holmberg *et al.*, 2006; Olofsson and Holmdahl, 2007; Smolen and Aletaha, 2009). It has been shown that T cells isolated from the PIA rat spleens transfer severe arthritis, which indicates that the critical arthritogenic change induced by pristane is present in the spleen (Holmberg *et al.*, 2006). Therefore, we focused on the spleen to study the immune reaction after pristane injection.

NO reflects an immune-activated state with upregulated activity of inducible nitric oxide synthase (iNOS) by inflammatory mediators (Reddy *et al.*, 2005). In RA patients, the activity of iNOS is increased and the plasma NO level is higher, in comparison with the normal control (Savnik *et al.*, 2001; Romas *et al.*, 2002). The synovial fluid nitrite, a breakdown product of NO, was also increased in RA patients. Synovial fluid nitrite concentration was significantly correlated with serum nitrite concentration in patients with RA (Farrell *et al.*, 1992). Previously, we have found that the plasma NO level is also increased in PIA rat models (Meng *et al.*, 2010).
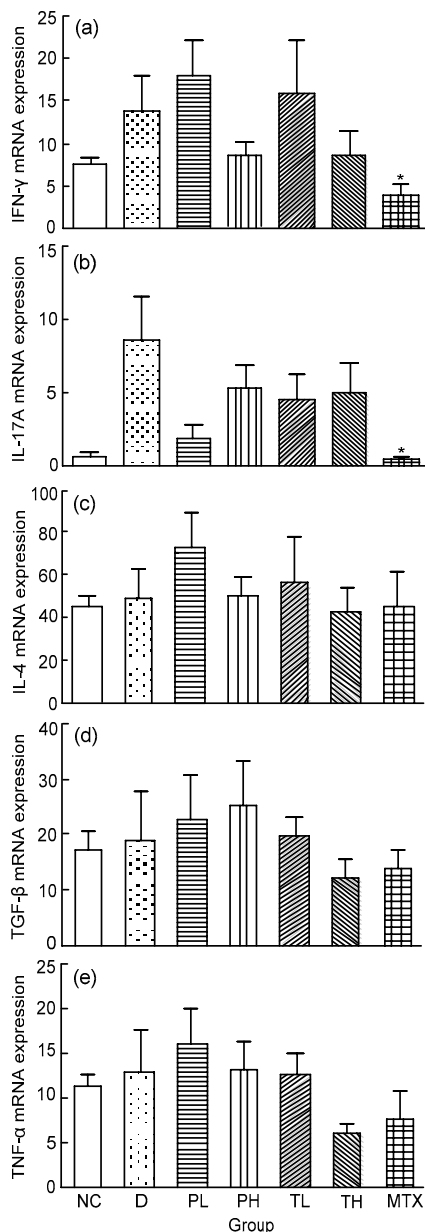
Fig. 2 Effect of MTX on IFN-γ (a), IL-17A (b), IL-4 (c), TGF-β (d), and TNF-α (e) mRNA expressions in PIA rat spleens by real-time quantitative PCR
MTX can decrease IFN-γ and IL-17A mRNA expressions in the spleens. Mann-Whitney test was used to analyze the data: * $P<0.05$ compared with the D group. All data are expressed as mean±SEM. NC: normal control group; D: disease group; PL: BSO prevention low dose group; PH: BSO prevention high dose group; TL: BSO treatment low dose group; TH: BSO treatment high dose group; MTX: MTX treatment group

In this study, our results showed that MTX reduced plasma NO levels in PIA rats, indicating that MTX can decrease the inflammatory level of the disease.

MTX is commonly known to directly inhibit dihydrofolate reductase, thymidylate synthase, and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, and also to indirectly inhibit methylenetetrahydrofolate reductase. Thus, it mainly prevents de novo pyrimidine and purine syntheses (Celtikci *et al.*, 2009; Patiño-García *et al.*, 2009; Shimura *et al.*, 2009). It has been reported that MTX efficiently suppresses PIA when given after the onset of arthritis, but the mechanism remains obscure (Lange *et al.*, 2005). Recently, many studies have shown that MTX can inhibit cell proliferation and induce apoptosis in a dose- and time-dependent manner, and this effect is different in different cell types (de Lathouder *et al.*, 2004; Wessels *et al.*, 2007). MTX can also influence cytokine production. MTX inhibits the upregulations of IL-6, IFN-γ, and IL-17A in the coculture of T cells and fibroblasts (Miranda-Carus *et al.*, 2004). Seitz *et al.* (2003) cultured the whole blood of healthy donors and RA patients, and found that MTX inhibited the productions of IL-4, IL-6, IL-13, IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factors which were secreted by activated T cells. Interestingly, MTX did not affect cytokines produced by monocytes (Gerards *et al.*, 2003). Thus, MTX plays the role of inhibiting activated T cells.

As we have shown here, MTX can decrease the over expressions of IFN-γ and IL-17A in PIA rats, which is consistent with the findings from in vitro studies (Seitz *et al.*, 2003). Traditionally, we know that Th1 cells, which mainly secrete IFN-γ, play an important role in the pathogenesis of RA (Herman *et al.*, 2008). Th17, a new designated T-helper cell type marked by IL-17A, IL-17F, and IL-22 secretions, has been shown to be closely related to RA (Harrington *et al.*, 2005; Park *et al.*, 2005). MTX can ameliorate RA by inhibiting the activation of not only Th1 but also Th17.

BSO has shown anti-inflammatory effects in asthma, experimental allergic encephalomyelitis, colitis, etc. (Abbas *et al.*, 2005; Salem, 2005). BSO can play the immunomodulatory role in vivo. We previously reported that BSO ameliorated allergic asthma by inhibiting T cell proliferation in E3 rats. The mRNA and protein expression levels of IL-4, IL-5, and IL-6 were reduced to normal in BSO treated group rats (Shahzad *et al.*, 2009). In this study, the

BSO groups had declining trends of IL-17A, but no effect on IL-4 and IFN-γ expressions. Budancamanak *et al.* (2006) and Tekeoglu *et al.* (2006) reported that TQ had a treatment effect on adjuvant induced or collagen induced arthritis rat models. This observation may be caused by the use of different arthritis models and different medicinal components.

In conclusion, our findings support that MTX inhibits activated Th1 and Th17 cells in the treatment of PIA, but BSO has little effect on this model.

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