Glucosides of *Chaenomeles speciosa* remit rat adjuvant arthritis by inhibiting synoviocyte activities[1]

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ABSTRACT

AIM: To investigate the effects of Glucosides of *Chaenomeles speciosa* (GCS) on rat adjuvant arthritis (AA) and to clarify the role of synoviocytes in this process. **METHODS:** Complete Freund's adjuvant was used to induce AA in rats. Secondary paw swelling of AA rats was measured with MK-550 volume meter. The pain response and polyarthritis index were scored. Synoviocytes were separated by incubation of collagenase and trypsin, and morphological changes of synoviocytes were observed by transmission electron microscope. Interleukin-1 (IL-1) production was measured by thymocyte proliferation assay. Tumor necrosis factor (TNFα) and prostaglandin E_2 (PGE_2) production were determined by radioimmunoassay. **RESULTS:** There were significant secondary inflammatory reactions in AA rats. The morphology of synoviocytes from AA rats was changed, companying the elevation of the level of IL-1,TNFα, and PGE_2 produced by synoviocytes from AA rats. GCS (60 and 120 mg/kg, ig, 8 d) suppressed secondary inflammatory paw swelling, pain response, and polyarthritis index. It also improved ultrastructural changes of synoviocytes and inhibited IL-1,TNFα, and PGE_2 production in AA rats. The inhibitory effect of GCS 120 mg/kg was more evident than that of Actarit 60 mg/kg. **CONCLUSION:** GCS reduced the secondary inflammatory in AA rats, which is associated with prevention of ultrastructural changes of synoviocytes and inhibition of secretion of proimflammatory cytokines.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint swelling, synovial membrane inflammation, and cartilage destruction. Adjuvant arthritis (AA) in rat is an experimental model that shares some features with human RA[1] , such as swelling, cartilage degradation and loss of joint function. The higher secretion of synovial cells activated by proinflammatory factors such as IL-1, TNFα, and PGE_2 is thought to be a key step in the destruction of cartilaginous and bony tissues in RA joints. IL-1, TNFα, and PGE_2 overproduction play potential pathogenic roles in the establishment of rheumatoid synovitis, in the formation of pannus tissue and in the process of joint destruction. Up to date, the cause of RA is not fully understood. Although there are a few anti-rheumatic drugs showing effectiveness on treating RA, their side effects and toxicity call for new and more effective

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natural drugs.

Chaenomeles speciosa (sweet) Nakai is one of the valuable traditional herbs used in treatment for RA with a long history in traditional Chinese medicine. Glucosides of *Chaenomeles speciosa* (GCS), extracted from the fructus of *Chaenomeles speciosa,,* is an active compound. Previous studies from our laboratory showed that GCS possesses anti-inflammatory and analgesic properties. However, it is unknown whether GCS exerts its effect on the chronic autoimmune diseases such as RA. The present study was therefore designed to investigate the effects of GCS on rat AA and its relative mechanisms by which GCS affects the process of AA, especially synoviocyte function.

MATERIALS AND METHODS

Drug and reagents GCS (yellow power, purity of glucosides beyond 50 %) and Actarit were provided by the Chemistry Lab of institute of Clinical Pharmacology of Anhui Medical University and suspended in 0.5 % sodium carboxymethylcellulose (CMC-Na). Bacillus Calmette Guerin (BCG) was obtained from Shanghai Biochemical Factory. Lipopolysacharide (LPS), Collagenase type II, trypsin, and 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St Louis, MO, USA). RPMI-1640 medium was purchased from Gibco Co (CA, USA). The RPMI-1640 medium was supplemented with Hepes 10 mmol/L, *L*-glutamine 2 mmol/L, 2-mercaptoethanol 50 μmol/L, penicillin sodium 100 kU/L, streptomycin 100 mg/L, and 10 % new born bovine serum and pH was adjusted to 7.2. ^{125}I-TNFαRIA kit was the product of Beijing Biotinge Biomedicine company. ^{125}I PGE_2 RIA kit was the product of Suzhou Medical College. All other reagents were of analytical purity.

Animal Male Sprague-Dawley (SD) rats weighing 160-180 g and C57BL/6J mice weighing 18-20 g were purchased from Shanghai BK Experimental Animal Center (Grade II, Certificate No D-65).

AA induction and evaluation AA was induced as previously described[2]. Briefly, rats were immunized on d 0 by intradermal injection of Complete Freund's adjuvant (FCA), containing 10 mg heat-inactive BCG in 1 mL paraffin oil, into the left hind paw in 0.1 mL for each rat. Right hind paw volume was determined with MK-550 volume meter (Muromachi Kikai Co Japan) before immunization (basic value, d 0) and repeated on d 14, d 17, d 21. The paw swelling (ΔmL) was defined as the paw volume evaluated after inflammation.

The polyarthritis severity was graded on a scale of 0-4[3]: 0=no swelling; 1=isolated phalanx joint involvement; 2=involvement of phalanx joint and digits; 3=involvement of the entire region down to the ankle; 4=involvement of entire paw, including ankle. The maximum joint score was 12 including 3 secondary arthritis paw for each rat.

A pain-related test was conducted. The "footbend" procedure evaluated the hyperalgesic state[4]. It involved holding the rat comfortably and gently extending the right hind paw. The test was repeated 5 times at 5 s intervals; a rating of 1 or 0 was given according to whether the animal vocalized (1) or not (0). For each animal, the rating ranged from 0 to 5.

Drug treatment The rats with AA were divided randomly into four groups which were GCS (30, 60, and 120 mg/kg) and Actarit (60 mg/kg) given intragastrically from d 7 to d 14 after immunization. For the normal and AA model, rats were given an equal amount of vehicle.

Synoviocytes culture Rats were killed on d 21 after immunization. Synoviocytes from rat knees were excised and dispersed with sequential incubation of 0.4 % (w/v) collagenase type II and 0.25 % (w/v) trypsin. Synoviocytes were resuspended in RPMI-1640 medium at a concentration of 1×10^9 cells/L. The cell suspension 500 μL and LPS 500 μL with the concentration of 10 mg/L were added to 24-well plate. After incubation at 37 ºC in 5 % CO_2 atmosphere for 48 h , the supernatant containing IL-1,TNFα, and PGE_2 was collected and stored at -20 ºC.

Ultrastructure of synoviocyte Harvested synoviocytes were fixed with 4 % para-formal dehyde solution overnight. Synoviocytes were washed with PBS after fixing with 1 % osmic acid for 2 h. Being embedded in an Epon/Araldite mixture and stained with uranyl acetate and lead citrate, the cells were observed under 1230 type transmission electron microscope (Electron Co, Japan) and photographed.

Analysis of IL-1 IL-1 activity was measured by thymocyte proliferation assay. Suspension of thymocytes (5×10^9 cells/L) taken from C_{57}BL/6J mice was distributed in a flat bottomed 96 -well plate exposed to ConA (3 mg/L). Then the cells were incubated at 37 ºC in 5 % CO_2 atmosphere for 48 h . MTT (5 g/L) 10 μL was added to each well. After an incubation at 37 ºC for an additional 4 h, the cells were centrifuged 1000×*g*

for 10 min and all the supernatants were discarded. The formazan crystals were dissolved in 120 μL isobutanol (containing 0.04 mol/L HCl) and the absorbance was examined at 570 nm using an enzyme-linked immunosorbent assay plate reader. The result was expressed as mean of triplicate samples.

TNFα and PGE_2 radioimmunoassay (RIA) TNFα and PGE_2 produced by cultured synoviocytes were measured according to the procedures offered in TNFα and PGE_2 ^{125}I RIA kit.

Statistical analysis Data were expressed as mean±SD. The analysis of variance and *t*-test were used to determine significant differences between groups. *P* values less than 0.05 were considered to be significant.

RESULTS

Effects of GCS on inflammatory and pain responses in rats with AA Inflammatory polyarthritis was induced in all immunized rats. The peak incidence occurred on d 14 after immunization. Treatment with GCS and Actarit diminished the right hind paw swelling and polyarthritic symptoms on d 14 after immunization. The same results were observed at dose of GCS 60 or 120 mg/kg on d 17. The suppressive effect of GCS 120 mg/kg lasted to d 21. GCS 120 mg/kg was more effective than Actarit on d 21 (Tab 1, 2). The analgesic effect of GCS at dose of 60 or 120 mg/kg were observed on d 14, d 17, and d 21. The inhibitory potency of GCS 120 mg/kg was higher than that of Actarit (Tab 3).

Effect of GCS on ultrastructure of synoviocytes Cartilage is a specialized connective tissue. Its degradation depends on synoviocytes activities. There are two types of synoviocytes. Type A synoviocyte derives from macrophage. Type B synoviocyte derives from fibroblast which was characterized by developed rough endoplasmic reticulum (RER) and free ribosomes in physiological state. It was found that the number of type A synoviocytes was increased significantly in AA rats compared with the normal. In contrast, the number of type B synoviocytes was decreased. Furthermore, the intracellular changes were observed in AA rats, including that Golgi bodies reduced in size and curled, mitochondria swelled with ridges decreasing, RER increased and dilated, dense bodies and vacuoles increased. GCS and Actarit repaired the injury mentioned above to some extent (Fig 1).

Effects of GCS on proinflammatory cytokines produced by synoviocytes Synoviocytes suspension 500 μL and LPS 500 μL with the concentration of 10 mg/L was cultured for 48 h. After incubation, the supernatants were centrifuged (1000×*g*, 10 min) and fil-

Tab 1. Effect of glucosides of *Chaenomeles speciosa* (GCS) on paw swelling in rats with adjuvant arthritis (AA). *n*=8. Mean±SD. $^{c}P<0.01$ *vs* normal. $^{e}P<0.05$, $^{f}P<0.01$ *vs* AA model. $^{h}P<0.05$ *vs* Actarit.

Group	Dose/ mg·kg^{-1}	Paw swelling/ΔmL d 14	d 17	d 21
Normal	-	0.07±0.04	0.12±0.02	0.09±0.05
AA	-	0.68±0.12^{c}	0.88±0.24^{c}	0.99±0.26^{c}
GCS	30	0.46±0.16^{e}	0.62±0.26	0.81±0.25
	60	0.43±0.22^{e}	0.56±0.17^{f}	0.77±0.21
	120	0.38±0.18^{f}	0.46±0.20^{f}	0.45±0.12^{fh}
Actarit	60	0.37±0.11^{f}	0.60±0.26^{e}	0.72±0.24

Tab 2. Effect of glucosides of *Chaenomeles speciosa* (GCS) on polyarthritis index in rats with adjuvant arthritis (AA). *n*=8. Mean±SD. $^{e}P<0.05$, $^{f}P<0.01$ *vs* AA model. $^{h}P<0.05$ *vs* Actarit.

Group	Dose/ mg·kg^{-1}	Polyarthritis index d 14	d 17	d 21
AA	-	9.9±1.2	11.4±2.7	11.8±2.4
GCS	30	8.5±1.0	10.8±1.1	11.1±1.2
	60	7.6±2.1^{e}	9.0±1.1^{e}	9.3±1.4^{e}
	120	6.0±2.1^{f}	7.1±2.4^{f}	7.1±1.6^{fh}
Actarit	60	7.2±2.7^{e}	9.3±1.8^{e}	9.9±2.3^{e}

Tab 3. Effect of glucosides of *Chaenomeles speciosa* (GCS)on pain response in rats with adjuvant arthritis (AA). *n*=8. Mean±SD. $^{c}P<0.01$ *vs* normal. $^{e}P<0.05$, $^{f}P<0.01$ *vs* AA model. $^{i}P<0.01$ *vs* Actarit.

Group	Dose/ mg·kg^{-1}	Pain response score d 14	d 17	d 21
Normal	-	0.25±0.06	0.13±0.05	0.13±0.06
AA	-	1.00±0.22^{c}	3.4±0.5^{c}	4.2±0.7^{c}
GCS	30	0.89±0.27	2.0±0.4	3.3±0.6
	60	0.83±0.13	1.4±0.5^{f}	2.8±0.8^{f}
	120	0.67±0.22^{f}	1.6±0.5^{f}	2.2±0.3^{fi}
Actarit	60	1.0±0.5	1.9±0.7	3.4±0.7

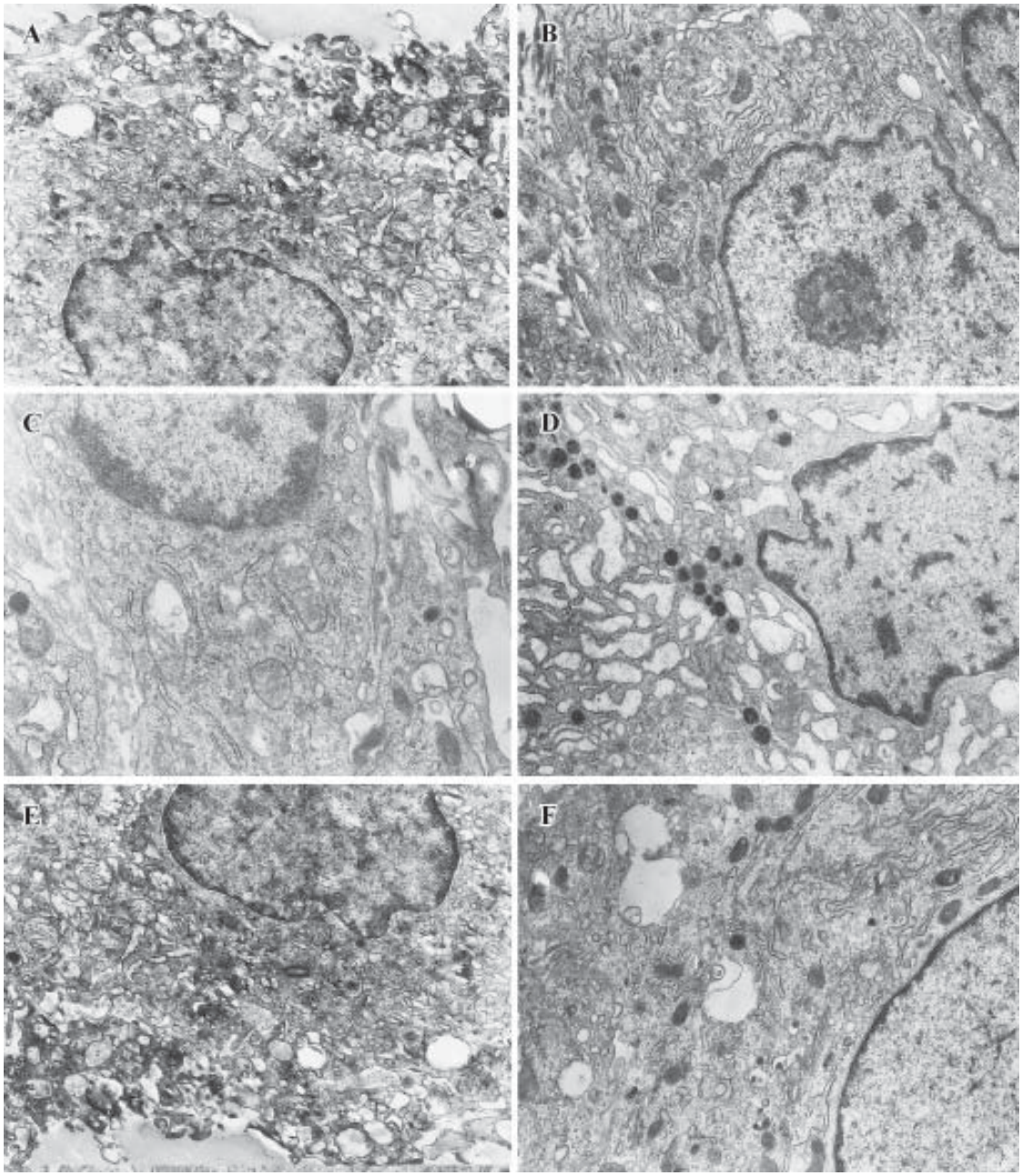

Fig 1. Effects of glucosides of *Chaenomeles speciosa* (GCS) on ultrastructure of synoviocytes. A: type A synoviocyte in normal rats; B: type B synoviocyte in normal rats; C: type A synoviocyte in adjuvant arthritis (AA) rats; D:type B synoviocyte in AA rats; E: type A synoviocyte in AA rats treated with GCS 60 mg/kg; F: type B synoviocyte in AA rats treated with of GCS 60 mg/kg. A, B, E, F: ×8000. C, D: ×10 000.

tered through 0.22 μm filter. Synoviocytes suspension containing IL-1, TNFα and PGE_2 was collected for assay. Synoviocytes from AA rats released a higher level of IL-1, TNFα, and PGE_2 than that from the normal rats. GCS (60 and 120 $mg \cdot kg^{-1} \cdot d^{-1}$, ig, 8 d) significantly inhibited the production of IL-1, TNFα, and PGE_2 of synoviocytes (Tab 4). Actarit also reduced the content of IL-1 and TNFα, but showed no effect on PGE_2 production.

DISCUSSION

Joint swelling, arthrodynia and deformity were main clinical symptoms of RA. Rat AA induced by CFA had similar characteristics to RA in the aspects of histology and immunology. The present study demonstrated that GCS markedly inhibited joint swelling and pain, as well as down-regulated the index of polyarthritis in AA rats. This suggested that GCS might be effective on chronic autoimmune disease such as RA. This

Tab 4. Effects of glucosides of *Chaenomeles speciosa* (GCS) on IL-1, TNFα, and PGE_2 production from the supernatant of cultured synoviocytes stimulated by LPS (5 mg/L). *n*=4-6. Mean±SD. $^{c}P<0.01$ *vs* normal. $^{e}P<0.05$, $^{f}P<0.01$ *vs* adjuvant arthritis (AA) model. $^{i}P<0.01$ *vs* Actarit.

Groups	Dose/ $mg\cdot kg^{-1}$	IL-1	TNFα/$ng\cdot L^{-1}$	PGE_2/$ng\cdot L^{-1}$
Normal	-	0.29±0.05	100±29	1.3±0.4
AA	-	0.48±0.04^{c}	347±47^{c}	6.4±0.8^{c}
GCS	30	0.37±0.09	314±53	5.5±0.8
	60	0.37±0.07^{e}	208±60^{e}	4.6±1.0^{e}
	120	0.36±0.04^{f}	148±79^{f}	3.6±0.8^{fi}
Actarit	60	0.38±0.03^{e}	210±68^{e}	5.6±0.9

provided the further consideration that GCS might be a new class of effective anti-inflammatory agents.

The main pathological changes of RA included synovitis and pannus formation, which lead to cartilage erosion and articular destruction. Synoviocytes were the ultimate effectual cells of pathologic change[5]. TNFα and IL-1 are considered master cytokines in the process of human RA, with a claimed cascade of TNFα inducing most of the IL-1 production. Studies in experimental models revealed that TNFα is indeed a pivotal cytokine in joint swelling, and IL-1 is the dominant cartilage destructive cytokine and its production may occur independent of TNFα. It was also found TNFα/IL-1 receptor antagonists were recently backed up with similar data in arthritis models in TNFα and IL-1 knockout mice[8,9]. In our study, synoviocytes of AA rats released a higher level of IL-1 and TNFα than that of the normal rats. GCS inhibited the production of IL-1 and TNFα. Therefore, GCS might ameliorate the secondary inflammatory reaction of AA via influencing secretory function of activated synoviocytes.

A variety of low-molecular-weight inflammatory mediators played an important role in mediating the local inflammation in RA, especially PGE_2, a major metabolite of arachidonic acid. PGE_2, produced by rheumatoid synovial tissue, causes inflammation as well as joint destruction and stimulates of bone resorption[10]. PGE_2 was reported as a potent stimulator of protein kinase A and an inducer of expressions of various genes, such as vascular endothelial growth factor[11], IL-1[12] and collagenase[13]. These reports suggested the new mechanisms of inflammatory reaction and demonstrated another biological role of PGE_2 in RA. In our experiment, the level of PGE_2, produced by synovial cells obtained from AA rats, was substantially reduced by GCS administration. These results suggest that GCS protected inflammatory joints from destroying partly by means of blocking PGE_2 production.

Golgi bodies in type A synoviocytes mainly take part in secretory function, while the mitochondrion is the energy provider to cell metabolism. Meanwhile, the rough endoplasmic reticulum locating to type B synoviocytes is the place where proteins are processed and secreted. Our results showed that the secretion and metabolism of synoviocytes of AA rats became hyperfunctional at the d 21 after immunization, which were improved by GCS. The morphologic changes of synoviocytes contributed to its secretory function which was related to the over-production of IL-1, TNFα and PGE_2. The effects of GCS on synoviocytes secretory function might provide an explanation for the mechanism of its action in AA rats. These pharmacological effects of GCS strongly suggested its potential therapeutic role for the autoimmune disease, especially RA.

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