Journal of Chromatography B, 1038 (2016) 118–126

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

^{1}H NMR-based metabonomic study on the effects of Epimedium on glucocorticoid-induced osteoporosis

Sina Pan[a,b], Ali Chen[c], Zhihui Han[a,b], Yaling Wang[a,b], Xin Lu[a], Yongxia Yang[a,*]

[a] School of Basic Courses, Guangdong Pharmaceutical University, Guangzhou, 510006, PR China
[b] Department of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, 510006, PR China
[c] School of Medicine and Chemical Industry, Guangdong Pharmaceutical University, Guangzhou, 510006, PR China

ARTICLE INFO

Article history:
Received 9 June 2016
Received in revised form 12 October 2016
Accepted 14 October 2016
Available online 14 October 2016

Keywords:
Metabonomics
Osteoporosis
Epimedium
^{1}H NMR
Multivariate data analysis

ABSTRACT

Glucocorticoids are widely used in clinical practice for the treatment of many immune-mediated and inflammatory diseases, and glucocorticoid-induced osteoporosis (GIO) is the most common type of secondary osteoporosis. Epimedium is one of the most commonly used traditional Chinese medicines for treating osteoporosis. In the present study, we systematically analysed the metabonomic characteristics of GIO model rats and elucidated the therapeutic effect of Epimedium by using a ^{1}H NMR-based metabonomic approach in conjunction with multivariate data analysis. Rats in treatment and model groups were injected with dexamethasone (0.1 mg/kg/day) for 5 weeks. Simultaneously, two treatment groups were orally administered Epimedium (10 g/kg/day) or Alendronate (1.2 mg/kg/day) for 5 weeks. In GIO model rats, lipid and lactate levels in serum were increased, while creatine/creatinine, PC/GPC, taurine, glycine and β-glucose levels were decreased. In urine, GIO rats had higher levels of phenylacetylglycine but lower levels of 2-oxoglutarate, citrate, creatine/creatinine, taurine, PC/GPC and hippurate than controls. Epimedium reversed the aforementioned metabolic alterations in multiple metabolic pathways involved in energy, lipid, amino acid and phospholipid metabolism and gut microbiota derangement. Our results indicated that Epimedium had significant effects in the prevention and treatment of osteoporosis. It is concluded that ^{1}H NMR metabonomics is a useful method for studying the metabolic effects of traditional Chinese medicine from a systematic and holistic view.

1. Introduction

Osteoporosis is a systematic metabolic bone disease characterized by low bone mass and microarchitectural deterioration of bone tissue, which leads to increased bone fragility and risk of fracture. Glucocorticoids have been widely used for treatment of organ transplantation, self-immunity diseases and kidney diseases in clinical practice. However, glucocorticoid-induced osteoporosis (GIO) has been confirmed to be the most frequently occurring type of secondary osteoporosis, and it has become a serious threat to public health. It is estimated that 30–50% of patients using glucocorticoids long-term (over 6 months) will develop osteoporosis [1]. After treatment with glucocorticoids for 3 months, the risk of fracture increases by 75% [2]. Therefore, prevention and treatment of GIO have become hot topics.

The effect of glucocorticoids on bone involves a complex pathological process, which can inhibit formation and differentiation of osteoblasts [3,4] and promote bone and bone cell apoptosis [5,6]. Moreover, glucocorticoids also affect the expression of insulin-like growth factor (IGF) I and suppress the release of gonadotropins [7,8]. Currently, the main treatments for GIO include supplementation with calcium, active vitamin D, bisphosphonates and hormone replacement therapy. However, there are some potential side effects, such as gastroesophageal irritation [9] and osteonecrosis of the jaw [10]. Therefore, looking for new therapeutic alternatives has become important with respect to treatment of GIO.

Traditional Chinese medicine has been applied for treatment of osteoporosis. It has been reported that Du-Zhong prevents bone loss and deterioration of bone trabecular structure in an ovariectomy-induced osteoporosis model [11]. Ginkgo biloba extract has been estimated to improve bone formation and increase bone mineral content in the proximal tibial epiphysis in GIO rats [12]. Epimedium (*Berberidaceae*) is one of the most commonly used Chinese herbal medicines for treating osteoporosis. It has been confirmed that Epimedium extract promotes proliferation of osteoblast-like UMR106 cells and increases osteoblastic activity [13,14]. Icariin was found to increase bone mass and prevent glucocorticoid-induced apoptosis in osteocytes [15]. In addition,

* Corresponding author.
E-mail address: sheepma@163.com (Y. Yang).

http://dx.doi.org/10.1016/j.jchromb.2016.10.015
1570-0232/

the total flavonoids of Epimedium can regulate the formation and activity of osteoclasts in vitro [16] and improve bone properties in ovariectomized mice [17]. However, the molecular mechanism of Epimedium in the treatment of GIO remains unclear, and it should be further analysed from a holistic perspective.

Metabonomics, an important component of systems biology, has been a good method for monitoring the holistic metabolic alterations caused by external and intrinsic factors. Currently, the main analytical techniques of metabonomics include nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC/MS) and gas chromatography-mass spectrometry (GC/MS). NMR analysis is an effective non-destructive method of detecting metabolic responses throughout entire organisms. Traditional Chinese medicine, characterized by "multi-component and multi-target", is consistent with the "integrity and systematization" of metabonomics. Therefore, metabonomics has been widely used to study the mechanisms of traditional Chinese medicine [18].

In this report, we established a GIO model by administering subcutaneous injections of dexamethasone, following a previously reported modelling method [3]. We adopted the NMR-based metabonomic approach to study the metabolic profile of the GIO model, evaluate the effect of Epimedium on GIO and further elucidate the metabolic regulation of this process.

2. Materials and methods

2.1. Preparation of Epimedium

Epimedium was purchased from Zhongzheng Chinese Herbal Pieces Co., LTD (Guangzhou, China) and identified by associate Prof. Suying Tian (Experiment Centre of Guangdong Pharmaceutical University). Epimedium (100 g) was extracted with distilled water (1200 mL) decoction for 30 min and then filtered. The extraction was repeated three times. The extracted solution was concentrated in a final volume 50 mL. The Epimedium extract was prepared at a concentration of 2 g/mL (expressed as the weight of raw materials) and then stored at 4 °C.

2.2. Animal experiments

Animal experiments were reviewed and approved by the Ethics Committee of Guangdong Pharmaceutical University. Forty male Sprague-Dawley (SD) rats (230–250 g, animal licence No.SCXK2013-0020) were purchased from the Laboratory Animal Institute of Guangzhou University of Chinese Medicine. These 40 rats were housed under standard animal conditions with a 12 h/12 h light/dark cycle, regulated temperature of 25 ± 1 °C and humidity of 50 ± 10%. The animals were allowed to have free access to food and pure water throughout the study period. After acclimatization for 10 days, the rats were randomly assigned to four groups with ten rats in each group: a control group, a GIO model group, and Epimedium and Alendronate (as positive control drug) treatment groups. The modelling method was in accordance with that of a previous publication [3]. All groups were subjected to subcutaneous injections of dexamethasone (0.1 mg/kg/day) for 5 weeks, while the control group received the same volume of saline. Simultaneously, Epimedium rats were also gavaged with extract of Epimedium at a dose of 10 g/kg/day for 5 weeks [19]; control and GIO rats received the same volume of distilled water. Another treatment group was gavaged with Alendronate (1.2 mg/kg/day) during the experimental period. The weights of the animals were measured weekly. The dosage was adjusted per week according to weight.

2.3. Sample collection

For each group, blood samples were collected from the orbital venous plexus at week 0 (before injections of dexamethasone), and the 3rd and 5th weeks. The blood samples were allowed to stand at 37 °C for 30 min to coagulate and then centrifuged at 5000 rpm for 10 min to collect serum. The serum samples were used for biochemical analysis and metabolite determination. For each group, urine samples were collected with metabolic cages at the end of weeks 0, 3 and 5. The collection container was supplemented with 1% sodium azide (50 μL) before urine collection. All rats were sacrificed by cervical dislocation at the end of the 5th week. Femurs were removed from each animal for bone mineral density (BMD) analysis. All samples were then stored in a −80 °C freezer for later analysis.

2.4. Biochemical analysis

Serum samples were analysed for alkaline phosphatase (ALP) levels using an automatic clinical chemistry analyzer (Beckman, Los Angeles, CA, USA). The levels of carboxy-terminal telopeptide of type I collagen (CTX) were measured with an enzyme-linked immunosorbent assay (ELISA) kit, which was purchased from Huijia Biotechnology Co., LTD (Xiamen, China).

2.5. BMD analysis

The entire femur BMD was measured using dual-energy X-ray diagnosis absorptiometry (Discovery-A, Holigic Co., USA). At the same time, the BMD of all rats was obtained using a Subregion Hi-Res software program.

2.6. Sample preparation for NMR spectroscopy

Serum and urine samples were thawed at room temperature and then prepared by mixing 300 μL of each sample with 150 μL PBS (0.2 M Na_2HPO_4/NaH_2PO_4, pH = 7.4). The supernatants were pipetted into NMR analysis tubes after centrifuging (5000 rpm, 10 min, 4 °C). 80 μL D_2O containing 0.05% TSP as internal standard for chemical shift reference (δ 0.00) was also added to each tube for the deuterium lock.

2.7. Data acquisition of NMR

The ^1H NMR spectra of all samples were recorded on a Bruker spectrometer (Avance III 500 MHz). The probe temperature was 298 K. For serum samples, the pulse sequence was one-dimensional Carr-Purcell-Meiboom-Gill (CPMG) (relaxation delay-90°-(τ-180°-τ)$_n$-acquisition) with water suppression in order to obtain the signals with low molecular weight selectively. A total of 128 scans were collected into 32 k data points and spectral width was 10 kHz, with relaxation delay of 3 s and the total echo time (2nτ) of 100 ms. Before Fourier transformation, the exponential function corresponding to a line broadening factor of 0.3 Hz was applied to all acquired free induction decays (FIDs). The ^1H NMR spectrum was collected for each urine sample using the water-presaturated standard one-dimensional NOESYPR1D pulse sequence (recycle delay-90°-t_1-90°-t_m-90°-acquisition). 128 transients were collected into 32 k data points and spectral width was 10 kHz with a relaxation delay of 3 s and a mixing time (t_m) of 100 ms. A series of two-dimensional NMR (2D NMR) spectra including ^1H-^1H correlation spectroscopy (COSY), ^1H-^1H total correlation spectroscopy (TOCSY) and ^1H-^{13}C hetero nuclear single quantum correlation (HSQC) were acquired for the purpose of signal assignments.