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Extracts from plastrum testudinis reverse glucocorticoid-induced spinal osteoporosis of rats via targeting osteoblastic and osteoclastic markers

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ABSTRACT

Extracts from plastrum testudinis (PTE), an important traditional Chinese medicine, have been demonstrated promotion of osteoblastic function in vitro. This study aims to investigate the protective effect of PTE on glucocorticoid-induced osteoporosis(GIOP) in vivo and analyze therapeutic targets of PTE on GIOP. SD rats were randomly assigned to two experiments: preventive and therapeutic experiments, in which rats respectively received oral PTE at the same time of glucocorticoid injection or after glucocorticoid injection inducing osteoporosis. BMD, microarchitecture, biomechanics, bone metabolism markers and histomorphology were evaluated. mRNA and protein expression of OPG, Runx2, CTSK and MMP9 were examined.Results showed bone quality and bone quantity were significantly elevated by PTE. Histomorphometry showed thicker and denser bone trabecularsand more osteoblasts and less osteoclasts in group of PTE intervention. The mRNA expression of OPG was significantly upregulated whereas expression of CTSK was significantly downregulatedin different groups of PTE intervention. Stronger immunostaining for Runx2 and weaker immunostaining for CTSK were observed in groups of PTE intervention. This demonstrated that PTE may reverse GIOP in prevention and management via targeting OPG, Runx2 and CTSK in mRNA and protein levels.

1. Introduction

Glucocorticoid-induced osteoporosis (GIOP), recognized as the most frequent secondary osteoporosis, with resultant fractures, leads to significant pain and disability and even reduces life expectancy [1]. Surveys estimated that 50% patients treated with glucocorticoids would suffer from an osteoporotic fracture which often occurs in spine and hip [2,3]. To improve quality of life, a variety of pharmacological treatments, including calcium supplementation, bisphosphonates, anabolic steroids, vitamin D and so on have been recommended for preventing risk of associated osteoporotic fractures [1,4], but GIOP remains clinically undertreated due to the inadequacies and side effects of the current management [5]. Therefore, new agents against GIOP still need to develop.

Plastrum testudinis (PT) is an important traditional Chinese medicine often used clinically to treat bone diseases in China. The previous study has acquired the extracts from plastrum testudinis (PTE) and identified its chemical component including Palmitic Acid Methyl Ester, Ethyl Palmitate, Methyl Stearate, Stearic Acid Ethyl Ester, Palmitic Acid, Stearic Acid, Cholesterol, Cholesterol Myristate and (+)4-cholesten-3-one,and then also have demonstrated that PTE could promote proliferation of MSCs [6]. However, study in vivo about PTE preventing bone loss in GIOP is still not performed. Although many active components of Chinese traditional medicines have been reported for candidate Pharmaceuticals of GIOP, most of studies in fact only focus on prevention of GIOP, but not treatment [7–9].

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To illuminate the preventive and therapeutic effect of PTE on glucocorticoid-induced osteoporosis in detail, this study was grouped into two experiments in which rats respectively received oral PTE at the same time of glucocorticoid injection or after glucocorticoid injections inducing osteoporosis. Bone mass, microarchitecture, biomechanics, bone metabolism and histomorphology were evaluated for the indicators of preventive and therapeutic effect. mRNA and protein expression levels of osteoprotegerin (OPG), Runx2, Cathepsin K (CTSK) and matrix metalloproteinase 9 (MMP9) were examined to identify the potential factor to the action mechanism of PTE on GIOP.

2. Materials and methods

2.1. Preparation of PT extracted with ethyl acetate

Extracts from Plastrum testudinis (PTE) were obtained according to a method established previously [6]. Briefly, Plastrum testudinis purchased from the first affiliated hospital of Guangzhou University of Chinese Medicine (Guangzhou, China) was extracted stepwise with solvent of ethyl acetate and water, and then solvents were recovered to obtain ethyl acetate (PTE) which was dissolved in dimethyl sulphoxide. Gas chromatography-mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) were performed for determining steroids, fatty acids and their ester components. This extract contained nine chemical compounds including Palmitic Acid Methyl Ester (PubChem CID: 8181), Ethyl Palmitate (PubChem CID: 12366), Methyl Stearate (PubChem CID: 8201), Stearic Acid Ethyl Ester (PubChem CID: 8122), Palmitic Acid (PubChem CID: 985), Stearic Acid (PubChem CID: 5281), Cholesterol (PubChem CID: 5997), Cholesterol Myristate (PubChem CID: 99486), (+)4-cholesten-3-one (PubChem CID: 91477).

2.2. Animals

Four-month-old female Sprague–Dawley rats (n = 48) were purchased from Guangzhou University of Chinese Medicine and all experiments performed were approved by the ethics committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (license no., 20130425). Rats were reared and kept in standard conditions and facilities which comply with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health. Food and water were available freely throughout the experiment.

Following an acclimation period of 1 week, rats were randomly assigned to two experiments: preventive and therapeutic experiments. In every experiment rats were randomly divided into three groups. In preventive experiment, grouping was as follows: (1) sham group (P-SHAM), which received vehicle intervention for eliminating system bias and interference factor. (2) Dexamethasone group (P-DEXA), received a sub-percutaneous injection of dexamethasone at a dose of 0.6 mg/kg body weight, twice per week for three months. (3) Extracts from plastrum testudinis group (P-PT), which received oral PTE at a dose of 30 mg/kg, once a day during injection of dexamethasone similar to P-DEXA group. The dose selection of PTE was based on previous studies [6]. In therapeutic experiment, grouping was as follows: (1) sham group (T-SHAM), which is similar to P-SHAM. (2) Dexamethasone group (T-DEXA), which received vehicle intervention after injection of dexamethasone at a dose of 0.6 mg/kg body weight, once a day for three months. (3) Extracts from plastrum testudinis group (T-PT), which were treated with PTE similar to P-PT after injections of dexamethasone inducing osteoporosis.

2.3. Sample preparation

In preventive experiment, rats were euthanized for experimental analysis at the end of three months of dexamethasone injection, while rats in therapeutic experiment were euthanized for experimental analysis at the end of six months (including three months of dexamethasone injection and three months of PTE treatment). Lumbar vertebrae devoid of soft tissues were isolated. Lumbar vertebrae 6 (L6) samples were preserved at −80 °C for mRNA expression analyses. En bloc lumbar vertebrae 1–3 (L1–3) samples were stored at −20 °C for dual-energy X-ray absorptiometry. Lumbar vertebrae 2 (L2) samples were isolated for micro-computed tomography (microCT) and biomechanical analysis. Lumbar vertebrae 4 (L4) samples were fixed in 4% phosphate-buffered paraformaldehyde for HE stain and Immunohistochemistry. Blood samples were collected and stored at −20 °C prior to assessment of bone metabolism markers, including serum β-CTX and serum PINP.

2.4. Dual-energy X-ray absorptiometry detection

According to a previous method [10], en bloc L1–3 samples were scanned by dual-energy X-ray absorptiometry with a small-animal high-resolution collimator (Discovery A/SL/W/C; Hologic, Bedford, MA) for detecting bone mineral content (BMC, g), bone mineral density (BMD, g/cm^2) and bone area (AREA, cm^2). The regions of interest (ROI) were marked across the entire L1–3 region. Analysis was performed using the small animal mode of the software supplied with the collimator (v. 13.2:3; Hologic) and was calibrated at each start of the experiment.

2.5. Bone microarchitecture

A cone beam-type desktop micro-CT system (μCT80; Scanco Medical, Brüttisellen, Zurich, Switzerland) with the supplied software (μCT80 Evaluation Program v. 6.5-1; Scanco Medical) was used to quantify the structural parameters of L2 vertebraes. The analytical conditions were 55 kvp with 80 μA, and the spatial resolution was 14 μm in all directions. The cancellous bone of the vertebrae was chosen as the volume of interest (VOI), which was restricted to an internal region of the vertebrae where trabecular and cortical bones were extracted by drawing cylinder contours (diameter 2 mm) with the CT analyzer software. Microstructure of cancellous bone was characterized using standardized techniques to determinethe relative bone volume (BV/TV, %), structural model index (SMI), trabecular number (Tb.N, 1/mm), tubercular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), volume bone mineral density (vBMD, mg HA/ccm). The reconstructed three-dimensional (3D) images were obtained through multi-planar reformation.

2.6. Compressive test for bone biomechanics

After micro-CT analysis, L2 vertebrae were subjected to compression testing to determine the mechanical parameters with a materials testing machine (ElectroPuls E1000 test system; Instron Corp., Norwood, MA). Both end plates of the vertebrae body and its appendix were removed for a central cylinder with plano-parallel ends and height of approximately 5 mm. The individual vertebrae were then tested along the longitudinal axis in the materials testing machine at a constant compression speed of 1 mm/min. After the compression test, the load-displacement curve was plotted using the supplied software (Bluehill 3; Instron Corp.) to analyze the compressive stiffness (in N/mm), compressive