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-α 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 al., 2016).
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 benzoylaconitin 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.
Therapeutic effects of benzoylaconitine and paeoniflorin in rats with collagen-induced arthritis

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 ≥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⁻¹; BG, 1.5 mg·kg⁻¹; CG, benzoylaconitine and paeoniflorin were 1.5 mg·kg⁻¹ and 24 mg·kg⁻¹ respectively; TGG, 8 mg·kg⁻¹. 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⁻¹, and CG was administered at a dose of 5 ml·kg⁻¹ 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⁻¹ 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 immnosorbent assay (ELISA) kits for rat TNF-α, IL-1β, 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 μm 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 diamiobenzidine (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 synovicytes. Fixed cells were postfixed 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 ± SD (X±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.
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<0.05). 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 BCG (P<0.01). On d34, TGG 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).
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).

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

The rats in the MCG group showed high levels of IL-1β, IL-6, TNF-α, 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-1β, IL-6, TNF-α, 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-1β and TNF-α levels. Furthermore, a better therapeutic effect could be obtained if benzoylaconitine and paeoniflorin were used cooperatively for IL-1β, VEGF and PGE2.
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.
Effect of benzoylactonine 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).

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 benzoylactonine and paeoniflorin on the expression of STAT1 and STAT3 in synovial tissues of CIA rats by immunohistochemical staining (×40).

Effect of benzoylactonine and paeoniflorin on the ultrastructure of synoviocytes 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 TNF-α, IL-1β, IL-6 and VEGF, causing synovial inflammation, hyperplasia, cartilage destruction and other pathological changes (Vaillancourt, et al., 2011; Brennan, Mcinnes, 2008). However, IL-10, a protective factor in RA, could inhibit the production of proinflammatory cytokines (Min, et al., 2004). The serum levels of IL-1β, VEGF, PGE2, TNF-α and IgG could be decreased significantly by BAC and PAE, while there was no influence on IL-6 and 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 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 STAT1 and STAT3 was inhibited by BG and CG, which showed that pathological changes in synovial cells were related to the higher expression of STAT1 and 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 IL-1β, VEGF, PGE2, TNF-α and 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.

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REFERENCES

Brennan FM, Mcinnes IB. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest. 2008;118(11):3537-45.

Chen Q, Zhang BL. Research Methods for Pharmacodynamics of Traditional Chinese Medicine (M), People’s Medical Publishing House, 1st edition. 2016.

Chen Y, Tong L, Qifu W. Effects of hanshibi oil pathology of ankle tissue in rheumatoid arthritis rats. TCM College of southern Medical University 2010; 12-14.

Chen Y, Wei W, Hong W, Zhang LL, Chen JY. Effects of paeoniflorin on the level of antibodies and cAMP produced by splenocytes in rats with adjuvant arthritis. Acta Pharm Sin. 2007;42(11):1147-1151.

Cho YG, Cho ML, Min SY, Kim HY. Type II collagen autoimmunity in a mouse model of human rheumatoid arthritis. Autoimmun Rev. 2007;7(1):65-70.

Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The global burden of rheumatoid arthritis:estimates from the global burden of disease 2010 study. Ann Rheum Dis. 2014;73(7):1316-1322.

De Roos A, Cooper G, Alavanja M, Sandler D. Rheumatoid arthritis among women in the Agricultural Health Study: risk associated with farming activities and exposures. Ann Epidemiol. 2005;15(10):762-70.

Deng YJ, Lv HY. Active ingredients from Chinese traditional medicine to see the traditional decoction of special medicine. Zhejiang J Integr Tradit Chin West Med. 2001;11:387.

Gao XYi, Wang YW, Xiao LJ, Guo YC, Song HR, Gao YJ. Effects of total saponin from Rhizoma Dioscoreae Nipponicae on the expression of AP-1in synovium of CIA rats. Lishizhen Medicine and Materia Medica Research. 2014;25:1043-1045.

Gong Y, Deng G, Zheng X, Qin J, Luo M, Fang W, et al. Study on the content of alkaloid and the raw of hydrolysis of radix aconite lateralis during decocting process. China Pharmaceuticals. 2017;26(4):9-15.

Gu P, Li JQ, Tong RS. Anti-inflammation and analgesia effects of combination therapy with benzoylaconine and paeoniflorin. Chin J Mod Appl Pharm. 2018;35:1212-1216.

Kallberg H, Padyukov L, Plenge RM, Ronnelid J, Gregersen PK, van der Helm-van Mil AH, et al. Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis. Am J Hum Genet. 2007;80(5):867-75.

Kochi Y, Suzuki A, Yamada R, Yamamoto K. Genetics of rheumatoid arthritis: underlying evidence of ethnic differences. J Autoimmun. 2009;32(3-4):158-162.

Kochi Y, Suzuki A, Yamamoto K. Genetic basis of rheumatoid arthritis: a current review. Biochem Biophys Res Commun. 2014;452(2):254-62.

Li TF, Gong N, Wang YX. Ester hydrolysis differentially reduces aconitine anti-hypersensitivity mediated by spinal microglial dynorphin A expression and neurotoxicity: Implications for the Aconitum processing. Front Pharmacol. 2016;7:367.

Lim L, Gibbins JR. Immunohistochemical and ultrastructural evidence of a modified microvasculature in the giant cell granuloma of the jaws. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1995;79(2):190-8.

Lin L, Chen J, Min B. Comparison of effects of paeoniflorin microemulsion and paeoniflorin in rats with adjuvant arthritis. Acta Universitatis Medicinalis Anhui. 2011;46:240-244.

Mackenzie AR, Dawson J. Could rheumatoid arthritis have an infectious aetiology? Drug Discovery Today Dis Mech. 2005;2(3):345-349.

Mennamee K, Williams R, Seed M. Animal models of rheumatoid arthritis: How informative are they? Eur J Pharmacol. 2015;759:278-86.

Min SY, Hwang SY, Park KS, Lee JS, Lee KE, Kim KW, et al. Induction of IL-10-producing CD4+CD25+ T cells in animal model of collagen-induced arthritis by oral administration of type II collagen. Arthritis Res Ther 2004;6(3):R213-9.

Moore KW, de Waal Malefyt R, Coffman RL, O’Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683-765.

Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2014. J Nat Prod. 2016;79(3):629-661.
Qin Z, Zhou H, Xiong W, Jia W, Hu P, Ming Y. Effects of paeoniflorin on aconitine transport behavior in Caco-2 cell model. World Chinese Med. 2015;10:322-326.

Symmons DP. Environmental factors and the outcome of rheumatoid arthritis. Best Practi Res Clin Rheumatol. 2003;17(5):717-727.

Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. J Exp Med. 1977;146(3):857-68.

Vaillancourt F, Silva P, Shi Q, Fahmi H, Fernandes JC, Benderdour M. Elucidation of molecular mechanisms underlying the protective effects of thymoquinone against rheumatoid arthritis. J Cell Biochem. 2011;112(1):107-17.

Vk V, Hallikeri K, Girish HC, Murgod S. Expression of CD34 and CD68 in peripheral giant cell granuloma and central giant cell granuloma: An immunohistochemical analysis. J Oral Maxillofac Pathol. 2014;18(3):341-8.

Walker JG, Smith MD. The Jak-STAT pathway in rheumatoid arthritis. J Rheumatol. 2005;32(9):1650-1653.

Yang J. The empirical study on the effects of resveratrol on CIA rats and its impact on the role of JAK-STAT signal transduction pathway. Tangshan: North China Coal Medical College; 2009.

Yanmiao MA, Wang Y, Yanyan LI, Gao L, Zhou R. Effects of fengshining capsule on the ultrastructure of synovial cells and the expressions of apoptosis-related genes in arthritic rats induced by collagen. J Trad Chin Med. 2013;(4):326-328.

Zhang S, Li JQ. Research progress on cardiotoxicity mechanism of Aconitine. Chin J Integr Med Cardio-/Cerebrovasc Dis. 2018;16:1366-1370.

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