Methionine attenuates the intensity of rheumatoid arthritis by downregulating NF- κ B and iNOS expression in neonatal rats

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Abstract

The present study investigated the anti-arthritic effects of methionine in neonatal rats. Rats were divided into four groups, with six rats in each group. The rats were administered methionine (150- or 300-mg/kg body weight) orally for 45 consecutive days. The expression levels of catalase, superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation, glutathione peroxidase (Gpx), prostaglandin E₂ (PGE₂), matrix metalloproteinase-3, uric acid, nitric oxide (NO), ceruloplasmin, inducible nitric oxide synthase (iNOS), and nuclear factor (NF)- κ B were determined in rheumatoid arthritis-induced neonatal rats. The levels of SOD, catalase, Gpx, and GSH were substantially reduced in control rats, while the levels of other parameters were increased in control neonatal rats. However, methionine supplementation significantly increased (more than 40%) the levels of SOD, catalase, Gpx, and GSH in neonatal rats. The levels of lipid peroxidation, uric acid, ceruloplasmin, NO, and PGE₂ were significantly reduced following methionine supplementation. Furthermore, NF- κ B mRNA expression was substantially reduced up to 51.7% in the 300-mg/kg methionine group, whereas the mRNA expression of iNOS was reduced up to 43.5% in the 300-mg/kg methionine group. NF- κ B protein expression was substantially reduced up to 45.8% in the 300-mg/kg methionine group, whereas the protein expression of iNOS was reduced up to 45.4% in the 300-mg/kg methionine group. Taken together, these data suggest that methionine supplementation was effective against rheumatoid arthritis.

Keywords Methionine · Neonatal rats · Inflammation · Antioxidants · Lipid peroxidation

Introduction

Rheumatoid arthritis is a well-known chronic autoimmune disorder of the synovial joints. Stiffness, swollen, painful, and inflamed joints are primary symptoms of rheumatoid arthritis (Majithia and Geraci 2007). Nakamura et al. (2007)

reported that nitric oxide (NO), tumor necrosis factor- α , interleukin-1, prostaglandin E₂ (PGE₂), and matrix metalloproteinases (MMPs) of the rheumatoid synovium induce catabolic events in synovitis and articular components. Sutton et al. (2009) reported that tissue tethering, joint deformity, and defective joint function are remarkable symptoms of synovitis. Wasserman (2011) reported that treatment with disease-modifying agents reduces the intensity of swelling and pain symptoms in the early stage disease. To date, no reliable therapeutic approach exists for rheumatoid arthritis (Saag et al. 2008).

Methylsulfonylmethane is a well-known sulfur-containing compound (Engelke et al. 2005). Several researchers have reported that methylsulfonylmethane with glucosamine is effective against osteoarthritis, and a remarkable decrease in swelling and pain, as well as improvement in joint function, was noted (Usha and Naidu 2004). Hasegawa et al. (2004) reported on the anti-inflammatory effect of methylsulfonylmethane in rheumatoid arthritis. Kim et al. (2009) reported that methylsulfonylmethane inhibits the production of NO and PGE₂ in lipopolysaccharide-stimulated RAW264.7 cells.

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Several researchers reported on another sulfur-containing compound (chondroitin sulfate) that prevents knee joint space narrowing and reduces joint pain (Iouv et al. 2008). Volpi (2011) reported that chondroitin sulfate inhibits the expression of nuclear factor (NF)- κ B in chondrocytes. Because methionine is a sulfur-containing amino acid, it is expected to have similar anti-inflammatory effects as chondroitin sulfate and methylsulfonylmethane. Thus, the present study investigated the anti-arthritic effects of methionine in neonatal rats.

Materials and methods

Animals

Neonatal rats were purchased from the animal house of Laboratory Animal Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The rats weighed 6–7 g and were used for the investigation. Standard food and water were provided to the neonatal rats with a 12-h light and dark cycle. All experiments involving rats were monitored and approved by the ethics committee of our institution.

Experimental groups

The rats were classified into four groups, with six rats in each group. The dose was given orally for 45 consecutive days.

- Group I: Normal control (negative control);
- Group II: Control rheumatoid arthritis (positive control);
- Group III: Methionine (150 mg/kg body weight); and
- Group IV: Methionine (300 mg/kg body weight).

Experimental rheumatoid arthritis

Experimental rheumatoid arthritis was induced by intradermal administration of complete Freund's adjuvant. Briefly, the emulsion was prepared by mixing complete Freund's adjuvant (*Mycobacterium tuberculosis*) and type II bovine collagen at an equal ratio. Rheumatoid arthritis was induced by the intradermal administration of the emulsion (100- μ g/100- μ l/body weight) at the tail region and was

allowed to continue for 3 weeks. Thereafter, a booster dose of the emulsion with incomplete Freund's adjuvant was given to induce rheumatoid arthritis (Zimmermann 1983).

Determination of biochemical markers

Superoxide dismutase (SOD) and catalase activities were determined as described previously (Meng et al. 2002; Iwase et al. 2013). Glutathione peroxidase (Gpx) activity, lipid peroxidation, and reduced glutathione (GSH) levels were determined as described previously (Power and Blumbergs 2009; Kaddour et al. 2016). The uric acid level in plasma was determined using the calorimetric assay (Mahajan and Tandon 2004). The nitric oxide (NO) level in the plasma was measured according to Van Beezooijen et al. (1988). The plasma levels of PGE₂ and MMP-3 were determined using the enzyme-linked immunosorbent assay method (RAB0311-1KT, Sigma-Aldrich, Shanghai). The ceruloplasmin level in plasma was measured according to Fossati et al. (1980).

Preparation of cell supernatant

Chondrocytes were isolated from neonatal rats according to Gartland et al. (2005). The enzymes such as collagenase, hyaluronidase, and trypsin were used for chondrocytes digestion. The digestion process was immediately performed after chondrocyte isolation and thawed for cell culture experiments. Then, chondrocytes were cultured in cell culture dish and allowed to differentiate for the expression of NF- κ B and iNOS.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (qRT-PCR) was used to quantify expression. Total RNA was isolated from chondrocytes and converted into cDNA using oligo (dT) primers. Next, qPCR quantified the mRNA expression with primers specific for NF- κ B and inducible nitric oxide synthase (iNOS; Table 1). GAPDH was used as a qPCR internal control. The $2^{-\Delta\Delta C_T}$ method was used to calculate the relative expression ratios (Borges et al. 2017).

Table 1 List of primers used in real-time polymerase chain reaction (qRT-PCR)

S. no.	Gene name	Sense primer	Anti-sense primer
1	iNOS	5'-GTTCTCAAGGCACAGGTCTC-3'	5'-GCAGGTCACCTATGTCACTTATC-3'
2	NF- κ B	5'-GAAATTCCTGATCCAGACAAAAAC-3'	5'-ATCACTTCAATGGCCTCTGTGTAG-3'
3	GAPDH	5'-TCCCTCAAGATTGTCAGCAA-3'	5'-AGATCCACAACGGATACATT-3'

Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate proteins in the chondrocyte, and the proteins were transferred to polyvinylidene difluoride membranes. Next, the membranes were probed with the primary antibodies NF- κ B (ab16502; Abcam) or iNOS (ab3523; Abcam) for 12 h. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (A0545-1ML; Sigma-Aldrich) for 1 h. The protein levels of NF- κ B and iNOS were determined by enhanced chemiluminescence (Dmitriev et al. 2005).

Statistical analysis

All experimental outcomes are presented as means with standard error of the mean. Analysis of variance test was used for multiple comparisons. Statistically, significance was taken when $P < 0.05$.

Results

Control groups

Compared with the normal control, SOD and catalase enzyme activities were reduced to 65.66 and 74.78%, respectively, in the rheumatoid arthritis group (Fig. 1; $P < 0.05$).

The malondialdehyde (MDA) content was substantially increased to 375.46% in the rheumatoid arthritis group compared with the normal control group (Fig. 2A, $P < 0.05$). Gpx activity and GSH content were reduced to 57.44 and 62.16%, respectively, in the rheumatoid arthritis group (Figs. 2B, 3; $P < 0.05$). The uric acid, NO, and ceruloplasmin acid contents were substantially increased to 371.43, 295.8, and 59.65%, respectively, in the rheumatoid arthritis group (Fig. 4; $P < 0.05$). The PGE₂ and MMP-3 contents were substantially increased to 522.5 and 279.5%, respectively, in the rheumatoid arthritis group (Fig. 5; $P < 0.05$). The NF- κ B and iNOS mRNA expression levels were substantially increased by 140 and 120%, respectively, in the rheumatoid arthritis group (Fig. 6; $P < 0.05$). The NF- κ B and iNOS protein expression levels were substantially increased by 115 and (Fig. 7; $P < 0.05$) 101%, respectively, in the rheumatoid arthritis group.

Effect of methionine on rheumatoid arthritis

Compared with the controls, methionine supplementation increased SOD enzyme activity up to 148.7% in group IV (Fig. 1; $P < 0.05$), whereas catalase activity was increased up to 246.8% in group IV (Fig. 1; $P < 0.05$). Compared with the controls, methionine supplementation reduced the MDA content up to 66.7% in group IV (Fig. 2A, $P < 0.05$). Methionine supplementation significantly increased Gpx activity up to 95% in group IV (Fig. 2B, $P < 0.05$), while the GSH

Fig. 1 Effect of methionine on superoxide dismutase and catalase activity in rheumatoid arthritis-induced neonatal rats. The activities are expressed as U/ml. ^a $P < 0.05$ vs. group I, ^b $P < 0.05$ vs. group II, and ^c $P < 0.05$ vs. group III

Fig. 2 Effect of methionine on lipid peroxidation and glutathione peroxidase activity in rheumatoid arthritis-induced neonatal rats. **A** Lipid peroxidation content is expressed as nmol/ml. **B** Gpx activities are

expressed as U/ml. ^a $P < 0.05$ vs. group I, ^b $P < 0.05$ vs. group II, and ^c $P < 0.05$ vs. group III

Fig. 3 Effect of methionine on the reduced glutathione (GSH) content in rheumatoid arthritis-induced neonatal rats. The GSH content is expressed as nmol/ml. ^a $P < 0.05$ vs. group I, ^b $P < 0.05$ vs. group II, and ^c $P < 0.05$ vs. group III

content was increased up to 142.8% in group IV (Fig. 3; $P < 0.05$).

Compared with the controls, methionine supplementation reduced the uric acid level up to 62.1% in group IV (Fig. 4A, $P < 0.05$), while NO was reduced up to 56.8% in group IV (Fig. 4B, $P < 0.05$). The ceruloplasmin content was reduced

up to 29.7% in group IV (Fig. 4C, $P < 0.05$). Compared with the controls, methionine supplementation reduced the PGE₂ level up to 69.7% in group IV (Fig. 5; $P < 0.05$), whereas MMP-3 was reduced up to 61.4% in the methionine-treated group (Fig. 5; $P < 0.05$). NF- κ B mRNA expression was substantially reduced up to 51.7% in group IV (Fig. 6; $P < 0.05$), whereas the mRNA expression of iNOS was reduced up to 43.5% in group IV (Fig. 6; $P < 0.05$). NF- κ B protein expression was substantially reduced up to 45.8% in group IV (Fig. 7; $P < 0.05$), whereas the protein expression of iNOS was reduced up to 45.4% in group IV (Fig. 7; $P < 0.05$).

Discussion

In the present study, we investigated the therapeutic effect of methionine against rheumatoid arthritis in neonatal rats. Baueroova and Bezek (1999) reported that the chronic inflammation of tissues and joints and infiltration of activated T cells and macrophages are primary symptoms of rheumatoid arthritis. Heliovaara et al. (1994) reported that the reduced level of cellular antioxidants is critical for rheumatoid arthritis, and accelerated free radical production from the inflammatory site leads to intensified rheumatoid arthritis. Lipid peroxyl radicals produced from membrane fatty acid oxidation and further chain reaction lead to cell membrane damage. Increased production of

Fig. 4 Effect of methionine on the uric acid (**A**), nitric oxide (NO) (**B**), and ceruloplasmin (**C**) levels in rheumatoid arthritis-induced neonatal rats. **A** Uric acid, **B** NO, and **C** ceruloplasmin levels are

expressed as mg/ml, ng/ml, and mg/ml, respectively. ^a $P < 0.05$ vs. group I, ^b $P < 0.05$ vs. group II, and ^c $P < 0.05$ vs. group III

prostaglandins from oxidative injury and the inflammatory site has been reported (Bae et al. 2003).

Methionine substantially reduced MDA, uric acid, NO, MMP-3, PGE₂, NF- κ B, and iNOS levels compared with those in the rheumatoid arthritis group. Ozturk et al. (1999) reported a higher rate of lipid peroxidation in the rheumatoid arthritis condition. Okada et al. (1989) reported that MMP-3 from synovial lining cells plays a crucial role in the activation of pro-collagenases and destruction of type IX collagen and cartilage proteoglycans. Yamanaka et al. (2000) indicated that the increased level of serum MMP-3 serves as an index for radiological damage and degradation of cartilage. Yamagishi et al. (2012) reported that the combined supplementation of methionine and glucosamine inhibited inflammatory mediators and synovial inflammation. Najm et al. (2004) reported that *S*-adenosylmethionine supplementation was useful in the management of osteoarthritis symptoms.

Gambhir et al. (1997) reported that cellular antioxidant levels and lipid peroxidation are negatively correlated. The levels of SOD, catalase, GSH, and Gpx were low in rheumatoid arthritis. However, the level of this antioxidant was found to be higher in methionine-treated rats than in normal control rats. In this study, the ceruloplasmin level was substantially higher in rheumatoid arthritis rats than in normal control rats. Methionine supplementation significantly reduced the ceruloplasmin level compared with the control. Amancio et al. (2003) reported a higher level of ceruloplasmin under rheumatoid arthritis conditions. An increased level of uric acid has been noted in rheumatoid arthritis due to activated xanthine oxidase (Nemeth et al. 2002).

Fig. 5 Effect of methionine on prostaglandin E₂ (PGE₂) and matrix metalloproteinase (MMP)-3 levels in rheumatoid arthritis-induced neonatal rats. The PGE₂ and MMP-3 levels are expressed as pg/ml and ng/ml, respectively. ^a*P* < 0.05 vs. group I, ^b*P* < 0.05 vs. group II, and ^c*P* < 0.05 vs. group III

Fig. 6 Effect of methionine on the mRNA expression of nuclear factor (NF)-κB and inducible nitric oxide synthase (iNOS) in rheumatoid arthritis-induced neonatal rats. ^a*P* < 0.05 vs. group I, ^b*P* < 0.05 vs. group II, and ^c*P* < 0.05 vs. group III

Conclusions

Our experimental data suggest that supplementation with sufficient methionine improves the antioxidant status and reduces inflammatory markers. Taken together, our

Fig. 7 Effect of methionine on the protein expression of NF-κB and iNOS in rheumatoid arthritis-induced neonatal rats. **A** Western blot bands of NF-κB and iNOS and **B** Densitometry analysis of **A**. ^a*P* < 0.05 vs. group I, ^b*P* < 0.05 vs. group II, and ^c*P* < 0.05 vs. group III

data suggest that methionine supplementation is effective against rheumatoid arthritis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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