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Original Article

Isorhamnetin attenuates collagen-induced arthritis via modulating cytokines and oxidative stress in mice

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Abstract: Inflammation and oxidative stress were involved in the development and progression of rheumatoid arthritis (RA). Isorhamnetin has anti-inflammatory and anti-oxidative activities, but its effects on RA have not been investigated. In order to observe the possible therapeutic effects of isorhamnetin on RA, we established a collagen-induced arthritis mouse model and treated the animal with isorhamnetin for 3 weeks. Besides, fibroblast-like synoviocytes (FLS) were treated with lipopolysaccharide (LPS) and isorhamnetin. The severity of arthritis was assessed by arthritis score, joint destruction score and inflammation score. Levels of cytokines TNF-α, IL-1β, IL-6, IL-17A, IL-17F, IL-10 and IL-35 in the joint tissue homogenate and cell culture medium as well as anti-type II collagen antibody in serum were measured using ELISA. Contents of H_2O_2 and malondialdehyde (MDA) in joint tissue homogenate were measured using assay kits. We found collagen immunization induced significant arthritis in mice and isorhamnetin at the dose of 10 and 20 mg/kg/day could significantly attenuate the collagen-induced arthritis. Isorhamnetin also modulated the production of cytokines and suppressed the oxidative stress in the mice with collagen-induced arthritis at the dose of 10 and 20 mg/kg/day. These data suggested that isorhamnetin might be a potential agent for the management of RA.

Keywords: Isorhamnetin, collagen-induced arthritis, rheumatoid arthritis, effect, inflammation, oxidative stress

Introduction

Rheumatoid arthritis (RA), a systemic autoimmune disease, is characterized by persistent synovial inflammation and progressive destruction of both joint cartilage and bone tissues [1]. If untreated, it may cause extra-articular involvements such as involvements of the cardiovascular system, haematological system, liver, respiratory system, eyes, muscles, kidneys and the neurological system [2] and it may seriously affect life quality of the patients and reduce life expectancy. Although some medications are available in clinic, the RA treatment is not satisfactory at present. Efforts should be made on the discovering of new agents targeting at the specific pathogenesis of RA.

Inflammation is the essential pathological change of RA [3]. It is well known that uncontrolled production of the pro-inflammatory cytokines can promote autoimmune pathology. Although Th1 cell is essential for driving autoimmune pathology, its cytokine interferon-γ was not considered as a driver of autoimmunity in RA. Among the multiple pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 have well been demonstrated to contribute to the development and progression of RA. Increased levels of these cytokines were reported in blood and joint tissue in many pre-clinical and clinical studies [4]. Some drugs are reported to ameliorate the collagen-induced arthritis via decreasing the overproduction of TNF-α, IL-1β and IL-6 in animals [4, 5]. Biological agents of anti-TNF, anti-IL-1β and anti-IL-6 such as etanercept, infliximab and adalimumab are clinically available and have showed some degree of efficacy in animals and patients [6, 7]. Recently, medications targeting at TNF-α IL-1β and IL-6 become popular in the clinical practice [8]. Besides, some recent studies demonstrated that TH17 cell also played an essential role in RA [9-13]. Elevated levels of IL-17 protein and mRNA were observed in serum and tissues of animals and patients with RA. A very recent study showed IL-17-deficient allogeneic bone marrow transplantation prevented the induction of collagen-

Table 1. Effects of isorhamnetin on the arthritis score of the mice

	Day 21	Day 25	Day 29	Day 33	Day 37	Day 41
Control	0.15±0.03	0.16±0.05	0.13±0.04	0.09±0.02	0.16±0.04	0.10±0.02
CIA	0.20±0.04	0.69±0.15[a]	2.03±0.50[a]	5.25±1.09[a]	6.19±1.05[a]	6.95±1.17[a]
Iso-2	0.22±8.4	0.59±0.08[a]	1.85±0.23[a]	4.87±0.82[a]	5.53±1.14[a]	6.07±1.09[a]
Iso-10	0.25±6.9	0.41±0.05[a,b]	0.92±0.15[a,b]	2.10±0.31[a,b]	2.86±0.48[a,b]	3.11±0.55[a,b]
Iso-20	0.21±6.1	0.40±0.07[a,b]	0.88±0.17[a,b]	1.93±0.35[a,b]	2.58±0.33[a,b]	2.66±0.37[a,b]

Data are presented as mean ± SD. [a]$P<0.05$, vs. the control group; [b]$P<0.05$, vs. the CIA group.

Table 2. Effects of isorhamnetin on joint destruction score, inflammation score andserum anti-C II antibody level

	Joint destruction score	Inflammation score	Anti-C II antibody (×10^4 Unites/ml)
Control	0.11±0.02	0.20±0.01	0.06±0.01
CIA	3.40±0.55[a]	3.60±0.44[a]	8.65±1.21[a]
Iso-2	2.97±0.49[a]	3.04±0.36[a]	8.31±1.33[a]
Iso-10	1.53±0.32[a,b]	1.60±0.22[a,b]	7.56±1.09[a]
Iso-20	1.40±0.35[a,b]	1.49±0.25[a,b]	7.39±1.26[a]

Data are presented as mean ± SD. [a]$P<0.05$, vs. the control group; [b]$P<0.05$, vs. the CIA group.

induced arthritis in DBA/1J mice, which confirmed the role of IL-17 in the development of RA. Furthermore, agents that could decrease IL-17 levels provided important benefits in the treatment of RA [14, 15]. Opposite to the above cytokines, Treg cell cytokines are believed to play a protective role in RA.

Besides inflammatory cytokines, oxidative stress also plays a role in RA. Excessive reactive oxygen species (ROS) is found in subjects with RA and the overproduction of ROS leads to various damage [16]. ROS can degrade isolated proteoglycans, and HOCl fragments collagen, inhibite cartilage proteoglycan synthesis, activate latent metalloproteinases, inactivae TIMPs, low levels of ascorbate in synovial fluid, promotes chondrocyte apoptosis, ultimately leads to the disruption of cartilage and bone tissue [17]. The activation of nicotinamide adenine dinucleotide phosphate- oxidase by inflammatory cytokines IFN-γ, TNF-α and IL-1β is one of the main sources of ROS [18], whereas the excessive ROS in turn can activate NF-κB which promotes the productions of cytokines TNF-α, IL-1β and IL-6 [19-21]. Theoretically, the interactions between inflammatory cytokines and oxidative stress would accelerate the progression of RA. Based on the understanding of oxidative stress in RA, anti-oxidative stress is thought to be a potential method in the treatment of RA.

Natural compounds extracted from plants are characterized by multiple pharmacological activities and mild adverse reactions and have been extensively used in many diseases [22]. Isorhamnetin is a plant flavonoid abundant in herbal medicinal plants such as *Hippophae rhamnoides* L. and *Ginkgo biloba* L. It has been recently reported for its activities of anti-inflammation and anti-oxidative stress in some preclinical studies [23-26]. But no studies investigated the potential effects of isorhamnetin on RA, and whether isorhamnetin can inhibit the collagen-induced inflammation and oxidative stress in animals is unknown to date. We intended to observe the effects of isorhamnetin on RA as well as on the inflammatory cytokines and oxidative stress in a mice model of collagen-induced arthritis.

Methods

Animals

C57BL/6 mice were fed in specific pathogen free conditions (4 mice per cage) and provided food and water ad libitum with a 12 hour light dark cycle. Room temperature and humidity were set at 22-25°C and 60-65%, respectively. Animals were randomly divided into control group, CIA group, CIA + isorhamnetin 2 mg group (Iso-2 group), CIA + isorhamnetin 10 mg group (Iso-10 group) and CIA + isorhamnetin 20 mg group (Iso-20 group) with 8 mice in each group. The procedures and protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by Animal Care and Use Committee of Weifang Medical College.

Table 3. Levels of TNF-α, IL-1β, IL-6, IL-17A, IL-17F, IL-10 and IL-35 in jointtissue homogenate of the mice (pg/ml)

	TNF-α	IL-1β	IL-6	IL-17A	IL-17F	IL-10	IL-35
Control	65.1±8.7	44.0±6.3	38.0±5.2	16.4±3.8	13.1±1.6	55.4±7.2	48.3±6.0
CIA	183.2±25.1[a]	115.5±14.0[a]	135.3±16.1[a]	51.0±7.7[a]	45.9±6.2[a]	23.2.0±4.0[a]	19.8±2.5[a]
Iso-2	153.8±20.2[a]	102.7±13.6[a]	119.6±13.3[a]	46.1±5.9[a]	39.0±5.0[a]	26.8±4.1[a]	23.1±3.2[a]
Iso-10	112.0±15.4[a,b]	71.5±10.8[a,b]	80.4±12.0[a,b]	32.7±5.4[a,b]	25.5±6.1[a,b]	38.6±5.2[a,b]	33.5±3.7[a,b]
Iso-20	101.6±12.5[a,b]	65.3±7.9[a,b]	73.1±10.1[a,b]	29.4±4.6[a,b]	21.8±3.3[a,b]	41.1±4.8[a,b]	36.6±4.6[a,b]

Data are presented as mean ± SD. [a]$P<0.05$, vs. the control group; [b]$P<0.05$, vs. the CIA group.

Induction of CIA

C57BL/6 mice were treated with chicken type II collagen (CII; Sigma-Aldrich) to establish the CIA mice model as described elsewhere [27, 28]. Briefly, the CII immunization comprised two times of CII injection. On day 1, the mouse was injected 2 mg/ml CII emulsion (dissolved in 0.5 M acetic acid and then emulsified with CFA) at two sites at the base of the tail. On day 21, the mouse received a second injection of CII emulsion with the same protocol as above.

Isorhamnetin treatment

The mice Iso-2 group, Iso-10 group and Iso-20 group were administrated with isorhamnetin (Shanghai Winherb Medical S&T Development, Shanghai, China) dissolved in saline, respectively at the dose of 2, 10 and 20 mg/kg/day by intraperitoneal injection for 3 weeks starting from day 21. The mice in the control group and the CIA group were treated with vehicle saline.

Assessment of arthritis

The severity of arthritis was assessed by arthritis score, joint destruction score and inflammation score as described elsewhere [29]. Criteria (0-4) for arthritis score is: normal (0), swelling in 1 joint (1), swelling in >1 joint (2), swelling in the entire paw (3), and deformity and/or ankylosis (4); the cumulative score for all 4 paws of each animal was used to represent the severity. Hind paws were used for radiographic evaluation and the joint destruction was scored on a scale of 0-4 as: no damage (0), demineralization (1), 1 or 2 erosions (2), severe erosions (3) and complete destruction of the joints (4). The hind paw was fixed in 10% buffered formalin, decalcified in 15% EDTA, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (HE). Inflammation was graded as: 0 (no inflammation) to 3 (severely inflamed joint) based on the infiltration extent of inflammatory cells into the synovium.

Cell culture

There were 3 groups: normal group, lipopolysaccharide (LPS) group and LPS + isorhamnetin group. The human fibroblast-like synoviocytes (FLS) were seeded at 2×10^5 cells/well in culture plates and cultured in Ham's F12 supplemented with 10% heat-inactivated fetal bovine serum and 50 units/ml penicillin/ streptomycin. After 2 days, the medium in plates of the LPS group was replaced by new medium containing LPS (1 μg/ml); the medium in plates of the LPS + isorhamnetin group was replaced by medium containing LPS (1 μg/ml) and isorhamnetin (10 μM). The cells were further cultured for 24 h and the medium was collected for the measurements of cytokines.

Tissue homogenate preparation

The dissected joints were washed in iced saline and homogenized in saline using a homogenizer. The homogenate was immediately centrifuged at 3000 rpm for 10 min twice. The liquid supernatant was used for the measurements of cytokines and oxidative markers and stored at -80°C prior to biochemical assays.

Cytokines analysis

Levels of cytokines TNF-α, IL-1β, IL-6, IL-17A, IL-17F, IL-10 and IL-35 in the joint tissue homogenate and cell culture medium were measured using a standard sandwich ELISA (CUSABIO, Wuhan, China) according to the manufacturer's instructions.

Oxidative markers analysis

Contents of H_2O_2 and malondialdehyde (MDA) in joint tissue homogenate were measured using commercially available assay kits (Ji-

Table 4. Levels of TNF-α, IL-1β and IL-6 in cell culture medium (pg/ml)

	TNF-α	IL-1β	IL-6
Normal	8.36±1.05	7.09±1.12	8.08±1.17
LPS	21.71±2.64[a]	17.84±2.31[a]	22.10±2.78[a]
LPS + isorhamnetin	13.85±2.01[a,b]	11.50±1.57[a,b]	14.39±2.15[a,b]

Data are presented as mean ± SD. [a]P<0.05, vs. the normal group; [b]P<0.05, vs. the LPS group.

ancheng Bioengineering Institute, Nanjing, China), according to the protocol provided by the manufacturer.

Anti-type II collagen antibody analysis

Blood was collected from the heart of the mouse on the last day of the isorhamnetin treatment and centrifuged to obtain serum. Anti-CII antibodies in serum were determined using a standard sandwich ELISA (Chondrex, Redmond, WA, USA) strictly according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with subsequent Students-Newman-Keuls (SNK) test. Differences between groups were considered statistically significant if P value is less than 0.05.

Results

Arthritis assessment

The severity of arthritis was assessed by arthritis score, joint destruction score and inflammation score. The mice in the CIA group had much higher arthritis score (on day 25, 29, 33, 37 and 41), joint destruction score and inflammation score than the control animals (all P<0.05), which meant CII immunization successfully induced arthritis in the animal of the CIA group. Iso-10 group and Iso-20 group showed much reduced arthritis score (on day 25, 29, 33, 37 and 41), joint destruction score and inflammation score if compared to the CIA group (all P<0.05). But the scores of the Iso-2 group were similar to the CIA group (all P>0.05). The data indicated that isorhamnetin at the dose of 10 and 20 mg/kg/day could significantly attenuate the CII induced arthritis (Shown in **Tables 1** and **2**).

Cytokines analysis

The results the Elisa assay showed mice in the CIA group had much increased levels of TNF-α, IL-1β, IL-6, IL-17A and IL-17F as well as decreased levels of IL-35 and IL-10 in the joint tissue homogenate than the control animals (all P<0.05). Compared to the CIA group, isorhamnetin treatment significantly reversed the changes of the cytokines in the Iso-10 group and Iso-20 group (all P<0.05), but not in the Iso-2 group (all P>0.05). The cell culture also showed isorhamnetin markedly inhibited the production of TNF-α, IL-1β and IL-6 in medium of the LPS + isorhamnetin group, if compared to the LPS group (all P<0.05) (Shown in **Tables 3** and **4**).

Oxidative markers analysis

Mice in the CIA group had much increased levels of H2O2 and MDA in the joint tissue homogenate than the control animals (both P<0.05). Compared to the CIA group, isorhamnetin treatment significantly reduced the elevation of H_2O_2 and MDA in the Iso-10 group and Iso-20 group (all P<0.05), but not in the Iso-2 group (P>0.05) (Shown in **Table 5**).

Anti-type II collagen antibody analysis

Mice in the CIA group had much increased levels of serum anti-CII antibody than the control animals (P<0.05). However, no significant differences in levels of serum anti-CII antibody among the CIA group, the Iso-2 group, the Iso-10 group and the Iso-20 group were observed (all P>0.05) (Shown in **Table 2**).

Discussion

The study provided evidence that isorhamnetin attenuates arthritis induced by collagen injection and modulates the production of cytokines and oxidative markers in the animal model, but has no effects on anti-CII antibody production.

RA is a chronic systemic autoimmune disease that affects millions of people worldwide. Although there are multiple drug choices in the treatment of RA, the effects are still not satisfactory and some patients are suffering from the adverse reactions of the dugs. For decades,

Table 5. Effects of isorhamnetin on oxidative stress markers in joint tissuehomogenate

	MDA (mmol/gprot)	H_2O_2 (mmol/gprot)
Control	0.39±0.06	0.41±0.06
CIA	1.15±0.21[a]	1.55±0.30[a]
Iso-2	0.91±0.10[a]	1.28±0.16[a]
Iso-10	0.73±0.11[a,b]	0.83±0.10[a,b]
Iso-20	0.64±0.08[a,b]	0.78±0.09[a,b]

Data are presented as mean ± SD. [a]$P<0.05$, vs. the control group; [b]$P<0.05$, vs. the CIA group.

natural compounds from medical plants have attracted people's great attention duo to their multiple pharmacological activities and mild adverse reactions. Isorhamnetin is a plant flavonoid abundant in some herbal medicinal plants. Duo to its various activities, it has been used in the management of several diseases [23-26]. But its effect on RA has not been valuated to date to our knowledge.

In order to detect its potential anti-RA effects, we firstly establish an arthritis mouse model by immunization of chicken type II collagen according to the literature. It is regarded that the collagen-induced arthritis (CIA) animal model exhibits joint swelling, synovitis, periosteal new bone formation, articular bone erosion, and osteopenia, which are similar to the clinical and pathological features of human RA. So CIA animal model is widely used to evaluate the anti-RA activity of new agents [29]. With two times of collagen immunization, we found the CII immunized mice in the CIA group had higher arthritis score, joint destruction score and joint inflammation score than control group, indicating the CIA animal model was successfully established. From the day 21, three groups of CII immunized mice were treated with isorhamnetin for 3 weeks. We found isorhamnetin at the dose of 10 and 20 mg/kg/day significantly decreased the arthritis score, joint destruction score and joint inflammation score if compared to the CIA group, but the dose of 2 mg/kg/day not. The decreases in the scores indicated that isorhamnetin significantly reduced the CII-induced injury of the joint.

As a systemic autoimmune disease, RA involves a significant immune imbalance [3]. The role of the overproduction of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 has been well demonstrated in RA. These cytokines are well known targets in RA management [4-7]. Th17 cell, a subset of T helper cell, plays an important role in host defense and the pathogenesis of various autoimmune and inflammatory diseases including RA [9-13]. IL-17 is an inflammatory cytokine secreted by Th17 cell. It can coordinate tissue inflammation by inducing the production of TNF-α, Il-1β, IL-6 and IL-8. Elevated levels of IL-17 were found in subjects with RA and the IL-17 overproduction was regarded as an important contributor leading to or exacerbating RA. Regulatory T (Treg) cell is another type of T cell which is involved in RA. Treg cell cytokines IL-35 and IL-10 are regarded as protective factors in RA. It is believed that autoimmune diseases often result from an imbalance between Treg cells and TH17 cells [10]. The restoration of Th17 cell/Treg cell balance is one of the targets in RA management [31]. We detected some cytokines in joint tissue and found mice in CIA group had significantly increased levels of TNF-α, IL-1β, IL-6, IL-17A and IL-17F as well as decreased levels of IL-35 and IL-10. The results were in coincident with the previous studies [4, 5]. We also found that isorhamnetin at the dose of 10 and 20 mg/kg/day could significantly reverse the changes of the cytokines induced by CII immunization, but the dose of 2 mg/kg/day not. Invasion of fibroblast-like synoviocytes (FLSs) is critical in the pathogenesis of RA. It was reported that LPS could induce the release of cytokines from FLSs, such as IL-1β and TNF-α [31]. In order to confirm the anti-cytokine effects of isorhamnetin in FLSs, we cultured FLSs with LPS and isorhamnetin. We found LPS promoted the production of IL-1β, IL-6 and TNF-α in medium in the LPS group, while isorhamnetin significantly abolished LPS-induced IL-1β, IL-6 and TNF-α in the LPS + isorhamnetin group. This further confirmed the anti-cytokine effect of isorhamnetin.

Excessive reactive oxygen species (ROS) and low oxidant defense activity were found in RA. There is some evidence suggesting the role of oxidative stress in the pathogenesis of RA [16]. Various forms of antioxidant managements have demonstrated promising effects in experimental arthritis models [32]. In this study, we also found the mice in the CIA group had elevated levels of MDA and H_2O_2 in joint tissue homogenate, which was in consistent with other studies. H_2O_2 is regarded as a substance which can activate NF-κB, while the activated NF-κB can

exacerbate inflammatory response via promoting the release of cytokines TNF-α, IL-1β and IL-6. Three weeks of isorhamnetin treatment at the dose of 10 and 20 mg/kg/day significantly decreased levels of MDA and H_2O_2 in joint tissue homogenate, but not at the dose of 2 mg/kg/day.

Anti-type II collagen antibody plays a crucial role in the development of RA [33, 34]. Suppression the production of the antibody was proved to be beneficial for RA control [35, 36]. In line with the other studies, we found the mice in the CIA group had markedly elevated levels of serum anti-C II antibody than the control animals. Although isorhamnetin attenuated the RA, it had no significant effects on levels of serum anti-C II. But there was a downtrend in its levels with isorhamnetin treatment. The result indicated the improvement of RA in this study was not benefit from the inhibition of anti-C II antibody production.

In conclusion, isorhamnetin treatment attenuated collagen-induced arthritis, modulated the production of cytokines and suppressed the oxidative stress, but had no effects on the anti-CII antibody production in mice. These data suggest that isorhamnetin might be a potential agent for the management of RA in clinic. However its effects in patients with RA should be further investigated.

Disclosure of conflict of interest

None.

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