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^{1}H NMR-based metabolomics approach to investigate the urine samples of collagen-induced arthritis rats and the intervention of tetrandrine

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ABSTRACT

Tetrandrine is an effective ingredient isolated from the roots of a frequently used medicinal plant *Stephania tetrandra* S. Moore. It has been used for the management of arthritis in China, but the precise mechanism remains unclear. In the present study, a metabolomics method based on the ^{1}H NMR was constituted to quantify the alterations of the endogenous metabolites in the urines of collagen-induced arthritis (CIA) rats treated with tetrandrine. Data showed that tetrandrine treatment could alleviate the ankle joint swelling and ameliorate histopathological changes in rats. The metabonomic analysis indicated that 23 potential biomarkers in urine were affiliated with CIA. They mainly participated in energy metabolism, amino acid metabolism, lipid metabolism and gut microbe metabolism. Moreover, our results implied that tetrandrine could reverse the pathological process of CIA through adjusting the unbalanced metabolic pathways. Thus, these metabolic pathways and potential biomarkers might be the potential therapeutic targets of tetrandrine, and these findings supplied new visions into the protective effect of tetrandrine against arthritis in rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with distinct signatures of persistent synovitis and hyperplasia, and followed by cartilage and bone destruction. It affects different parts of the body, and results in multi-organ disorders inevitably. RA patients suffer from probable disability, reduced life expectancy, and risen risk of other diseases [1–3]. What's more, RA is one of auto-immune diseases for which predominate in females, which female are three times more affected than men [4], it affects about 1% of the global population [1,5]. However, the pathogenesis of RA is multi-factorial and to a large degree, remains unknown.

At present, the disease modifying anti-rheumatic drugs (DMARD), glucocorticoids (GC) and biological agents are widely used for the treatment of RA. However, their clinical use has been limited due to the high costs and adverse effects with a high frequency [6]. Therefore, it is necessary to find novel candidate agents for the treatment of RA. Traditional Chinese medicine (TCM) has been used in China for centuries and has shown efficacy in RA treatment [7]. In recent years, a lot of researches have shown that some TCMs and their functional ingredients have observable therapeutic effects on RA. And most key effective ingredients in TCM that can treat RA belong to alkaloids, flavonoids, phenols, terpenoids and quinines.

Tetrandrine, a bis-benzylisoquinoline alkaloid that is isolated from the roots of a frequently used herb medicine *Stephania tetrandra* S. Moore, has been used to treat patients with arthritis in China for many years [6,8]. Tetrandrine, reported as a potent immunomodulator used to treat rheumatic disorders, could prevent the degradation of IκB-α and inhibit nuclear translocation of p65 by blocking IκB-α kinases α and β activities [9]. Recent research indicated that tetrandrine ameliorates CIA in mice by restoring the balance between Th17 and Treg cells via the aryl hydrocarbon receptor, and inhibits migration and invasion of RA fibroblast-like synoviocytes through down-regulating the expressions of Rac1, Cdc42, and RhoA GTPases and activation of the PI3K/Akt and JNK signaling pathways [10,11]. Its chemical structure was shown in Fig. 1. As an important functional ingredient from nature-derived drugs, tetrandrine has been demonstrated to attenuate adjuvant-induced arthritis in rats [12].

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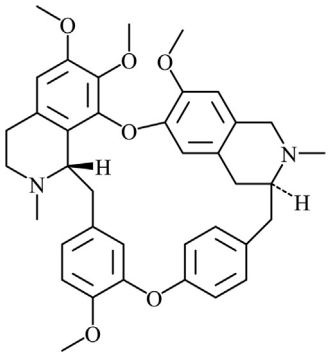

Fig 1. The chemical structural of tetrandrine.

Metabolomics is an important technique using NMR and MS to analyze metabolites or tissue extracts for detection of biomarkers and biochemical effects induced by a disease or its therapeutic intervention [13]. The disturbances caused by a disease in a biological system are usually accompanied by the changes in the concentrations of relevant metabolites. The research of metabolomics is based on these changes. In some diseases, it is probably possible to identify a single diagnostic metabolite. But most metabolic diseases (including RA) involve the metabolism of multiple pathways [14]. Multivariate statistics is used on experimental spectra obtained by NMR or MS, which possibly describes the changes of spectra biomarkers that are highly discriminatory for diseases [15]. Among these approach, NMR analysis is nondiscriminating, nondestructive, and highly reproducible and requires minimal sample preparation. NMR is advantageous in providing detailed structural information on metabolites. Based on the above, the changes of the metabolic profiles in RA model rats and tetrandrine-treated rats were investigated using ^{1}H NMR-based metabonomic approaches in this study. The results may provide potential biomarkers that can improve diagnostic accuracy, and predict therapeutic efficacy in RA.

2. Materials and methods

2.1. Chemicals and reagents

Tetrandrine with a purity of 98% was purchased from Jingzhu Bio-Technology Co., Ltd. (Nanjing, China). 3-(Tri-methyl-silyl) propionic-2, 2, 3, 3-d4 acid sodium salt (TSP, 89%) was provided by Cambridge Isotope Laboratories. D_2O (99.9%) was bought from Sigma-Aldrich USA. Other chemicals and agents are commercially available.

2.2. Animals and treatment

Female rats (140–160 g) were obtained from the Comparative Medical Centre of Yangzhou University (Yangzhou, China). Freund's incomplete adjuvant (Becton Dickinson, Franklin Lakes, USA) (1.5 mg/mL) and chicken type II collagen (CII, Sigma-Aldrich, Saint Louis, USA) (4 mg/mL) were mixed with an equal volume, then an emulsion was prepared on ice and kept at 4 °C of short-term preservation. On the first day, the CII emulsion (0.2 mL) was intradermally injected at the base of the tail of rats under slight anaesthesia with ether. On the seventh day, a booster immunization was managed with 0.1 mL of emulsion at the base of the tail of rats. On the fourteenth day, the 20 CIA rats were randomly divided into two groups, the model group (M) and treatment group (T). Other 10 healthy rats were chosen as the normal group (N). Only females were used due to higher disease susceptibility [16,17]. The pathogenesis of CIA model shares several pathological features with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and, like RA, susceptibility is linked to the expression of specific MHC class II genes. These pathological features have enabled researchers to study a wide range of pathogenic mechanisms in this model. Collectively, CIA has been the most commonly studied autoimmune model of RA.

The studies were approved by the animal ethics committee of the China Pharmaceutical University, and were in conformity with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

2.3. Sample and collection

Three groups of rats were housed under controlled conditions of temperature (22 ± 2 °C) and relative humidity (50 ± 20%) with a 12 h light/12 h dark cycle. Water and a nutritionally adequate diet were provided *ad libitum*. In treatment group, the rats orally received 40 mg/kg of tetrandrine suspended in 0.5% CMC-Na for 7 days. Normal and model groups of rats were concurrently subjected to oral gavage with only 0.5% CMC-Na. The 24 h urine of each rat was collected starting from 0 h after the first and seventh gavage, respectively. The urine samples were collected on wet ice on every two hours and centrifuged (4 °C, 12000 rpm, 10 min) immediately, and the supernatants were stored frozen in 2 mL PE tube and kept in −80 °C until analysis. It's worth noting that the urine samples should be collected on ice and rats were fasted overnight with free access to water before experiments.

2.4. Histopathological examination

After the collection of samples, all rats were sacrificed by decapitation. Their joints were fixed in 10% buffered phosphate formalin for at least 1 week, before being subjected to acid decalcification. Then, the joints were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for two weeks at 4 °C, and were dehydrated by processing in different grades of alcohol, chloroform mixture and embedded in paraffin wax. Serial paraffin sections (5 mm) were stained with hematoxylin and eosin (HE). The injury of synovial tissues, articular bone and cartilage were then analyzed under the light microscope. The histopathological alterations were observed in regard of diminished joint space, pannus formation, inflammation, and infiltration of inflammatory cells in the ankle joints.

2.5. Sample preparation and ^{1}H NMR spectroscopy

Preparation of the samples for metabolomic analysis by ^{1}H NMR was performed as described previously [18]. After defrosting at room temperature, 400 μL of urine was mixed with 200 μL of phosphate buffer (0.2 mol/L Na_2HPO_4, 0.2 mol/L NaH_2PO_4 in 99.9% D_2O, pH = 7.4) and then centrifuged at 12000 rpm for 10 min at 4 °C. After that, 450 μL of the supernatant was added to a NMR tube which contained 80 μL internal standard solution (TSP, 0.1% w/v, in 99.9% D_2O). The TSP acted as a chemical shift reference (δ 0.0) and the D_2O provided a lock signal. ^{1}H NMR spectra of the urine samples were recorded on a BRUKER AVANCE III–500 MHz NMR spectrometer. The following parameters: controlling temperature of 299 K, spectral width of 10 KHz, data points of 64 K.

2.6. Data processing and analysis

The data were Fourier transformed, the ^{1}H NMR spectra of the samples were than processed using MestReNova 6.1 software with an exponential weighting function corresponding to a line-broadening of 1.0 Hz in order to improve the signal to noise ratio. And all raw spectra were baseline corrected and referenced