

## Therapeutic effects of benzoylaconitine and paeoniflorin in rats with collagen-induced arthritis

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To explore the effects and mechanisms of benzoylaconitine and paeoniflorin on collagen-induced arthritis (CIA) rats. Weight, paw swelling, arthritis index and joint pathologic changes were examined in each group after CIA induction. PGE<sub>2</sub>, IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , VEGF, MMP-3, IgG and anti-CII Ab were assessed by ELISA; STAT1 and STAT3 expressions were analyzed immunohistochemically, and the ultrastructure of synovial cells was observed by transmission electron microscopy. Therapeutic effects were determined in CIA rats via injecting benzoylaconitine and paeoniflorin, which could alleviate the degree of swelling and arthritis index (AI) and pathological lesions of the sacroiliac gland; decrease the levels of PGE<sub>2</sub>, IL-1 $\beta$ , TNF- $\alpha$ , VEGF and IgG in serum; reduce STAT1 and STAT3 expression in the membrane tissue; and inhibit the secretion and proliferation of synovial cells. These results showed that benzoylaconitine and paeoniflorin could significantly palliate the arthritic symptoms of CIA rats, and better therapeutic effects could be achieved if the two components were used in combination.

**Keywords:** Benzoylaconitine. Paeoniflorin. Cellular Mechanisms. Collagen-induced Arthritis.

### INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease of the joints characterized by synovial hyperplasia and inflammation affecting approximately 0.24% of the human population worldwide with high morbidity (Cross, *et al.*, 2014). Therapeutic drugs, including glucocorticoids, nonsteroidal anti-inflammatory drugs, anti-rheumatic drugs and biological agents such as TNF- $\alpha$  inhibitors and IL-1 antagonists, have significant side effects that affect patients' medication compliance (Gao, *et al.*, 2014). Therefore, it is essential to find novel drugs for treatment to satisfy different demands with higher efficacy and lower toxicity. CIA is a chronic,

multiple arthritis caused by immunization of animals for either homologous or heterologous CII stimulation (Chen, Zhang, 2016), which closely resembles the genetic background and histological and immunological features of RA (Yang, 2009; Kochi, *et al.*, 2009).

Natural compounds derived from traditional Chinese medicine have been an abundant source in the process of novel drug discovery (Newman, Cragg, 2016). Radix Aconiti, the root of *Aconitum carmichaelii* Debx., is widely used with Radix Paeoniae Alba (the root of *Paeonia lactiflora* Pall.). This compatibility is a traditional Chinese cold medicine pair for the treatment of rheumatic arthralgia and modernly used for the treatment of RA (Symmons, 2003). Previous studies demonstrated that the decoction of Radix Aconiti and Radix Paeoniae Alba or the compatibility of their ingredients had both synergism and attenuation efficacy (anti-inflammatory and analgesic activity, treatment of RA) (Kochi, Suzuki, Yamamoto, 2014; Kallberg, *et*

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*al.*, 2007). Aconitum alkaloids are the distinctly active components of Radix Aconiti, but diester-type alkaloids are highly toxic, including aconitine, hypaconitine and mesaconitine. Monoester-type alkaloids have much lower toxicity (Deng, Lv, 2001), such as benzoylaconitine, benzoylhypaconitine and benzoylneoaconitine. In the process of decoction, the content of diester-type alkaloids was significantly reduced, while the content of monoester-type alkaloids was increased. In addition, monoester alkaloids are the main hydrolysates of diester alkaloids (Gong, *et al.*, 2017). Benzoylaconitine, the most abundant monoester alkaloid, can reduce primary and secondary foot swelling in adjuvant arthritis (AA) rats (Trentham, Townes, Kang, 1977). Meanwhile, it was more effective than aconitine in ED50 and less toxic in LD50 (Li, Gong, Wang, 2016), which indicated that aconitine can be a prodrug of benzoylaconitine. Paeoniflorin, a well-known active component of Radix Paeoniae Alba, has dramatic anti-inflammatory and immunomodulatory effects, especially in RA (Lin, Chen, Min, 2011; Chen, *et al.*, 2007). Regarding monomer compatibility, aconitine combined with paeoniflorin can increase the effect and decrease toxicity (Qin, *et al.*, 2015; Zhang, Li, 2018), but now the evidence showed that benzoylaconitine was more advantageous than aconitine evaluated by the range of safety indices. In addition, we previously determined the anti-inflammatory and analgesic effects of the two components with benzoylaconin and paeoniflorin (Gu, Li, Tong, 2018).

Thus, benzoylaconitine (BAC) and paeoniflorin (PAE) are the active monomer ingredients of Radix Aconiti and Radix Paeoniae Alba, respectively. As their effects and mechanisms in RA remain to be identified, we extended our strategy to investigate the pharmacodynamic effect of BAC and PAE on CIA and explore some of its mechanisms, which would provide a new theoretical basis for further research.

## MATERIAL AND METHODS

### Ethics statement

All experimental procedures regarding specimen collection from SD rats were approved by the Medical

Ethics Committee of Sichuan Provincial People's Hospital Ethics Committee, China.

### Animals

SD rats, male, body weight ( $140 \pm 10$  g), were provided by the Institute of Laboratory Animals, Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, production license number: SCXK (Chuan) 2013-15. All experimental rats were subjected to experiments after 5 days of adaptive feeding at the Experimental Animal Center of Sichuan Academy of Medical Sciences before the experiment. Rearing environment: temperature  $22^{\circ}\text{C}\sim 23^{\circ}\text{C}$ , humidity 30%~50%; feeding method: feed separately in cages according to groups, 5 per cage, free food and drink.

### Experimental Drugs

Benzoylaconitine (purity: 99.96% (HPLC), batch number: MUST-16012815) and paeoniflorin (purity: 99.30% (HPLC), batch number: MUST-16041901) were developed by Chengdu Mansite Biotechnology Co., Ltd. and Chengdu Institute of Biology, Chinese Academy of Sciences; Tripterygium glycoside tablets, Hunan Qianjin Xieli Pharmaceutical Co., Ltd., specifications 10 mg/tablet 100, approval number: CFDA approval No. Z43020138, product lot number: 20160704.

### CIA induction and assessment

Seventy SPF male SD rats weighing  $140\pm 10$  g were randomly selected as a normal control group after 10 days of adaptive feeding. The rest was used as the CIA model. The modeling method was based on the reported literature (Mackenzie, Dawson, 2005; De Roos, *et al.*, 2005) and the preliminary experimental results. 0.1 M acetic acid solution was used to dissolve the type II collagen (CII) to  $2.0\text{ mg}\cdot\text{ml}^{-1}$  before stirring overnight at  $4^{\circ}\text{C}$ . An emulsion was made with an equal volume of complete Freund's adjuvant (CFA). The trial rats were injected with the emulsion containing  $1.0\text{ mg}\cdot\text{ml}^{-1}$  CII intradermally into the back and tail at four points (two points on the back and two points at the base of the tail). A week later, the

rats were managed with an identical protocol. The day of CIA induction was designated day 0 (d0). The arthritis index (AI) was scored on the 14th day (d14). According to the “Methodology for the Study of Pharmacodynamics of Traditional Chinese Medicine” (Cho, *et al.*, 2007), the clinical process of CIA was scored via daily observations where the inflammation of the whole four paws was graded from 0 to 4 as represented below: grade 0, no paw swelling; grade 1, one red swollen paw; grade 2, two red swollen paws and ankle; grade 3, three or more red swollen paws and ankle; grade 4, all paws and ankle were red and swollen. Each paw was graded, and the four scores were added so that the maximum possible score was 16 per rat. Arthritis induction was successful with at least an AI score  $\geq 2$  in 1 foot. Fifty successful models were selected and randomly divided into a CIA model control group (MCG), Tripterygium glycoside group (positive control group, TGG), Paeoniflorin group (PG), Benzoylaconitine group (BG), and Benzoylaconitine combined with Paeoniflorin group (Compatibility group, CG).

### Administration Methods

On day 14 after the first immunization, each rat was administered a shot once by way of the stomach. The concentrations were PG, 24 mg·kg<sup>-1</sup>; BG, 1.5 mg·kg<sup>-1</sup>; CG, benzoylaconitine and paeoniflorin were 1.5 mg·kg<sup>-1</sup> and 24 mg·kg<sup>-1</sup> respectively; TGG, 8 mg·kg<sup>-1</sup>. The specific dosing regimen was as follows: the blank control group (BCG) and the MCG were given distilled water. BCG, MCG, TGG, BG and PG were administered at a dose of 10 ml·kg<sup>-1</sup>, and CG was administered at a dose of 5 ml·kg<sup>-1</sup> once a day for 4 weeks.

All concentrations and dosages were selected according to a previous anti-inflammatory and analgesic study (Gu, Li, Tong, 2018), and the acute toxicity of the test items was assessed before employing the doses for the study.

### General Observation

Starting at the first immunization (d0) and continuing until every 7 d after the first immunization (d7, d14)

and every 4 d after administration (d18, d22, d26, d30, d34, d38, d42), the body weight was measured, and the thickness of the right hind paw was measured with an electronic digital caliper (SF2000, Guilin Guanglu Digital Measurement and Control Co., Ltd.). The AI scores were determined from d14 to d42.

### Histopathological Investigation of the Joints

At the end of the experiment, rats were anesthetized with 10% chloral hydrate at a dose of 3 ml·kg<sup>-1</sup> i.p., and the hind paws were removed and fixed in 10% buffered formalin and then embedded in paraffin. After staining with hematoxylin and eosin, five-micrometer midline sagittal sections were observed under a micrometer-attached microscope. Pathological changes, including hyperplasia of the synovium, inflammatory cell invasion, pannus formation and destruction of cartilage and bone, were scored as follows (Chen, Tong, Qifu, *et al.*, 2010): grade 0, no abnormality; grade 1, slight lesion; grade 2, mild lesion; grade 3, medium lesion; grade 4, severe lesion. The highest pathology score of each ankle joint was 12 points.

### Cytokine and antibodies assay

All rat sera were obtained at once by abdominal aortic bleeding 28 days after CIA induction. Cytokine levels in the serum were quantified using enzyme-linked immunosorbent assay (ELISA) kits for rat TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, PGE2, MMP-3, IgG, anti-collagen antibodies (Elabscience, Wuhan, China) and vascular endothelial growth factor (VEGF, Shin Bo Shing Bio-Technology Co., Ltd.) per manufacturer’s instructions.

### Immunohistochemical detection

The signal transducer and activator of transcriptions 1 and 3 (STAT1 and STAT3) expression in the synovium were measured using immunohistochemistry. Briefly, 5  $\mu$ m sections of routinely processed paraffin-embedded blocks were cut and mounted on adherent glass slides. The sections were deparaffinized in xylene, rehydrated in a graded ethanol series and then treated

with 3% hydrogen peroxide. Microwave pretreatment for 15 minutes in citrate buffer (pH 6.0, 10 mM) was performed to retrieve antigen. The sections were then incubated for an hour at room temperature with primary antibodies as follows: 1) STAT1(1:100) monoclonal antibody (SAB), 2) STAT3(1:100) monoclonal antibody (SAB), followed by incubation with secondary antibody (Anti-Rabbit IgG (1:1000), SAB) for 30 minutes and finally diaminobenzidine (DAB) for 3-5 minutes. The slides were counterstained with hematoxylin and eosin (HE, Zhuhai Bezo Biotechnology Co., Ltd.). Clear brown-yellow particles in the cytoplasm and/or nucleus were used as positive cells. The result was assessed by the proportion of stained cells and staining intensity (Mcnamee, Williams, Seed, 2015; Vk, *et al.*, 2014; Lim, Gibbins, 1995). Staining intensity was graded as follows: 0, negative; 1, light staining; 2, moderate staining; 3, intense staining. The proportion score of stained cells for STAT1 and STAT3 was assessed as follows: 0, no stained cells; 1, <25% stained cells; 2, 25-50% stained cells; 3, 51% -75% stained cells; 4, >75% stained cells. The total score was obtained by multiplying the two parts.

### Ultrastructure of Synovial Cells

Transmission electron microscopy (TEM) was used to observe mitophagy and ultrastructural changes in synovocytes. Fixed cells were postfixated in 3%

glutaraldehyde, dehydrated in graded alcohol and flat-embedded in Epon 812 (Electron Microscopy Sciences, USA). Ultrathin sections (100 nm) were prepared, stained with uranyl acetate and lead citrate, and inspected under an electron microscope (H-600IV; Hitachi, Japan).

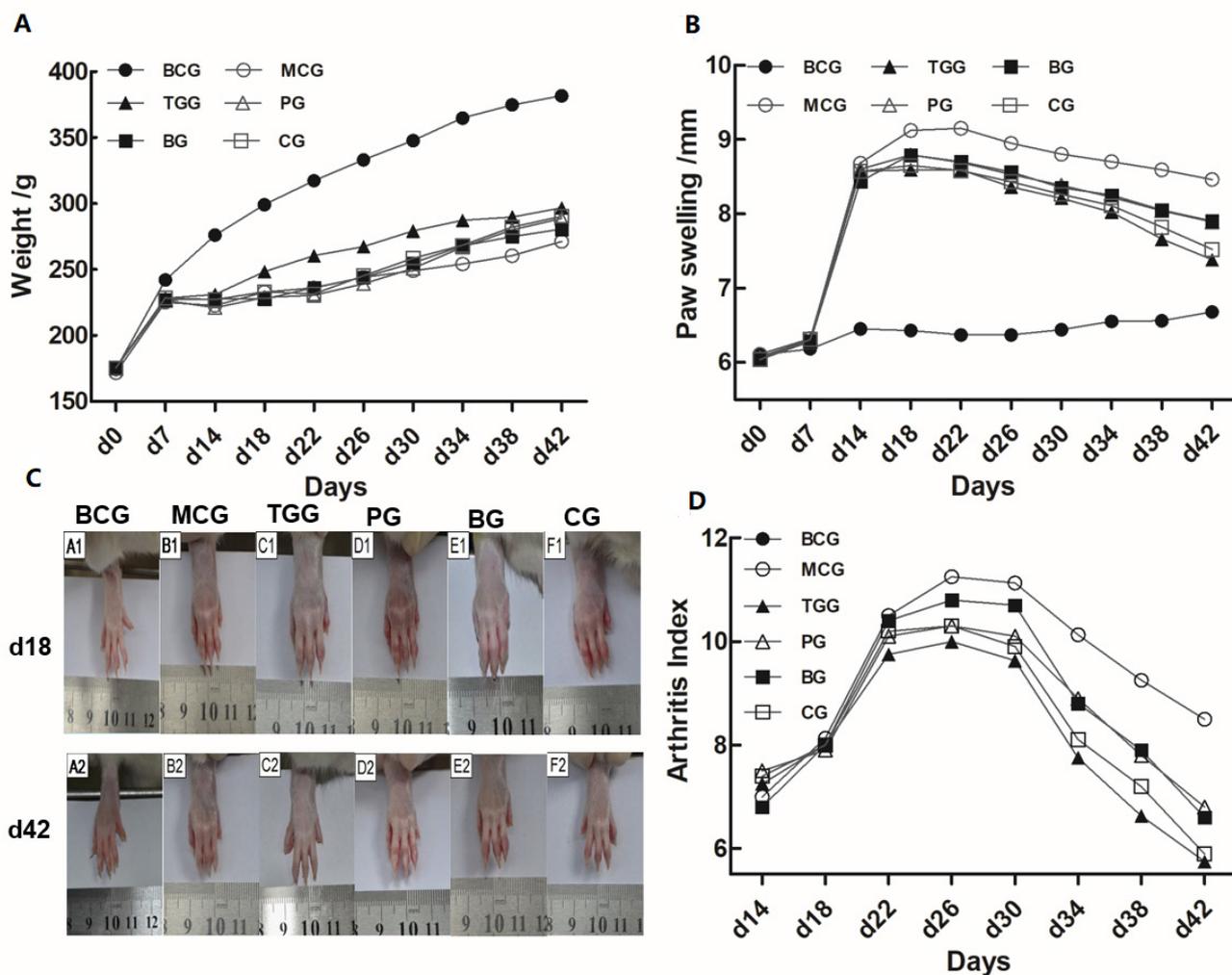
### Statistical analysis

Data are expressed as the mean  $\pm$  SD ( $\bar{x} \pm s$ ) and were analyzed with SPSS 17.0 Software Package (SPSS, Inc, Chicago, IL, USA). Comparisons of numerical data between groups were performed by one-way ANOVA. Significance was established at P-value < 0.05.

## RESULTS

### Effects on body weight of CIA rats

Normal rats had a healthy diet and movement, whose hair color was smooth and shiny, and their weight increased rapidly. CIA rats had less activity, dark coats and a reduction in food intake. As shown in Figure 1A, after treatment for 16 days (d30), the diet state of each treatment group was improved compared with that of the MCG group. The hair condition improved, and the growth in weight showed an upward trend ( $P > 0.05$ ). The weight of rats in the TGG increased quickly early, but later, the weight gain slowed. During the course of treatment, 2 rats died in the MCG and TGG.



**FIGURE 1** - Effect of benzoylaconitine and paeoniflorin on the articulus of CIA rats. A. The weight in each group. There was no weight difference on d0, and after modeling and treatment, the weight of MCG was significantly decreased compared with BCG ( $p < 0.05$ ). B-C. The change of paw swelling. On d14, the CIA group was significantly swollen compared with the BCG group ( $P < 0.01$ ). On d30, TGG showed a significant decrease compared with MCG; on d34, TGG and CG showed a significant decrease compared with MCG; on d38, TGG, CG, PG and BG showed a significant decrease compared with MCG ( $P < 0.05$ ). D. The change of AI. On d14, the AI in each CIA group was significantly higher than that of BCG ( $P < 0.01$ ). On d34, TGG showed a significant decrease compared with MCG; on d34, TGG and CG showed a significant decrease compared with MCG; on d42, TGG, CG, PG and BG showed a significant decrease compared with MCG ( $P < 0.01$ ,  $P < 0.05$ ).

### Effects on foot swelling of CIA rats

With the induction of CIA, paw swelling increased gradually and reached its peak levels on d22. After treatment, compared to MCG, TGG showed a significant decrease by d30; by d34, TGG and CG showed a significant decrease compared with MCG; by d38, the TGG, CG, PG and BG showed a significant decrease compared with the MCG ( $P < 0.01$  or  $P < 0.05$ ). The changes in the right hind paws in each group and pictures of paw

swelling before and after administration are shown in Figures 1B and 1C.

### Effects on AI of CIA rats

The changes in AI in CIA rats in each group are shown in Figure 1D. By d14, the AI in each CIA group was significantly higher than that of the BCG group ( $P < 0.01$ ). By d34, TCG showed a significant decrease compared with MCG; by d38, TGG and CG showed a

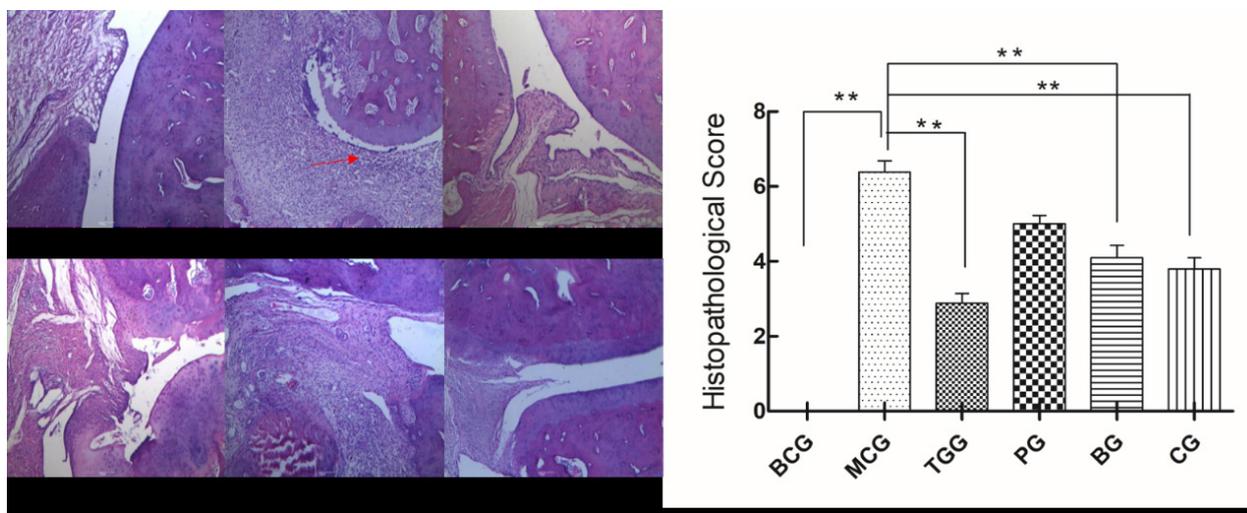
significant decrease compared with MCG; by d42, TGG, CG, PG and BG showed a significant decrease compared with MCG ( $P < 0.01$  or  $P < 0.05$ ).

### Effect on pathological changes of ankle joints in CIA rats

In BCG, the synovial membrane was thin and complete, consisting of 1 to 2 layers of synovial cells. The joint structure was intact, and the cartilage surface was smooth without any tissue edema, inflammatory cell infiltration, vascular proliferation or bone destruction. Otherwise, in the MCG, the synovial tissue proliferated significantly and formed a multilayer synovial membrane (up to 10 to 15 layers) with disordered arrangement and vasospasm formation. The joint structure was unclear, and in the joint cavity, there was massive infiltration

of inflammatory cells. Worse, joint cartilage and bone were destroyed with local fibrosis and adhesion.

The number of synovial cells in the TGG was slightly greater than the normal level, and inflammatory cell infiltration and vasospasm generally disappeared. It was rare to see rough cartilage surfaces or destroyed bone tissue. In the PG, synovial cell proliferation and inflammatory cell infiltration were observed, as well as vascular proliferation, rough articular cartilage, and destruction of bone tissue. In the BG, the number of synovial cell layers was decreased to 5-8 layers with less inflammatory cell infiltration, vascular proliferation, and cartilage and bone destruction. In the CG, synovial cell proliferation was notably reduced, showing 3 to 6 layers; little inflammatory cell infiltration, vascular hyperplasia, and slightly rough articular cartilage were observed, and almost no destruction of cartilage and bone was observed (Figure 2).

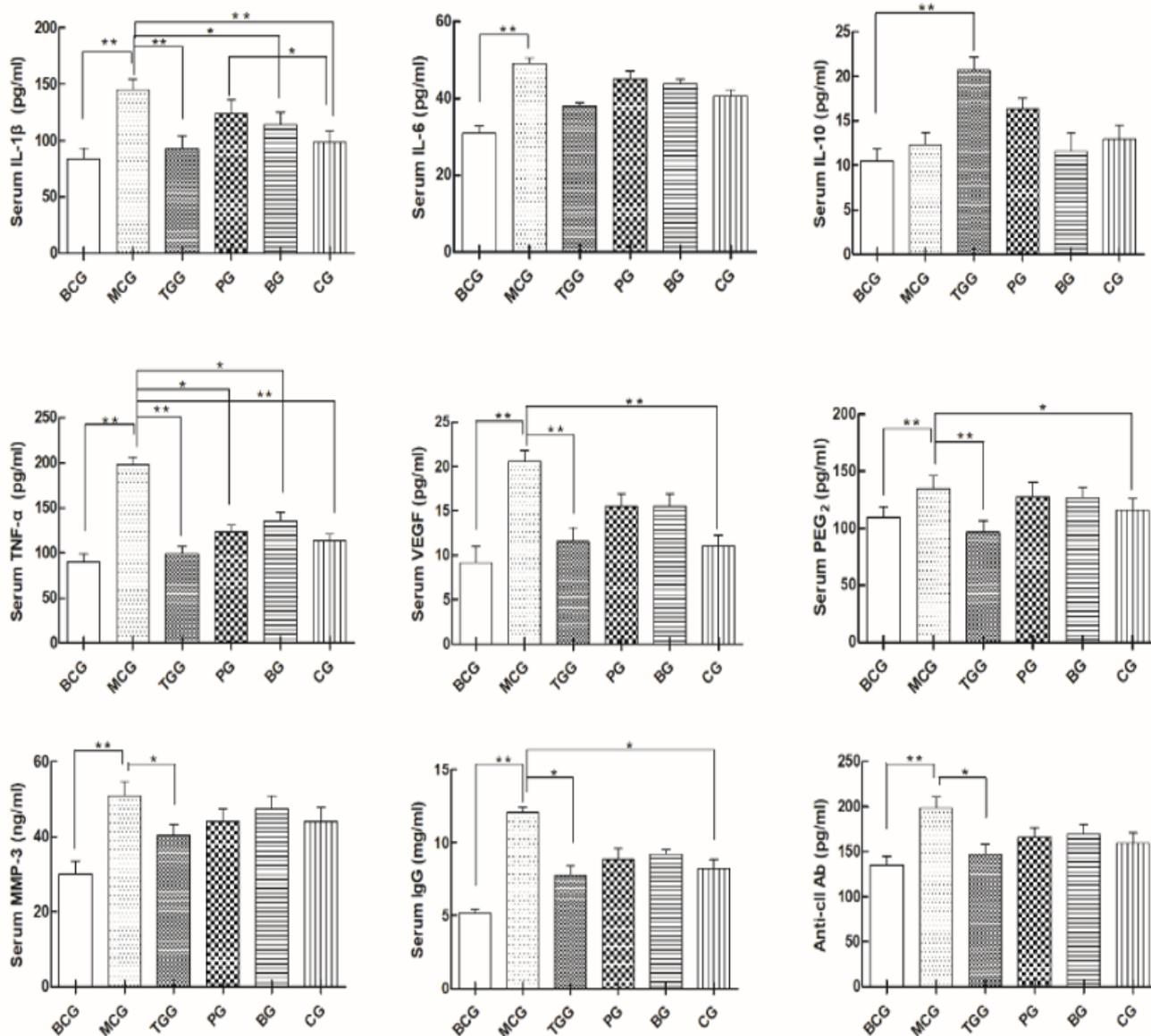


**FIGURE 2** - Histological changes of CIA rats (H. E  $\times 10$ ). Great synovial tissue proliferation, vasospasm formation, massive infiltration of inflammatory cells and destruction of joint cartilage and bone were observed in the MCG, as indicated by the red arrows. TGG, BG and CG showed a remarkable treatment effect compared with MCG. \*\*,  $p < 0.01$ .

### Effect of benzoylaconitine and paeoniflorin on serum cytokines and antibodies in CIA rats

The rats in the MCG group showed high levels of IL- $1\beta$ , IL-6, TNF- $\alpha$ , VEGF, PGE2, MMP-3, IgG and anti-cII Ab expression compared with those in the BCG group ( $p < 0.01$ ) (Figure 3). However, no differences were observed between PG, BG, CG and MCG in IL-

6, IL-10, MMP-3 and anti-cII Ab. The results implied that IL- $1\beta$ , IL-6, TNF- $\alpha$ , VEGF, PGE2, IgG and anti-cII Ab were relevant to the pathogenesis of CIA and that the use of benzoylaconitine and paeoniflorin could decrease IL- $1\beta$  and TNF- $\alpha$  levels. Furthermore, a better therapeutic effect could be obtained if benzoylaconitine and paeoniflorin were used cooperatively for IL- $1\beta$ , VEGF and PGE2.



**FIGURE 3** - Effect of benzoylaconitine and paeoniflorin on serum cytokines and antibodies in CIA rats. Benzoylaconitine and paeoniflorin decreased the levels of typical serum cytokines, such as IL-1β and TNF-α; their combined use had a better effect on IL-1β, VEGF and PGE<sub>2</sub>. \*\* p<0.01, \* P<0.05.

### Effect of benzoylaconitine and paeoniflorin on the expression of STAT1 and STAT3 in synovial tissue of CIA rats

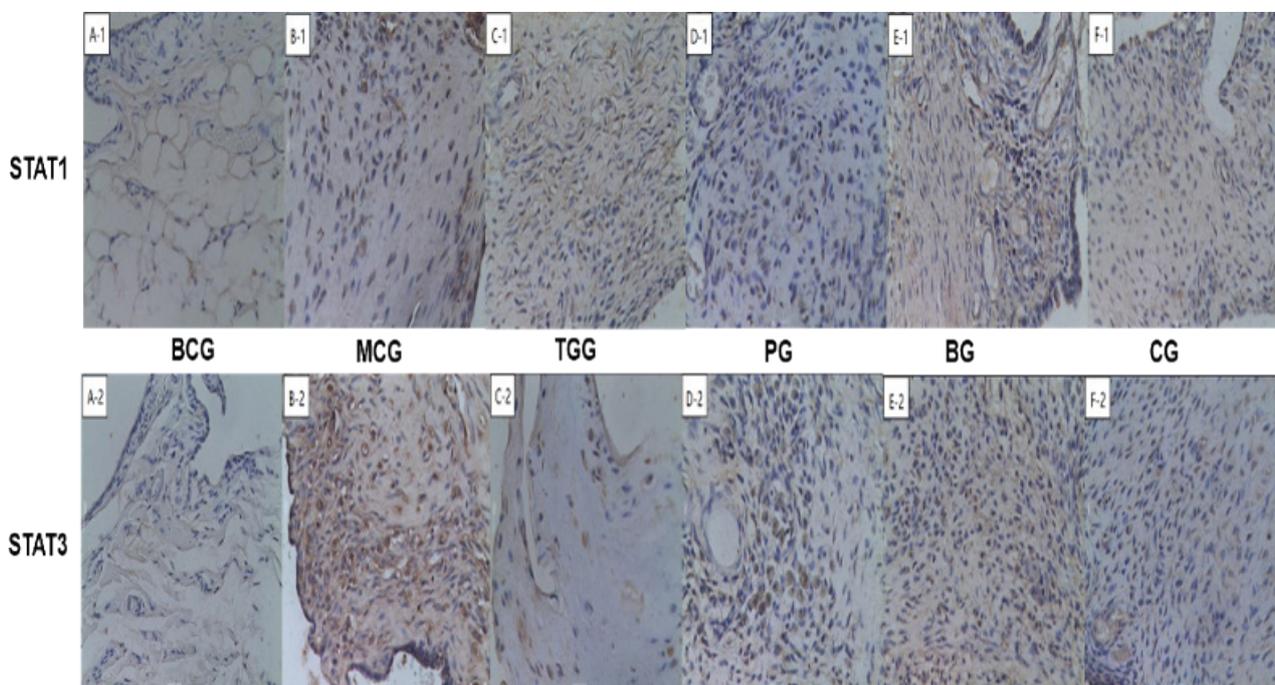
Immunohistochemistry results on the synovial tissue of ankle joint in rats in each group showed that

STAT1 and STAT3 expression in MCG was increased significantly compared with BCG, and the TGG, BG and CG showed a significant decrease compared with MCG (P <0.01 or P <0.05) (Table I and Figure 4), which indicated that a better effect could be reached with combination use of two components.

**TABLE I** - Immunohistochemical score for synovial membrane

Group	STAT1	STAT3
BCG	0.00±0.00	0.00±0.00
MCG	6.00±2.07**	6.38±2.33**
TGG	2.75±1.75##	2.88±1.89##
PG	4.50±2.27	4.60±2.27
BG	3.80±1.48#	3.90±1.79#
CG	3.00±1.63##	2.90±1.73##

\*\*Compared with BCG,  $p < 0.01$ ; #compared with MCG,  $p < 0.05$ ; ##compared with MCG,  $p < 0.01$ .

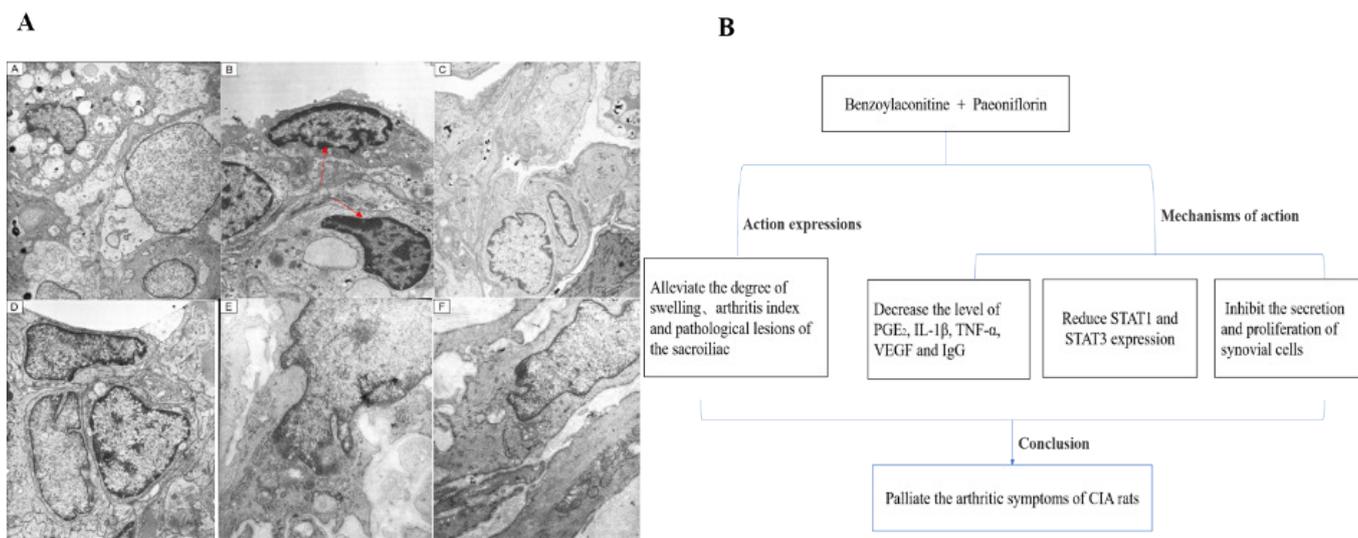


**FIGURE 4** - Effect of benzoylaconitine and paeoniflorin on the expression of STAT1 and STAT3 in synovial tissues of CIA rats by immunohistochemical staining ( $\times 40$ ).

### Effect of benzoylaconitine and paeoniflorin on the ultrastructure of synoviocytes in CIA rats

In BCG, cell morphology was intact, the distribution of chromatin was uniform, and there was no abnormality in the number and structure of the dense body, Golgi apparatus, mitochondria or rough endoplasmic reticulum. In MCG, nuclear heterochromatin collapsed into a shape of high electron density with an uneven distribution; rough endoplasmic reticulum showed a visible expansion with

a disordered arrangement; the number of dense bodies, Golgi apparatus, mitochondria was increased significantly, at meantime, mitochondria swelled partially, and nuclear chromatin condensed. In each treatment group, nuclear heterochromatin shrank slightly; the shape of high electron density was decreased and rough endoplasmic reticulum expanded slightly with a normal arrangement; the number of dense bodies, Golgi bodies, and mitochondria had a decreasing trend compared with MCG. The chromatin distribution in CG was more uniform (Figure 5).



**FIGURE 5** - Ultrastructure of synoviocytes ( $\times 40$ ) and a drug action map A. The effect of benzoylaconitine and paeoniflorin on the ultrastructure of synoviocytes in CIA rats: (A) BCG; (B) MCG; (C) TGG; (D) PG; (E) BG; (F) CG. The red arrow shows the shape of high electron density. B. The therapeutic effects and mechanisms of benzoylaconitine and paeoniflorin were observed in CIA rats.

## DISCUSSION

RA is an autoimmune disease mediated by T cells and is characterized by destructive polyarthritis. In this study, the therapeutic effects of benzoylaconitine and paeoniflorin were observed in CIA rats by successfully replicating the CIA rat model, and better therapeutic effects could be achieved if these two components were used in combination. Briefly, rat paw swelling and AI compatibility were significantly reduced compared with monotherapy; moreover, CG and BG effectively reduced synovial hyperplasia, vasospasm formation, inflammatory cell infiltration, bone destruction and the joint pathology index in CIA rats. In the process of RA, a great number of inflammatory cytokines are released, such as  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-6}$  and  $\text{VEGF}$ , causing synovial inflammation, hyperplasia, cartilage destruction and other pathological changes (Vaillancourt, *et al.*, 2011; Brennan, McInnes, 2008). However,  $\text{IL-10}$ , a protective factor in RA, could inhibit the production of proinflammatory cytokines (Min, *et al.*, 2004). The serum levels of  $\text{IL-1}\beta$ ,  $\text{VEGF}$ ,  $\text{PGE}_2$ ,  $\text{TNF-}\alpha$  and  $\text{IgG}$  could be decreased significantly by BAC and PAE, while there was no influence on  $\text{IL-6}$  and  $\text{IL-10}$ , indicating that these components are a source

of antiangiogenic agents and have no effect on anti-inflammatory cytokines.

The expression of various cytokines in the synovial tissue of RA is regulated by the JAK-STAT signaling pathway (Walker, Smith, 2005). More researchers (Moore, *et al.*, 2001) proposed that  $\text{STAT3}$  could promote the development of RA by inhibiting the apoptosis of synovial fibroblasts. Hence, the pathological changes in synovial cells coincided with a previous report (Yanmiao, *et al.*, 2013) that nuclear heterochromatin wrinkled to a shape of high electron density with uneven distribution and rough endoplasmic reticulum dilated with disordered arrangements. After treatment, synovial damage was improved dramatically. The expression of  $\text{STAT1}$  and  $\text{STAT3}$  was inhibited by BG and CG, which showed that pathological changes in synovial cells were related to the higher expression of  $\text{STAT1}$  and  $\text{STAT3}$  in the synovium of CIA rats.

In conclusion, benzoylaconitine and paeoniflorin can significantly relieve the symptoms of swelling and AI in CIA rats and downregulate the expression of  $\text{IL-1}\beta$ ,  $\text{VEGF}$ ,  $\text{PGE}_2$ ,  $\text{TNF-}\alpha$  and  $\text{IgG}$ , which is achieved by blocking the JAK-STAT signaling pathway. These findings suggested that benzoylaconitine and paeoniflorin could be potent inhibitors for RA treatment. Whether their

compatibility has effects on other signaling pathways or cytokines is still unknown. This study provides some indications for further exploration of the therapeutic effects of benzoylaconitine and paeoniflorin in vitro and in vivo against CIA and other autoimmune diseases.

## ACKNOWLEDGEMENTS

This research was supported by the Project of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (303050305519).

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Received for publication on 24<sup>th</sup> March 2020

Accepted for publication on 05<sup>th</sup> April 2021