Therapeutic effects of glucosides of *Cheanomeles speciosa* on collagen-induced arthritis in mice[1]

Ling-ling ZHANG, Wei WEI[2], Shang-xue YAN, Xiang-yang HU, Wu-yi SUN

Institute of Clinical Pharmacology, Anhui Medical University, Hefei 230032, China

KEY WORDS glucosides; *Cheanomeles speciosa*; experimental arthritis; inflammation; immunity

ABSTRACT

AIM: To investigate the therapeutic effect of the glucosides of *Cheanomeles speciosa* (GCS) on the collagen-induced arthritis (CIA) in mice. **METHODS:** Mice were divided randomly into six groups, including normal, CIA, CIA+GCS (60, 120, and 240 mg/kg) and CIA+glucosides of *Tripterygium wilfordii* (GTW) groups. CIA model was based on mice. The effect of GCS in CIA mice was measured by paw-swelling, arthritis scores, and histopathological assessment of synovium. Indices of thymus and spleens were measured. Thymocytes and splenocytes proliferation, activity of interleukin-1 (IL-1), and interleukin-2 (IL-2) were assayed by MTT and [^{3}H]TdR method. The level of anti-collagen type II (CII) antibody in serum and prostaglandin E (PGE) in ankle were assayed by ELISA and ultraviolet spectrophotometer method, respectively. **RESULTS:** The onset of paw-swelling was on d 24 after injection of emulsion. The peak of secondary inflammation appeared on d 36 and then declined after d 40. GCS and GTW significantly reduced paw-swelling and arthritis scores, reduced the increase of spleen indices of CIA mice, suppressed the ConA or LPS-induced thymocyte or spleen cell proliferation, and the production of IL-1 and IL-2 in CIA mice. GCS reduced the level of anti-CII antibody and PGE. Histological pathology analysis demonstrated that the synovium of CIA mice was hyperplastic, pannus was formed, and inflammatory cells infiltrated into synovium. The pathological changes were significantly reduced by GCS. **CONCLUSION:** GCS had anti-inflammatory effect on CIA mice, which might be related to the modification of the abnormal immunological function of CIA mice.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic disorder characterized by synovial inflammation and subsequent destruction and deformity of synovial joints. The articular lesions start with synovitis, focal erosion of cartilage, and then culminate in the destruction of subarticular bone by pannus tissue[1]. Growing evidence suggest that immune responses in RA may be driven by activated Th1 cells with insufficient Th2 activity[2]. Cytokines are the main inflammatory mediators in RA. The Th1 cytokines, such as IL-2 and IFN-gamma are supporting proinflammatory microenvironment in joints from patients with RA. The imbalance of Th1/Th2 cytokine steady state may play an important role in the pathogenesis of RA. The evaluation of this imbalance leads up to the possibility of discrimination in this disease[3]. Collagen-induced arthritis (CIA) is an experimental model of autoimmune-mediated polyarthritis that has been used to dissect the pathogenesis of human RA[4].

[1] Project supported by the Natural Science Foundation of Anhui Province, No 00044411.
[2] Correspondence to Prof Wei WEI.
Phn & Fax 86-551-516-1208. E-mail wwei@ahmu.edu.cn
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The pathogenesis of CIA in many ways resembles RA in human. It is a useful model to test and develop new drugs and elucidate the mechanisms of human autoimmune disease[5]. The glucosides of *Cheanomeles speciosa* (GCS) was one part of the *Cheanomeles* of Chinese herbal medicine produced in Xuanzhou, Anhui province. GCS had anti-inflammatory and immunomodulatory effects. GCS could restrain the secondary arthritis of adjuvant induced arthritis (AA) rats and CIA rats and alleviate the pathological damage of joint. GCS suppressed the proliferation of synoviocytes, reduced the level of tumor necrosis factor alpha, IL-1 and PGE_2 produced by synoviocytes, and up-regulated level of the cAMP and the stimulated G protein (Gs) mRNA, and down-regulated the level of inhibitory G protein (Gi) mRNA and tumor necrosis factor alpha mRNA[6,7]. Glucosides of *Tripterygium wilfordii* (GTW), as an immunosupressant, was used to treat autoimmune diseases. In the present study, the anti-inflammatory and immunomodulatory effects of GCS and GTW were studied in mice with CIA and their possible mechanisms were also investigated.

MATERIALS AND METHODS

Materials Kunming mice, male, weighing 18-22 g, and C_{57}BL/6J male mice, weighing 18-22 g (Grade II, Certificate No 01), were obtained from Experimental Animal Center of Anhui Medical University. They were housed at a temperature of 18-20 ℃ and humidity of 65 %-70 %, and were submitted to a 12 h light/dark cycle. In the present study, the incidence of CIA is about 50 %-60 %. All the CIA mice were randomized into 6 groups, and there were 13-15 mice in each group.

Chicken CII powder was provided by Institute of Bencao Biological Medicine, Shanghai. GCS was isolated from Institute of Clinical Pharmacology, Anhui Medical University. GTW was obtained from Hongqi Pharmaceutical Factory, Shanghai. GCS (purity was 51.47 %) and GTW were made into required concentration respectively with 0.5 % CMC-Na before use.

The following reagents were also used. Concanavalin A (ConA), lipopolysaccharide (LPS) and Hepes (Sigma Co); RPMI-1640 powder (Gibco Co, USA); bacillus Calmette Guerin (BCG, Shanghai Biological Products Factory); PPO and POPOP(Merck Co, USA); MTT (Merck Co, USA); [^{3}H]-TdR (China Institute of Atomic Energy, Beijing). Goat anti-mouse IgG-HRP (Haoyang Biological Medicine Ltd, Tianjin). Neonatal bovine serum was provided by Department of Microbiology, Anhui Medical University, heat-killed before use.

Methods Preparation and assessment of CIA in mice[8] were performed. Chicken CII was dissolved in acetic acid 0.1 mmol/L and emulsified with an equal volume of complete Freund's adjuvant (CFA) in ice-bath. The final concentration of CII and BCG were both 2 g/L. By immunization with heterologous CII emulsified with CFA, CIA was induced in Kunming mice, emulsion 0.1 mL was injected intradermally into the base of the tail, followed by a booster injection on d 21. The mice of control group were received the injection of an equal volume of acetic acid 0.1 mmol/L at the same location.

After the secondary immunization, CIA was assessed in mice by measuring the swell degree of paws with capacity measurement method and the occurrence of the arthritis was observed by the third person every 4 d by scoring all paws for severity of erythema and swelling using scores of 0 (normal appearance), 1 (redness and swelling in a part of the paw; mild), 2 (redness and swelling in the whole paw; moderate), 3 (redness and swelling below the ankle joints; severe), or 4 (redness and swelling including the ankle joints, very severe). The score of each paw was then added, with 12 as maximal arthritis score per animal. Mice were considered as arthritis when at least one entire paw was inflammed[9].

GCS at three doses of 60, 120, and 240 mg/kg×7 d were given intragastrically (ig) from the onset of paw swelling (d 24). During the same time, GTW (80 mg/kg×7 d) was given (ig) to another group. Normal group and CIA model group were both treated with 0.5 % CMC-Na.

Indices of thymus and spleens assay[10] On d 44 after immunization, mice were killed by cervical dislocation. Thymus and spleens were removed and weighed. The avoirdupois of mice divided by the weight of thymus were the indices of thymus, the indices of spleen were expressed with the value of avoirdupois divided by the weight of spleen also.

Thymocyte and spleen cell proliferation assay[10] On d 44 after immunization, mice were killed by cervical dislocation. Thymus and spleens were removed in sterile condition and thymocytes and splenocytes were collected. Then cells were suspended in RPMI-1640 medium at a concentration of 1×10^{10}cells/L. The cell suspension (100 μL) and ConA (100 μL with a final concentration of 3 mg/L) or LPS (100 μL with a final concentration of 4 mg/L) were seeded into 96-well cul-

ture plate simultaneously. Triplicates were designed. The cultures were incubated at 37 ºC in an atmosphere of 5 % CO_2 for 48 h. Two hours before the end, MTT (5 g/L) 10 μL was added to each well. The absorbance was measured on EJ301 ELISA Microwell Reader (Bio-Tek Co, USA) at 570 nm. The results were described as the average of triplicate absorbance (A) $A_{570\ nm}$.

Production and assay of IL-1[11] On d 44 after immunization, mice were killed by cervical dislocation. Peritoneal macrophages (PM) of Kunming mice were collected in D-Hanks' medium. Then PM was resuspended in RPMI-1640 medium at 1×10^9 cells/L and the cell suspension was seeded onto 24-well culture plate at a total volume of 1 mL per well. After incubation for 2 h at 37 ºC in 5 % CO_2 atmosphere, supernatants were removed and the adherent cells were washed with D-Hanks' medium containing 5 % neonatal bovine serum for 3 times. Thus the monolayer of PMΦ was obtained. LPS, with a final concentration of 4 mg/L, was added to each well and RPMI-1640 was also added to make a final volume per well up to 1 mL. Then the plate was incubated at 37 ºC in air with 5 % CO_2 for 48 h. After centrifugation (500×*g*, 10 min) all the supernatants containing extracellular IL-1 were collected and stored at -20 ºC until assay. IL-1 activity was measured by ConA-induced thymocytes proliferation assay. Suspension of thymocytes (2×10^6 cells per well) taken from C_{57}BL/6J mice were distributed over a flat-bottom 96-well plate. PM culture supernatants 50 μL were added to the cell suspension. The cultures were incubated in the presence of ConA (3 mg/L) for 48 h at 37 ºC in a 5 % CO_2 incubator. Six hours before the end, [^{3}H]TdR 20 μL was added to each well. The [^{3}H]TdR incorporation was measured on liquid-scintillator (Beckman Co, USA) counting technique. The results were described as the average of triplicate Bq.

Production and assay of IL-2[10] Kunming mice thymuses were removed in a sterile condition and thymocytes were collected and suspended in RPMI-1640 medium at a concentration of 1×10^{10} cells/L. Cell suspension 100 μL and ConA 100 μL with a final concentration of 3 mg/L were added to each well of 24-well culture plate. And RPMI-1640 medium was added to each well to make final volume of 1 mL. IL-2 activity was measured by ConA-induced thymocytes proliferation assay with [^{3}H]-TdR intake method (see above). The results were described as the average of triplicate Bq.

Assay of serum anti-CII antibody[12] Serum anti-CII antibody level was analyzed by ELISA. Blood sample from eyeball vein was centrifuged at 800×*g* (4 ºC) for 15 min, then serum was gained. Anti-CII antibody level was determined by goat anti-mouse IgG-HRP. The *A* was measured on EJ301 ELISA Microwell Reader at 490 nm. The results were described as the average of ten wells $A_{490\ nm}$.

Assay of PGE in ankle[13] PGE located in ankle level was analyzed by ultraviolet spectrophotometer method. On d 44 after immunization, mice were killed by cervical dislocation. Inflamed paw was scissored from 0.5-cm upside ankle joints, then the tissue was weighed and cut into 0.1-mm pieces. These tissue was marinated in 5 mL 0.9 % saline solution for 1 h and centrifuged at 500×*g* (4 ºC) for 10 min, then supernatant 0.1 mL was gained and mixed with 2 mL KOH-methanol (0.5 mL/L). The medley solutions were put into water at 50 ºC for 20 min and finally the medley solutions were diluted with methanol at a final volume of 20 mL. The *A* was measured on ultraviolet spectrophotometer at 278 nm. The results were described as the average of ten value of $A_{278\ nm}$.

Assay of pathology in anklebone[12] On d 44 after immunization, mice were killed by cervical dislocation. Ankle was scissored and marinated in 10 % formaldehyde , then the tissue was dehydrated, dehycalcium, and stained with HE and observed with a optic microscope.

Statistical analysis All data were expressed as mean±SD. Difference between groups were evaluated by one-way ANOVA.

RESULTS

Effects of GCS on polyarthritis in collagen-induced arthritis in mice The onset of paw-swelling was on d 24 after injection of emulsion. CIA mice were divided randomly into five groups, including CIA, CIA+GCS (60, 120, and 240 mg/kg) and CIA+GTW (80 mg/kg) groups. CIA+GCS and CIA+ GTW groups were given (ig) with GCS or GTW from d 24 to d 30, normal and CIA groups were given 0.5 % CMC-Na simultaneously, paw-swelling, and arthritis scores were measured from d 24 every 4 d. Results showed that the swelling peak appeared on d 36 and then declined after d 40. GCS (60, 120, and 240 mg/kg ×7 d) and GTW (80 mg/kg ×7 d) were able to reduce the paw-swelling and arthritis scores of CIA mice (Tab 1, 2).

Effects of GCS on indices of thymus and spleens of CIA mice The indices of thymus and

Tab 1. Effects of GCS on polyarthritis in collagen-induced arthritis in mice. *n*=10. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* CIA group, $^{f}P<0.01$ *vs* normal group.

Groups	Dose/ mg·kg^{-1}	The cubage of paws on d 0 /mL	Swelling rate/% d 24	d 28	d 32	d 36	d 40
Normal	–	0.209±0.019	11±3	14±4	17±3	20±4	21.2±2.9
CIA	–	0.199±0.015	30±12^{f}	43±10^{f}	50±12^{f}	40±11^{f}	36±8^{f}
CIA+GCS	60	0.204±0.020	17±7^{b}	35±5^{b}	33±6^{b}	26±4^{c}	21.8±2.4^{c}
	120	0.199±0.014	24±8^{b}	27±7^{c}	27±8^{c}	25±10^{c}	21±6^{c}
	240	0.207±0.020	17±10^{c}	24±8^{c}	28±9^{c}	22±7^{c}	21±5^{c}
CIA+GTW	80	0.202±0.018	20±10^{c}	28±8^{c}	30±6^{c}	29±10^{c}	24±3^{c}

CIA: collagen-induced arthritis; GCS: glucosides of *Cheanomeles speciosa;* GTW: glucosides of *Tripterygium wilfordii.*

Tab 2. Effects of GCS on polyarthritis in collagen-induced arthritis in mice. *n*=10. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* CIA model group.

Groups	Dose/ mg·kg^{-1}	Arthritis scores d 24	d 28	d 32	d 36	d 40	d 44
CIA	–	4.9±1.7	6.7±1.4	8.3±1.6	10.1±1.9	8.4±1.9	7.7±1.6
CIA+GCS	60	5.3±1.3	7.0±1.5	6.5±0.8^{c}	6.6±1.7^{c}	7.9±2.6	5.9±1.0^{c}
	120	5.3±1.5	6.2±1.6	6.5±1.4^{c}	6.2±1.5^{c}	7.2±2.2	5.3±0.8^{c}
	240	5.7±1.0	6.2±1.0	6.3±1.2^{c}	6.2±1.6^{c}	6.9±2.6	5.8±1.5^{c}
CIA+GTW	80	5.1±1.0	6.5±1.0	7.6±2.6	7.5±1.4^{c}	8.1±0.8	6.3±1.0^{b}

CIA: collagen-induced arthritis; GCS: glucosides of *Cheanomeles speciosa;* GTW: glucosides of *Tripterygium wilfordii.*

spleens were assayed on d 44. Results showed that spleens indices of CIA mice were increased, thymus indices were not changed. GCS (60, 120, and 240 mg/kg×7 d) and GTW (80 mg/kg×7 d) reduced the increase of spleens indices of CIA mice (Tab 3).

Effects of GCS on PGE located in ankle of CIA mice The level of PGE located in ankle was analyzed by ultraviolet spectrophotometer method on d 44 after immunization. PGE level was increased significantly in CIA model. GCS (120 and 240 mg/kg×7 d) and GTW (80 mg/kg ×7 d) could reduce the level of PGE (Tab 3).

Effects of GCS on serum anti-CII antibody of CIA mice The level of serum anti-CII antibody was assayed on d 44 by ELISA. Results showed that the level of serum anti-CII antibody was increased significantly in CIA model. GCS (120 and 240 mg/kg×7 d) and GTW (80 mg/kg×7 d) could reduce the level of serum anti-CII antibody (Tab 3).

Effects of GCS on thymocyte and splenocyte proliferation of CIA mice Thymocytes and splenocytes proliferation were assayed on d 44. Results showed that thymocyte and splenocyte proliferation in CIA mice were increased. GCS (60, 120, and 240 mg/kg×7 d) and GTW (80 mg/kg×7 d) could reduce ConA-induced thymocytes proliferation. GCS (120 and 240 mg/kg) and GTW (80 mg/kg) could reduce LPS induced splenocyte proliferation in CIA mice (Tab 4).

Effects of GCS on production of IL-1 and IL-2 in CIA mice The production of IL-1 and IL-2 were assayed on d44 after inmmunization. The production of IL-1 by PMΦ and IL-2 by T cell were up-regulated significantly in CIA model. GCS (120 and 240 mg/kg) and GTW (80 mg/kg) decreased the production of IL-1 and IL-2 (Tab 4).

Effects of GCS on anklebone in CIA mice Histological pathology analysis demonstrated that compared with those of normal mice (A, HE, ×200) the synovium of CIA mice was hyperplastic, synovial cell was increased, pannus was formed, and inflammatory cells infiltrated into synovium (B, HE, ×200). Hyperplastic synovium,

Tab 3. Effects of GCS on thymus index, spleens index, PGE located in ankle, and serum anti-CII antibody of CIA mice. *n*=10. Mean±SD. $^{c}P<0.01$ *vs* CIA group. $^{e}P<0.05$ *vs* normal group.

Group	Dose/mg·kg^{-1}	Index		PGE/ absorbance	Anti-CII antibody/ absorbance
		Thymus	Spleens		
Normal	–	0.96±0.09	3.52±0.45	0.37±0.07	0.096±0.004
CIA	–	1.27±0.24	5.13±0.11^{e}	0.55±0.22^{e}	0.119±0.014^{e}
CIA+GCS	60	1.14±0.19	4.64±0.17	0.38±0.12	0.109±0.018
	120	1.11±0.13	3.67±0.25^{c}	0.32±0.07^{c}	0.091±0.006^{c}
	240	1.44±0.22	3.72±0.55^{c}	0.33±0.03^{c}	0.083±0.009^{c}
CIA+GTW	80	1.52±0.25	3.65±0.28^{c}	0.35±0.06^{c}	0.083±0.011^{c}

CIA: collagen-induced arthritis; GCS: glucosides of *Cheanomeles speciosa;* GTW: glucosides of *Tripterygium wilfordii.*

Tab 4. Effects of GCS on T and B cell proliferation and production of IL-1, IL-2 of CIA mice. *n*=5. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* CIA group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* normal group.

Group	Dose/mg·kg^{-1}	$A_{570\ nm}$		[^{3}H]TdR incorporation/Bq	
		T cell	B cell	IL-1	IL-2
Normal	–	0.115±0.007	0.150±0.017	147±25	47±6
CIA	–	0.25±0.03^{f}	0.34±0.02^{f}	303±56^{e}	281±17^{e}
CIA+GCS	60	0.14±0.08^{b}	0.28±0.08	225±53	244±14
	120	0.13±0.04^{b}	0.20±0.03^{b}	200±83^{b}	178±69^{b}
	240	0.12±0.04^{b}	0.190±0.025^{c}	106±56^{b}	131±50^{c}
CIA+GTW	80	0.15±0.28^{b}	0.192±0.021^{c}	53±17^{c}	153±8^{b}

CIA: collagen-induced arthritis; GCS: glucosides of *Cheanomeles speciosa;* GTW: glucosides of *Tripterygium wilfordii.*

inflammatory cells infiltration, and pannus formation were significantly reduced in CIA after administration of GCS and GTW (C, D, HE, ×200) (Fig 1).

DISCUSSION

RA is an autoimmune disease and characterized by synoviocytes proliferation, synovial lining thickening, various type of cells invasion, and pannus construction. This pathological changes result from many factors, such as gene mutation, oncogene activation, synoviocyte transformation, cell invasion, cytokine production, *et al*[14]. Immune and inflammatory systems are controlled by multiple cytokines, including ILs and INFs[15]. Inflammation resulted in a profile of increased gene expression of matrix metalloproteinases, cell adhesion molecules, plasma levels of the proinflammatory cytokines, such as tumor necrosis factor, interleukin-1beta and interleukin-6, the proinflammatory cytokines involved in cell division and transcription, signal transduction, and protein synthesis and metabolism.

CIA is an experimental animal model of RA being characterized by synovitisand progressive destruction of cartilage and bone[5,16]. In this study, Kunming mice developed an erosive and hind paw arthritis when immunized with chicken CII in CFA. The incidence of CIA was 50 %-60 %. Clinical evidence of CIA first appeared as peri-articular erythema and edema in the hind paws. The onset of paw-swelling was on d 24 after injection of emulsion, and the swelling peak appeared on d 36-40 and then declined after d 40. GCS (60, 120, 240 mg/kg×7 d) and GTW (80 mg/kg×7 d) were able to reduce the paw-swelling and arthritis scores of CIA mice.

As cytokines play an important role in the pathogenesis of CIA, considerable interest has been directed at exploring the role of IL-1 in CIA[9]. IL-2, a Th1 lymphocyte-derived cytokine, is thought to play an im-

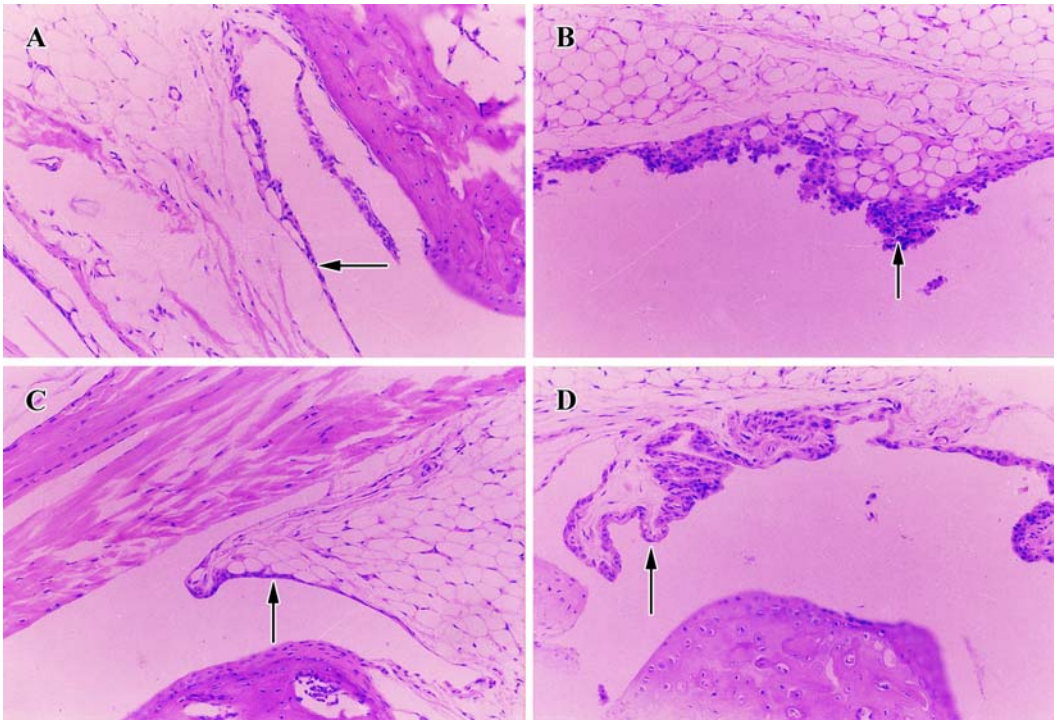

Fig 1. A) The arrowhead showed monolayer synovial cell; B) The arrowhead showed hyperplastic synovial cell and pannus; C) The arrowhead showed synovial cell was less and synovium was nearly monolayered; D) The arrowhead showed monolayer or double layer thin synovium.

portant role in the pathogenesis of RA[17]. T cells initiate the inflammatory cascade through secretion of either IL-2 or IFN-γ, or through direct cellular interaction with macrophages and synoviocytes. IL-2 production was higher in the long-lasting RA[18]. In the research, thymocyte and splenocyte proliferation in CIA mice were increased, and the production of IL-1 by PM and IL-2 by T cells were up-regulated significantly in CIA. GCS and GTW could inhibit thymocytes and splenocytes proliferation, reduced IL-1 and IL-2 production.

Abundant PGE_2 production at inflammation sites such as RA synovium is caused by the coordinated up-regulation of PGs and COX-2[19]. In our study, the level of PGE located in ankle and serum anti-CII antibody were increased significantly in CIA mice. GCS (120, 240 mg/kg×7 d) and GTW (80 mg/kg×7 d) could reduce the level of PGE and anti-CII antibody.

On all counts, the fact that administration of GCS and GTW efficiently ameliorated the disease severity assessed by clinical arthritis score and histological analysis demonstrated that inflammatory cell infiltration and pannus formation were significantly reduced. Thymocyte and splenocyte proliferation were decreased and IL-1 and IL-2 production were decreased after CIA mice were given with GCS and GTW compared to those of controls. At the same time, GCS and GTW could reduce the level of serum anti-CII and PGE located in ankle. These results suggested that the effects of GCS exerted anti-inflammatory and immunomodulatory effects. The mechanisms of GCS may be related to inhibition of Th1 activity and suppression of the function of macrophage and B cell. The results imply that GCS has a high potential therapeutic value for clinical treatment of RA.

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