Journal of Ethnopharmacology 201 (2017) 91–99

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Effects of the combined Herba Epimedii and Fructus Ligustri Lucidi on bone turnover and TGF-β1/Smads pathway in GIOP rats

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ARTICLE INFO

Chemical compounds studied in this article:
Icariin (PubChem CID: 5318997)
Oleanolic Acid (PubChem CID: 10494)
Rutin (PubChem CID: 5280805)

Keywords:
Herba Epimedii/Fructus Ligustri Lucidi
Active fractions
Glucocorticoid-induced osteoporosis
TGF-β1/Smads
Bone formation
Bone resorption

ABSTRACT

Ethnopharmacological relevance: Kidney deficiency is the main pathogenesis of osteoporosis based on the theory of "kidney governing bones" in traditional Chinese medicine (TCM). Combined Herba Epimedii and Fructus Ligustri Lucidi, based on traditional Chinese formula Er-Zhi pills, were frequently used in TCM formulas that were prescribed for kidney tonifying and bone strengthening. However, it is unclear whether the combination of the two herbs may have a protective influence on glucocorticoid-induced osteoporosis (GIOP). The objective of this study was to evaluate the therapeutic effects and the underlying molecular mechanism of the decoction and the active fractions of the combined herbs in GIOP rats.

Materials and methods: Male Sprague-Dawley rats were divided into seven groups, including the normal control (NC), GIOP model (MO), active fractions low (100 mg/kg, LAF), active fractions high (200 mg/kg, HAF), decoction low (3.5 g/kg, LD), decoction high (7 g/kg, HD) and Calcium with Vitamin D3 (0.2773 g/kg, CaD)-treated group. The GIOP model was established by intramuscular injection of dexamethasone (1 mg/kg) twice a week for 8 weeks. Different kinds of indicators were measured, including bone mineral density (BMD), bone biomechanical properties, serum bone alkaline phosphatase (b-ALP), serum bone γ-carboxyglutamic acid-containing protein (BGP), serum bone morphogenetic protein-2 (BMP-2), serum tartrate-resistant acid phosphatase (TRACP) and serum carboxy terminal cross linked telopeptide of typeIcollagen (ICTP), bone mineral content (BMC) and bone structured histomorphometry. The protein and mRNA expression of TGF-β1, Smad2, Smad3, Smad4 and Smad7 were detected by Western blotting (WB) and quantitative real time polymerase chain reaction (qRT-PCR), respectively.

Results: Administration of combined Herba Epimedii and Fructus Ligustri Lucidi decoction and combined active fractions could significantly prevent GC-induced bone loss by increasing the contents of serum b-ALP, BGP and BMP-2 as the markers of bone formation, reducing the serum TRACP and ICTP contents to inhibit bone resorption and enhancing BMC. They could also attenuate biomechanical properties and BMD reduction, deterioration of trabecular architecture in MO rats. The mRNA and protein expressions of TGF-β1, smad2, smad3 and smad4 were up-regulated, and the mRNA and protein expression of Smad7 was down-regulated following combined Herba Epimedii and Fructus Ligustri Lucidi treatment.

Conclusion: Combination of Herba Epimedii and Fructus Ligustri Lucidi exhibited protective effects on promoting bone formation and precluding bone resorption. The underlying mechanism may be attributed to its regulations on TGF-β1/Smads pathway. The substance bases of the combined herbs on anti-osteoporosis were total flavonoids of Herba Epimedii, total iridoids and flavonoids of Fructus Ligustri Lucidi.

Abbreviations: TCM, traditional Chinese medicine; GIOP, glucocorticoid-induced osteoporosis; GC, glucocorticoid; qRT-PCR, quantitative real-time polymerase chain reaction; WB, western blotting; HE, hematoxylin-eosin; i.p., intraperitoneally; NC, normal control; MO, GIOP model; DEX, Dexamethasone; BMD, bone mineral density; BMC, bone mineral content; b-ALP, bone alkaline phosphatase; BGP, bone γ- carboxyglutamic acid-containing protein; BMP-2, bone morphogenetic protein-2; ICTP, carboxy terminal cross linked telopeptide of typeIcollagen; TRACP, tartrate-resistant acid phosphatase; HETF, total flavonoids of Herba Epimedii; FLLTIF, total iridoids and flavonoids of Fructus Ligustri Lucidi; Tb.Ar, trabecular bone area; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TGF-β, transforming growth factor-beta

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http://dx.doi.org/10.1016/j.jep.2017.02.033
Received 17 October 2016; Received in revised form 6 February 2017; Accepted 13 February 2017
Available online 27 February 2017
0378-8741/

1. Introduction

Glucocorticoids (GCs) are widely used in clinical for their exceptional anti-inflammatory and immunomodulatory effects in numerous diseases including rheumatic, pulmonary, gastrointestinal and autoimmune diseases (Buttgereit et al., 2011; Rizzoli et al., 2012; Seibel et al., 2013). However, long-term high-dose administration of GCs leads to serious adverse effects. Osteoporosis is among the most devastating side effects of GCs therapy, which is characterized by rapid loss of bone mass, significant decrease of bone strength and increase of fracture risk (Whittier and Saag, 2016). Glucocorticoid-induced osteoporosis (GIOP) is recognized as the most common iatrogenic cause of secondary osteoporosis, and is now third in frequency after postmenopausal and senile osteoporosis (Rizzoli et al., 2012). Surveys estimated that 50% patients would suffer from an osteoporotic fracture in glucocorticoids therapy (Weinstein, 2011). Early intervention with bone protective therapy is important in individuals receiving chronic glucocorticoid therapy (Grossman et al., 2010). The clinical management of GIOP relies on medications similar to those used for treatment of post-menopausal osteoporosis, such as calcium, vitamin D, bisphosphonates, raloxifene, and PTH. But their side effects often severely limit the long-term usage of these drugs, such as gastrointestinal side effects, osteonecrosis of the jaw, musculoskeletal discomfort, hot flushes, increased blood pressure, renal calculi (Rossini et al., 2016). Therefore, novel and efficient agents for the treatment of GIOP are needed.

According to TCM theories, kidney governs bones, which means that kidney plays an important role in growth and formation of bones. Osteoporosis, also named as “bone atrophy”, is related to kidney deficiency (An et al., 2016). Thus, the treatment of osteoporosis follows the basic rules of strengthening the kidney function. Er-Zhi pills, which was described in “Fu Shou Jing Fang” in the year 1530, used for enriching the functions of liver and kidney, and nourishing marrow and essence (Cui et al., 2011). It has been used to strengthen skeleton and treat osteoporosis for thousands of years in China (QY, 2009; Yu et al., 2011). The combination of Herba Epimedii and Fructus Ligustri Lucidi is based on Er-Zhi pills. Herba Epimedii (*Yinyanghuo*) and Fructus Ligustri Lucidi (*Nvzhenzi*) were documented as replenishing kidney-*yang* and kidney-*yin* agents, respectively. Besides, combined Herba Epimedii and Fructus Ligustri Lucidi have been prescripted to treat osteoporosis for almost 50 years by Professor *Shizeng Li*, a famous doctor of TCM in China, and received positive therapeutic effects.

Our previous study noticed that combination decoction of Herba Epimedii with Fructus Ligustri Lucidi exhibited an osteoprotective effect by increasing bone mineral density (BMD) and biomechanical properties, as well as improving bone pathological structure in retinoic aci-induced osteoporosis rats (Kang et al., 2013, 2014). We also found that the combination extracts of Herba Epimedii and Fructus Ligustri Lucidi were able to enhanced the bone mineral content (BMC), retarded bone turnover, and exerted estrogen-like function by upregulating the expression of sex hormone receptors in osteoporosis rats (Liu et al., 2015a, 2015b). Growing *in-vivo* and *in-vitro* researches have demonstrated that osteoprotective effects of the total flavonoids of Herba Epimedii (Indran et al., 2016; Zhao et al., 2016) and the total iridoids and flavonoids extracted from Fructus Ligustri Lucidi (Feng et al., 2014; Li et al., 2015b). However, their combined effects on GIOP were still unclear.

In recent years, studies found that the transforming growth factor-β1 (TGF-β1)/Smads signal transduction is a key pathway for orientation, differentiation, development and proliferation of osteoblasts, playing a key role in bone formation (Yamaguchi and Weitzmann, 2009). The exact underlying mechanism of GIOP has not been determined completely, but the inhibition of bone formation by GCs has been suggested to play a crucial role in the pathogenesis of GIOP (Liu et al., 2015c). The aim of this work was to investigated whether combined Herba Epimedii and Fructus Ligustri Lucidi could exert anti-osteoporosis effects on GIOP, explored the active component to account for the beneficial effects of GIOP and illuminated its underlying molecular mechanism. Therefore, we examined the effects of the combined herbs and combined active fractions on bone formation and bone resportion, and explored the effects on TGF-β1/Smads signaling pathway in GIOP rats.

2. Materials and methods

2.1. Reagents and medicines

Dexamethasone Sodium Phosphate injection was obtained from Rongsheng pharmaceutical CO., Ltd (Henan, China). Caltrate calcium D3 tablets were provided by wyeth pharmaceutical CO., Ltd (Jiangsu, China). Serum calcium and phosphorus assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Rat bone alkaline phosphatase (b-ALP), bone γ- carboxyglutamic acid-containing protein (BGP) , bone morphogenetic protein-2 (BMP-2), carboxy terminal cross linked telopeptide of typeIcollagen (ICTP) and tartrate-resistant acid phosphatase (TRACP) ELISA kits were purchased from Shanghai BlueGene Biotech CO., Ltd. (Shanghai, China). Quantitative bromochloroacetate (BCA) protein kit was purchased from Beijing Biosynthesis Biotechnology CO., Ltd. (Beijing, China). Rabbit anti-TGF-β1 and rabbit anti-Smad4 were purchased from Abcam Co. Ltd. (Cambridge, UK). Rabbit anti-p-smad2/3 and rabbit anti-Smad7 were purchased from Santa Cruz Biotechnology Inc. (CA, USA). Mouse monoclonal anti-GAPDH, peroxidase-conjugated affinipure goat anti-rabbit IgG and peroxidase-conjugated affinipure goat anti-mouse IgG were purchased from Zhong Shan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Immobilon-P PVDF transfer membrane was purchased from Millipore Corporation (Billerica Ma, USA). Fastquant RT kits, SuperReal premix plus (SYBR Green) were from Tiangen Biotech Co., Ltd. (Beijing, China). PCR primers were synthesized by Takara Biotechnology Co. Ltd (Dalian, China).

2.2. Preparations of Herbal Materials and Samples

Herba Epimedii (*Epimediium brevicornum Maxim.*) and Fructus Ligustri Lucidi (*Ligustrum lucidum Ait.*), were purchased from Beijing *Tongrentang* pharmaceutical Co. Ltd. (Beijing, China). They were authenticated by an expert herbalist, Shiyuan Jin, Honorary Professor, School of Traditional Chinese Medicine, Capital Medical University. Voucher specimens were deposited at the Traditional Chinese medicine (TCM) Endocrine and Metabolic disease laboratory of TCM School of Capital Medical University, China.

Combined Herba Epimedii and Fructus Ligustri Lucidi decoction was prepared as follow. Herba Epimedii and Fructus Ligustri Lucidi crude herbs were mixed together at a ratio of 4:3. They were soaked in eight times volumes (V/W) of distilled water for 2 h, and then boiled for 1 h two times. Filtered and evaporated the decoction to a concentration at containing crude drugs 0.35 g/ml. The decoction was store at 4 ℃ before use.

The combination of Herba Epimedii and Fructus Ligustri Lucidi active fractions were prepared according to our previous report (Liu and Kang, 2015a). And the extraction process of the combined active fractions has been protected by the Chinese patent (20140037992.5). The extractive yield of Herba Epimedii was 2.5%, which was regarded as the total flavonoids of Herba Epimedii (HETF). The content of HETF was 80% calculated by Icariin. The extractive yield of Fructus Ligustri Lucidi extract was 5%, which was regarded as total iridoids and flavonoids of Fructus Ligustri Lucidi (FLLTIF). The content of FLLTIF was more than 80% calculated by Oleanolic Acid and Rutin. All components contents detection method was in accordance with the phytochemical test listed in the Chinese Pharmacopoeia 2010. The active fraction combinations of Herba Epimedii and Fructus Ligustri Lucidi were mixed at a ratio of 2 to 3 (HETF to FLLTIF), equivalent to