



Cholinergic system is involved in the therapeutic effect of madecassoside on collagen-induced arthritis in rats

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ABSTRACT

Our previous studies demonstrated that oral administration of madecassoside could markedly attenuate collagen-induced arthritis in rats, a rodent model of rheumatoid arthritis. As the autonomic nervous system is critically involved in the modulation of peripheral inflammation and immune response, the present study aims to explore the possible involvement of adrenergic and cholinergic nerves in the effect of madecassoside on rheumatoid arthritis. Arthritis was induced by chicken collagen in rats, and madecassoside was orally administered daily for two weeks from day 14 after the primary immunization. The antagonists of adrenoceptor and cholinergic receptors were co-administered with madecassoside, respectively. Unilateral cervical vagotomy was performed four days before the arthritis induction. The results showed that madecassoside (30 mg/kg) treatment markedly ameliorated arthritis symptoms in rats, mainly evidenced by the reduction of paw swelling and arthritis index scores. Co-administration of madecassoside with atropine (an antagonist of the muscarinic acetylcholine receptor) or hexamethonium (an antagonist of the nicotinic acetylcholine receptor) markedly diminished the therapeutic effects of madecassoside in arthritis. However, co-administration with phentolamine (an antagonist of the α -adrenoceptor) or propranolol (an antagonist of the β -adrenoceptor) did not alter the effect of madecassoside on arthritis. Furthermore, unilateral cervical vagotomy significantly reduced the anti-arthritis efficacy of madecassoside, including the amelioration of clinical symptoms, as well as the inhibition of the production of pro-inflammatory cytokines except T lymphocytes-related cytokines. These findings suggest that madecassoside exerts inhibitory effects on collagen-induced arthritis through, at least partially, the peripheral cholinergic system.

1. Introduction

Rheumatoid arthritis (RA), mainly characterized by chronic synovitis, is a systemic autoimmune and inflammatory disease with unknown etiology [1]. It is generally acknowledged that the immune dysfunction in RA results in excessive production of helper T cells-derived inflammatory cytokines, such as IL-17A and IFN- γ . These cytokines activate macrophages, synovial fibroblasts and osteoclasts [2,3], which causes the initiation and persistence of synovial inflammation, and eventually leads to the swelling of the joints and the damage of cartilage and bones.

In addition to the immune system, the nervous and endocrine systems also play active and important roles in the initiation and development of RA. Accumulative evidence indicates that the autonomic

nervous system, which consists of two parts, the sympathetic and the parasympathetic nervous systems, participates in the regulation of immune response and inflammation in RA [4]. A clinical report demonstrated that the treatment of infliximab (Remicade), a TNF- α inhibitor, resulted in a significant decrease of ambulatory blood pressure, plasma norepinephrine level and renin activity in RA patients, indicating the reduction of sympathetic nerve activity [5]. In collagen-induced arthritis (CIA), although there are conflicting reports with regard to the pro-inflammatory or anti-inflammatory effect of the sympathetic nervous system [6], it is certain that the activity of the parasympathetic nervous system is decreased. Stimulation of the vagus nerve or nicotinic receptors (especially $\alpha 7$ subunit) [7] or inhibition of acetylcholine esterase activity [8] could attenuate inflammation in endotoxemia, CIA and other inflammatory diseases [9–12]. Data of clinical trials also

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support that the autonomic nervous system was imbalanced in RA patients [13].

Madecassoside (Mad), one of the major active triterpenoid constituents of *Centella asiatica* herb, has previously been demonstrated to have a variety of bioactivities, including anti-scar, anti-pulmonary fibrosis, anti-oxidizing, memory enhancing, neuroprotective, and anti-inflammatory effects [14–17]. Recently, we found that Mad, orally administered, could effectively ameliorate the systemic immune abnormalities, and suppress synovial hyperplasia and bone erosion in CIA mice and rats [18,19]. However, pharmacokinetic studies showed that the plasma concentration of Mad by oral administration was extremely low [20]. *In vitro*, Mad exhibited very weak effects on immune and inflammation effector cells even at higher concentrations (> 1000 folds of C_{max}) that were hardly reached in tissues *in vivo* [18]. It was suggested that Mad, unlike conventional anti-arthritis agents, might exert anti-arthritis efficacy through mechanisms rather than directly affecting the effector cells after absorption.

There was a report indicating that Mad could restore cholinergic function by improving the abnormalities of acetylcholine level and acetylcholine esterase activity in cognitive impairment mice [21], which prompts us to explore the possible involvement of autonomic nervous system in the anti-arthritis action of Mad.

2. Materials and methods

2.1. Animals

Specific pathogen-free female Wistar rats, weighing between 130 and 150 g, were purchased from Shanghai Super B & K Laboratory Animal Corp. Ltd. (Shanghai, China). They were kept on standard laboratory chow with tap water *ad libitum* and under climate-controlled conditions. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the current ethical regulations of China Pharmaceutical University.

2.2. Chemicals and reagents

Mad (Fig. 1) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China), and the purity was determined to be higher than 98% detected by high-performance liquid chromatography.

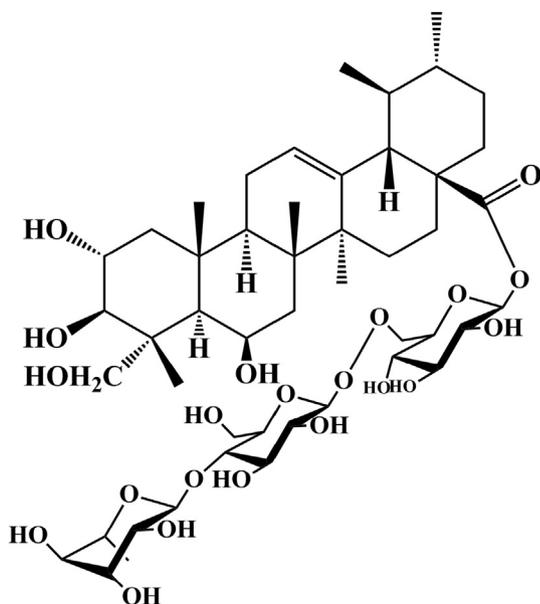


Fig. 1. Chemical structure of madecassoside (Mad).

Phentolamine Mesilate (Phe) was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China); Propranolol Hydrochloride (Pro) was purchased from Changzhou Kangpu Pharmaceutical Co., Ltd. (Changzhou, China); Atropine Sulfate (Atr) was purchased from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China); Hexamethonium Chloride (Hex) was purchased from Sigma-Aldrich Co. (St. Louis, USA); Leflunomide (Lef) was purchased from Suzhou Changzheng-Xinkai Pharmaceutical Co., Ltd. (Suzhou, China); Nicotine (Nic) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). Chicken type II collagen (CII) was purchased from Sigma Chemical Co. (St. Louis, USA). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Becton Drive Co., Ltd. (New Jersey, USA). Rat TNF- α , IL-1 β , IL-6, IFN- γ , IL-4, IL-17A, IL-10 and TGF- β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Dakewe Biotech Co., Ltd. (Shenzhen, China). All the other chemicals and reagents used were of analytical grade.

2.3. Induction of arthritis and treatment

2.3.1. Collagen II-induced arthritis (CIA) in rats

On day 0, primary immunization was performed. The rats were intradermally injected with 200 μ l of an emulsion of chicken type II collagen (CII) and CFA at the base of the tail. Then rats were boosted with an emulsion of CII and IFA seven days later. On day 14, the rats were randomly assigned to the following groups: Model group, Mad (30 mg/kg) group, Mad (30 mg/kg) plus Phe (1 mg/kg) group, Mad (30 mg/kg) plus Pro (20 mg/kg) group, Mad (30 mg/kg) plus Atr (1 mg/kg) group, Mad (30 mg/kg) plus Hex (4 mg/kg) group, Phe (1 mg/kg) group, Pro (20 mg/kg) group, Atr (1 mg/kg) group, Hex (4 mg/kg) group and Lef (2 mg/kg) group. Normal group, as control, included rats without application of CII and CFA. Mad and Lef were orally administered for consecutive two weeks from day 14. Phe, Pro, Atr or Hex was intraperitoneally injected 10 min before Mad administration. Rats in normal and model groups were orally given vehicle in the same schedule.

2.3.2. Vagotomy (Vgx) in CIA rats

Vgx was performed as previously described [22]. Briefly, anesthetized rats were subjected to a ventral cervical midline incision to expose the left cervical vagus trunk, which was ligated with 4-0 silk sutures and divided. Subsequently, the skin was closed with three sutures. Other rats underwent sham operation, in which the left nerve was merely exposed and isolated from surrounding tissue but not transected. Four days later, CIA was induced in all rats except for six rats in the Sham group. On day 14 after induction of CIA, rats underwent sham operation were randomly divided into 5 groups: Sham group (without application of CII and CFA), Sham-Model group, Sham-Mad (30 mg/kg) group, Sham-Lef (2 mg/kg) group, and Sham-Nic (300 μ g/kg) group. The rats underwent Vgx surgery were randomly divided into 3 groups: Vgx group, Vgx-Model group and Vgx-Mad (30 mg/kg) group. Mad and Lef were orally administered and Nic was intraperitoneally injected from day 14 to day 27. Other groups were orally given vehicle in the same schedule.

2.4. Assessment of arthritis

Arthritis was assessed by body weight, hind paw swelling and arthritis index (AI) scores. The volume of hind paws was measured using a plethysmometer. The clinical severity of arthritis in each paw of rats was evaluated double-blindly as follows: 0 = no arthritis; 1 = swelling in one type of joint; 2 = swelling in two types of joint; 3 = swelling in three types of joint; and 4 = swelling of the entire paw. The clinical score for each rat was the sum of four paws, with the maximum score of 16 [23].

2.5. Histopathologic analysis

After two weeks' treatment, all rats were sacrificed by inhalation of ether. The right knee joints were fixed for 48 h in 10% buffered formalin and decalcified in 10% EDTA. The joints were then embedded in paraffin, and cut into 5 μ m serial sections and stained with hematoxylin and eosin (H&E). The histopathological changes in the joints including inflammatory cell infiltration, synovial hyperplasia, pannus formation, cartilage and bone erosion were examined under optical microscope (Olympus DX45, digital camera DP72) (Tokyo, Japan) and graded on a scale of 0 = none, 1 = mild, 2 = moderate and 3 = severe by a pathologist blinded to the experimental groups [24].

2.6. Measurement of cytokine levels in serum

Peripheral blood samples were collected from rats after they were sacrificed. After clotting for 20 min at room temperature, blood samples were centrifuged for 20 min at a speed of 3000 rpm, and then the supernatants were collected. The levels of cytokines (TNF- α , IL-1 β , IL-6, IFN- γ , IL-4, IL-17A, IL-10 and TGF- β) were measured with ELISA kits according to the manufacturer's instructions.

2.7. Statistical analysis

All data were expressed as mean \pm S.E.M. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. *p* values < 0.05 was considered as statistically significant.

3. Results

3.1. Adrenergic receptors are not involved in the therapeutic effect of Mad on CIA

Arthritis was induced in Wistar rats using CII emulsified in CFA on day 0, and a booster seven days later. On day 14, several arthritic symptoms appeared and the indicated treatments started. After two weeks of treatments, rats in the Model group evidently developed arthritis, showing symptoms such as body weight loss, paw swelling, erythema, and ankylosis. As illustrated by Fig. 2, Mad markedly decreased the severity of paw swelling and AI scores during the experiment period, similar to the effects of the positive control Lef, a commonly-used disease modifying anti-rheumatic drug with immunosuppressant activity. The ameliorative effect of Mad on body weight loss was even greater than that of Lef. The results indicated that Mad at a dose of 30 mg/kg exerted significantly therapeutic effect on rat CIA. Both Phe (α -adrenoceptor antagonist) and Pro (β -adrenoceptor

antagonist) themselves did not affect rat CIA (data not shown). When co-administered with Mad, either Phe or Pro only slightly attenuated the action of Mad, suggesting that the anti-arthritis effect of Mad might be irrelevant to the sympathetic nervous system.

3.2. Cholinergic receptors are involved in the therapeutic effect of Mad on CIA

Following the onset of arthritis, the body weight, volume of hind paws and AI scores of rats were measured to evaluate the severity of arthritis. Neither Atr (muscarinic cholinergic receptor antagonist) nor Hex (nicotinic cholinergic receptor antagonist) itself markedly affected CIA in rats (data not shown). In contrast, the co-administration of the two antagonists with Mad resulted in marked reduction of the anti-arthritis efficacy of Mad, suggesting that the cholinergic receptors might be important mediator for the action of Mad (Fig. 3).

3.3. Vagotomy attenuates the therapeutic effect of Mad on CIA

To further ascertain the involvement of cholinergic nerve system in the anti-arthritis action of Mad, some rats were subjected to unilateral cervical vagotomy (Vgx) operation four days before CIA induction. Mad (30 mg/kg) was orally administered for two weeks from day 14, and its anti-arthritis potency in Sham- and Vgx-operated rats was compared. Lef (2 mg/kg, oral administration) and nicotinic receptor agonist Nic (300 μ g/kg, intraperitoneal injection) were chosen as positive controls. As illustrated by Fig. 4, Mad showed little anti-arthritis effect in Vgx rats in contrast to showing a markedly therapeutic effect in Sham rats, as evaluated by paw swelling and AI scores. It may be interpreted to mean that the integrity of vagus nerve is essential for the anti-arthritis effect of Mad.

3.4. Vgx attenuates the inhibitory effect of Mad on pathomorphological changes of ankles in CIA rats

It is well known that pathological changes in joints are vital to assess the arthritis severity. After treatment for 14 days, ankles of rats were removed, fixed, decalcified and then stained with H&E to evaluate histological changes. The ankle joints of the rats in Model and Vgx-Model groups presented prominent synovial hyperplasia, monocyte-macrophages and neutrophils infiltration, and severe erosion of cartilage and bone in comparison with Sham and Vgx groups, respectively. Mad, Lef and Nic treatments showed beneficial effects on the histological changes of joints in CIA rats. In CIA rats that underwent Vgx operation, the inhibitory effect of Mad on the inflammatory cell infiltration, synovial hyperplasia and pannus formation in the ankle joints significantly decreased, but the ameliorative effect of Mad on the

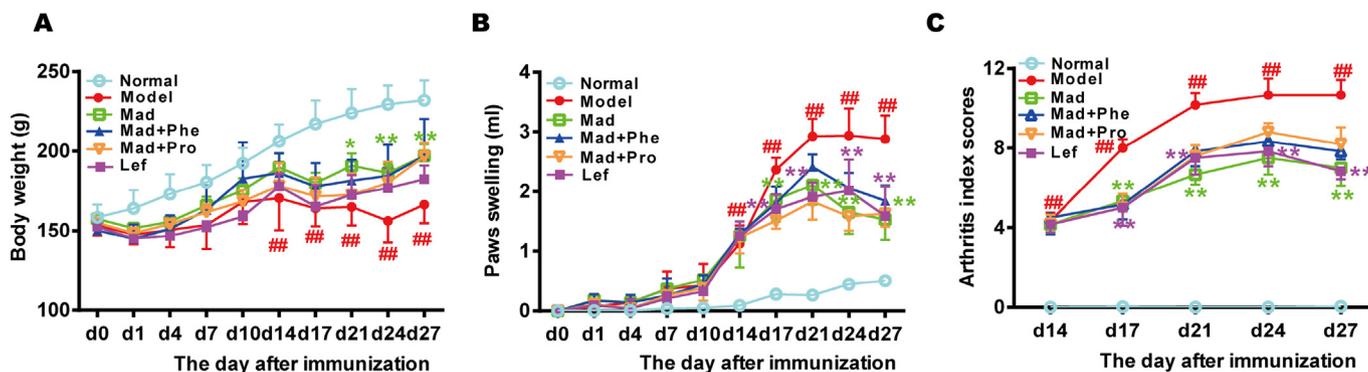


Fig. 2. Effect of madecassoside (Mad) on collagen II-induced arthritis (CIA) is not related to the adrenergic receptors. Rats were injected with CII and CFA emulsion to induce CIA on day 0, and were treated with Mad (30 mg/kg, i.g.) alone or combined with Phentolamine (Phe, 1 mg/kg, i.p.) or Propranolol (Pro, 20 mg/kg, i.p.) daily from day 14 to day 27. (A) Body weight changes. (B) Paw swelling. The volumes of hind paws were measured using a plethysmometer. (C) Arthritis index scores. Data were shown as means \pm S.E.M. for each group (n = 6). ##*p* < 0.01 vs. Normal group; **p* < 0.05, ***p* < 0.01 vs. Model group.

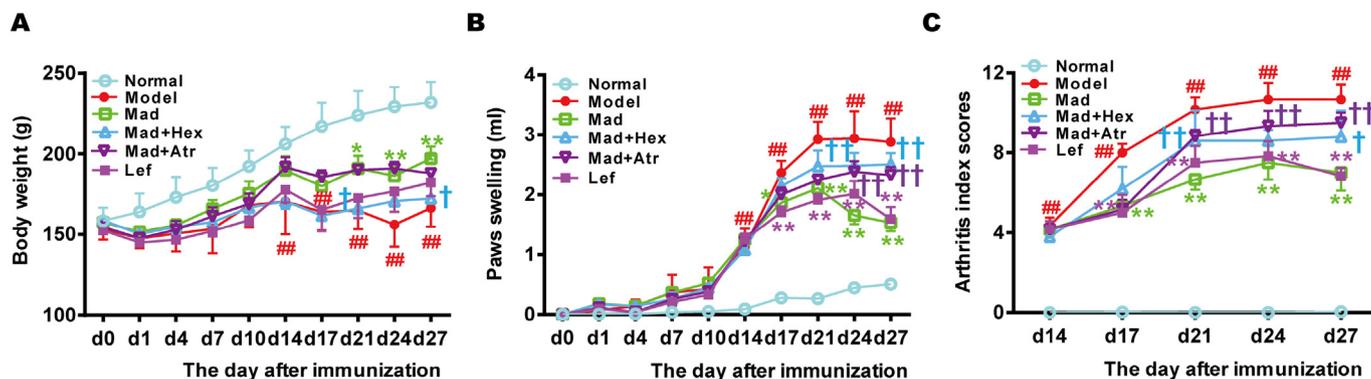


Fig. 3. Effect of madecassoside (Mad) on collagen II-induced arthritis (CIA) is mediated by the cholinergic receptors. Two weeks after CIA induction, rats were treated with Mad (30 mg/kg, i.g.) alone or combined with Hexamethonium (Hex, 4 mg/kg, i.p.) or Atropine (Atr, 1 mg/kg, i.p.) daily for two weeks. (A) Body weight changes. (B) Paw swelling. The volumes of hind paws were measured using a plethysmometer. (C) Arthritis index scores. Data were shown as means \pm S.E.M. for each group (n = 6). ## $p < 0.01$ vs. Normal group; * $p < 0.05$, ** $p < 0.01$ vs. Model group; † $p < 0.05$, †† $p < 0.01$ vs. Mad group.

erosion of cartilage and bone was not affected (Fig. 5A–D). These findings indicate that peripheral cholinergic nerve mediates the anti-inflammatory but not bone-protective effect of Mad in CIA rats, which was in line with the results in 3.3.

3.5. Vgx attenuates the down-regulation effect of Mad on the levels of pro-inflammatory cytokines but not T cell-related cytokines in serum of CIA rats

As a chronic, inflammatory and auto-immune disease, the pathogenesis of RA is very complex, and a range of cytokines are involved in the course of the disease. In this study, we detected the levels of

cytokines (pro-inflammatory cytokines and T cells (Th/Treg)-related cytokines) in rat sera. As illustrated in Fig. 6, Mad significantly decreased the levels of TNF- α , IL-1 β , IL-6, IFN- γ and IL-17A, and increased the levels of IL-10 and TGF- β , but did not affect the level of IL-4. This could be interpreted as that Mad markedly attenuated systemic pro-inflammatory cytokine response. Moreover, it might play a crucial role in modulating the disorder of effector T cell responses by adjusting the T cell-related cytokine concentrations, which reflects the balances of Th1/Th2 and Th17/Treg. Interestingly, Vgx markedly attenuated the down-regulatory effect of Mad on the levels of pro-inflammatory cytokines, but only slightly affected the regulatory effect of Mad on T cell-

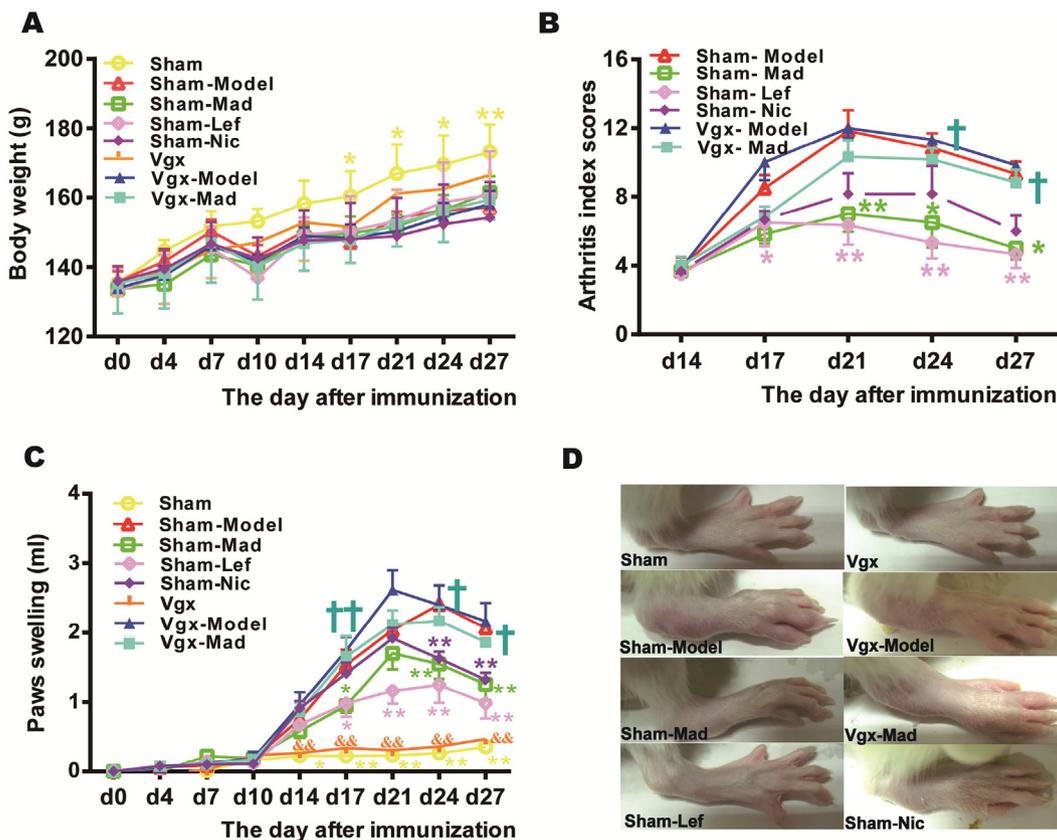


Fig. 4. Vagotomy attenuates the therapeutic effect of madecassoside (Mad) on collagen II-induced arthritis (CIA). Some rats were subjected to Vgx four days before CIA was induced. (A) Body weight changes. (B) Arthritis index scores. (C) Paw swelling. The volumes of hind paws were measured using a plethysmometer. (D) Ankle morphology. After treatment continuously for two weeks, the ankles of CIA rats were photographed, respectively. Data were shown as means \pm S.E.M. for each group (n = 6). * $p < 0.05$, ** $p < 0.01$ vs. Sham-Model group; && $p < 0.01$ vs. Vgx-Model group; † $p < 0.05$, †† $p < 0.01$ vs. Mad group.

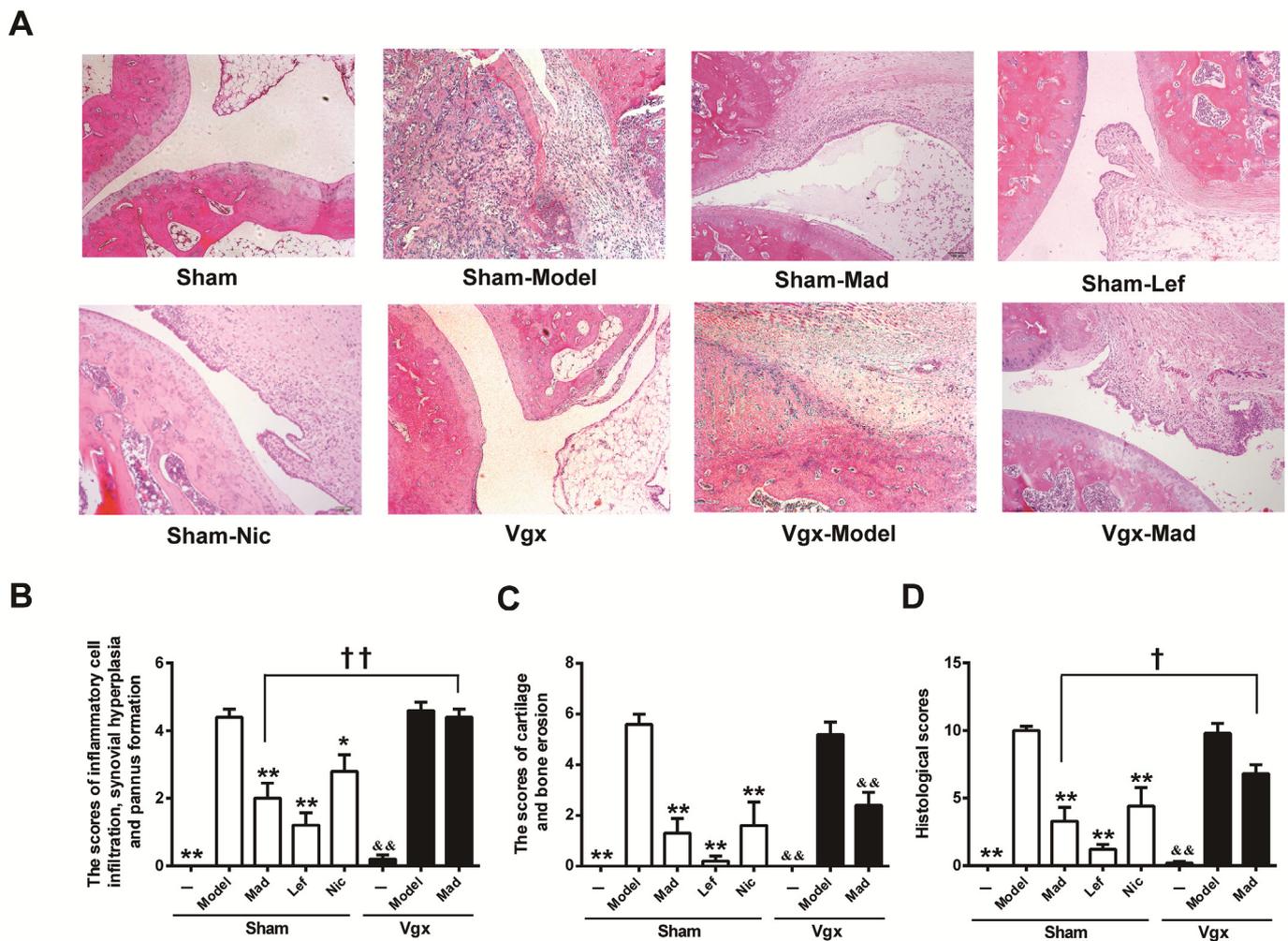


Fig. 5. Vgx attenuates the inhibitory effect of madecassoside (Mad) on pathomorphological changes of ankles in collagen II-induced arthritis (CIA) rats. (A) Histological changes of the ankle joint sections (H&E staining, original magnification 100×). (B) The scores of inflammatory cell infiltration, synovial hyperplasia and pannus formation, (C) the scores of cartilage and bone erosion, and (D) the total histological scores of ankle joint sections were summarized, respectively. Data were shown as means ± S.E.M. for each group (n = 6). **p* < 0.05, ***p* < 0.01 vs. Sham-Model group; &&*p* < 0.01 vs. Vgx-Model group; †*p* < 0.05, ††*p* < 0.01 vs. Sham-Mad group. The symbol “-” represents Sham group without application of CII and CFA.

related cytokines in serum of CIA rats. The findings indicated the anti-inflammatory effect in CIA rats, but not immunomodulatory effect, of Mad was dependent on vagus nerve.

4. Discussion

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterized by innate immune cells infiltrating into synovium, leading to chronic synovial inflammation and hyperplasia, pannus formation and subsequent cartilage and bone destruction, and it is usually accompanied by systemic function disorder [25]. Recently, there is mounting evidence indicating that neural-endocrine-immune system participated in the development and progression of RA [26]. Among these biological systems, the cholinergic neuronal system has drawn more and more attention by researchers in the past several years. Experimental and clinical researches demonstrated that the activity of the parasympathetic nervous system was explicitly reduced under RA condition, and stimulation of the vagal nerve and nicotinic acetylcholine receptor could attenuate CIA [13,22,27]. It seems reasonable to suppose that reagents or medical procedures capable of increasing the parasympathetic tone might be a satisfactory approach for the treatment of RA.

Our previous studies had confirmed the therapeutic effect of oral

madecassoside (Mad) on rodent CIA, but the poor plasma exposure of Mad implied that it might not directly affect immune and inflammation cells. The underlying anti-arthritis mechanism of Mad has intrigued us for a long time. The evidence that Mad could restore cholinergic function [21] threw us a preliminary hypothesis that Mad might exerts the anti-arthritis effect through the nervous system by regulating the functions of neurotransmitters, receptors, enzymes etc. To verify the prediction, related receptor antagonists were used in this study to certify whether adrenergic system or cholinergic system participates in the process. To our surprise, the sympathetic nervous system may not take part in the anti-arthritis function of Mad, while the cholinergic system was proved to be of the essence. Cholinergic antagonists could effectively attenuate the effects of Mad on AI scores and paw swelling in CIA rats. These findings indicate that cholinergic system is involved in the therapeutic effect of Mad on CIA.

The integrity of vagus nerve, an essential component of the cholinergic system, is so important in inhibiting the inflammatory and immune responses that vagotomy (Vgx) would attenuate the peripheral cholinergic anti-inflammatory pathway, resulting in the aggravation of arthritis symptoms [22,28,29]. To further investigate the involvement of peripheral cholinergic nerves in the therapeutic effect of Mad, the operation of unilateral cervical Vgx was conducted. In the present study, Vgx could markedly diminish the inhibitory effect of Mad on

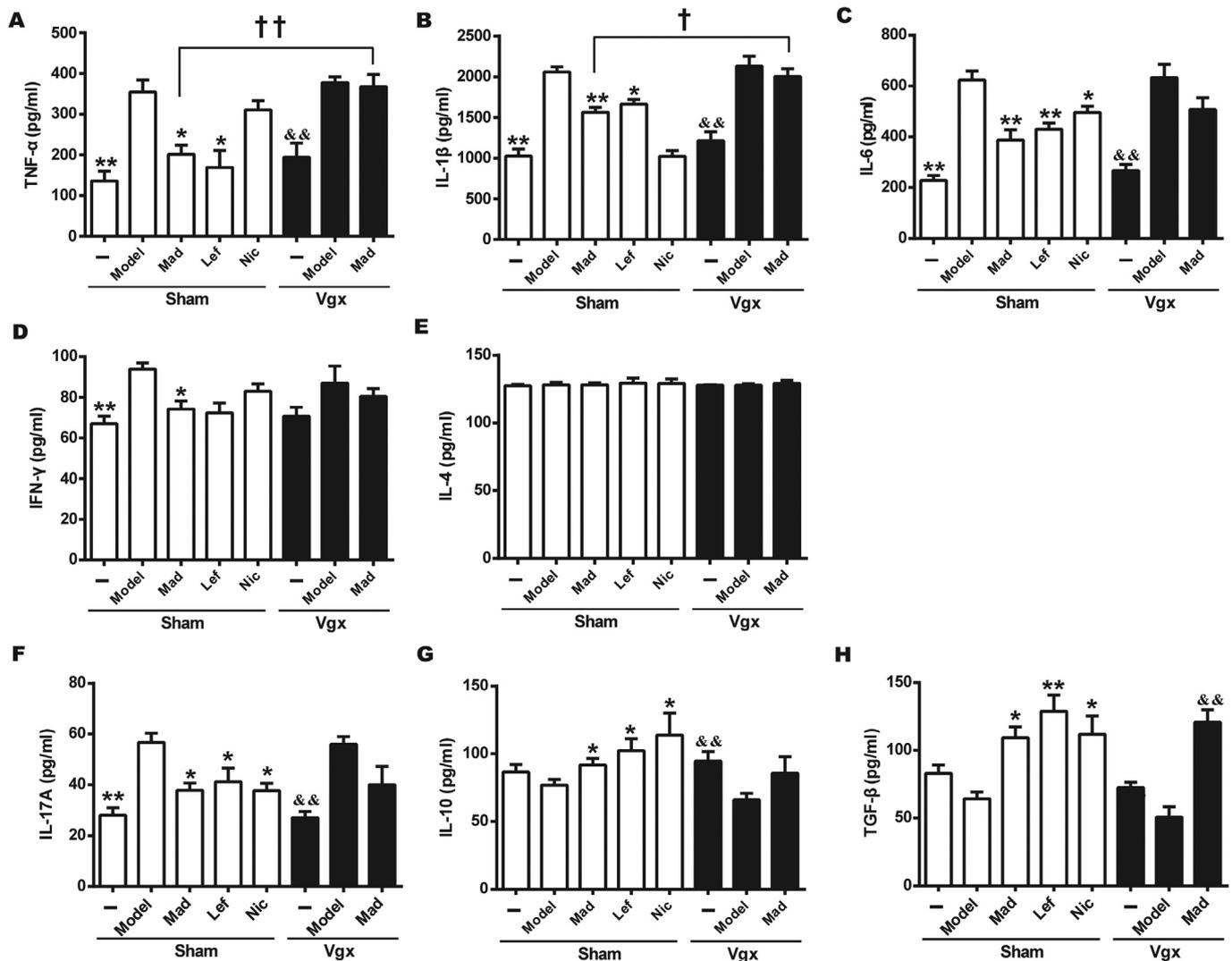


Fig. 6. Unilateral cervical vagotomy (Vgx) attenuates the down-regulatory effect of madecassoside (Mad) on levels of pro-inflammatory cytokines but not T cell-related cytokines in serum of collagen II-induced arthritis (CIA) rats. (A–C) Serum concentrations of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6). (D–H) Serum concentrations of T cell (Th17/Treg) - related cytokines (IFN- γ , IL-4, IL-17A, IL-10 and TGF- β). Data were shown as means \pm S.E.M. for each group (n = 6). *p < 0.05, **p < 0.01 vs. Sham-Model group; &&p < 0.01 vs. Vgx-Model group; †p < 0.05, ††p < 0.01 vs. Sham-Mad group. The symbol “-” represents Sham group without application of CII and CFA.

clinical symptoms, particularly on synovial inflammation, and the expression of pro-inflammatory cytokines, indicating that vagus nerve integrity is required for the anti-inflammatory effect of Mad in rat CIA.

The neural circuit of inflammatory reflex can regulate the immune response and inflammation. Once sensory vagus fibers are activated by injury stimuli in the peripheral organs, the afferent vagus nerve detects the peripheral inflammation, and transmits the signals to the brainstem. Subsequently, efferent vagus nerve signals travel to the celiac plexus and suppress innate immune responses [30]. The disturbance of the subsets of T cells (Th1/Th2, Th17/Treg) is believed to be a principal cause of synovial inflammation. The imbalance of Th17/Treg aggravates the disorder of systemic immune response by activating macrophages and synovial fibroblasts, and causing the production of pro-inflammatory cytokines. High level of inflammatory cytokines leads to synovial inflammation, loss of cartilage proteoglycans, joint destruction, etc. [2,31,32]. Currently, there is growing evidence indicating that correcting the imbalance between Th1/Th2 and Th17/Treg or blocking the production of pro-inflammatory cytokines is a reliable therapeutic strategy for RA [33]. Our previous studies provided evidence that Mad displayed anti-arthritis properties which was accompanied by modulating the dysregulated effector T cell responses and attenuating

systemic pro-inflammatory cytokine responses [14,19]. Although it has been described in the literature that the vagus nerve regulates the abnormal activation of T cells [26,34], in the present study we found that there was no obvious change in production of T cell-related cytokines in CIA rats with Vgx operation. Meanwhile, in Vgx rats, Mad could also regulate the expression of T cell-related cytokines. These data suggested that the vagus nerve might mediate the inhibitory effect of Mad on macrophages, fibroblast-like synoviocytes and other inflammatory cells, but not the T cells.

5. Conclusions

In summary, oral administration of Mad exhibited obvious therapeutic effects on rat CIA. The evidence presented indicates that the anti-arthritis mechanism involves the peripheral cholinergic system.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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