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Research Paper

Protective effects of water fraction of Fructus Ligustri Lucidi extract against hypercalciuria and trabecular bone deterioration in experimentally type 1 diabetic mice

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ABSTRACT

Ethnopharmacological relevance: Fructus Ligustri Lucidi (FLL), the fruit of *Ligustrum lucidum* Ait, is a commonly prescribed herb to nourish the endocrine and renal systems and to strengthen the bones in Traditional Chinese Medicine. This study was aimed to determine the effects of water fraction of FLL ethanol extract (WF-EE) on urinary calcium excretion and trabecular bone properties in type 1 diabetic mice.

Materials and methods: The DBA/2J mice with type 1 diabetes induced by streptozotocin injection were orally administered with WF-EE. After 6 weeks of treatment, the level of biomarkers, including serum calcium, parathyroid hormone (PTH), and fibroblast growth factor-23 (FGF-23) and urine calcium, was measured. Micro-CT was performed to detect trabecular bone properties of the proximal tibial metaphysis. The expression of active calcium transporting proteins in kidney and duodenum was determined by RT-PCR, immunoblotting and immunostaining.

Results: Type 1 diabetes induced hypercalciuria and trabecular bone deterioration. The WF-EE could significantly inhibit hypercalciuria and ameliorate the micro-structure of trabecular bone as well as increase serum PTH and FGF-23 levels in type 1 diabetic mice. The gene expressions of active calcium transporting proteins in duodenum were up-regulated, and the gene and protein expressions of calcium-sensing receptor (CaSR) in kidney were dramatically down-regulated in diabetic mice in response to the treatment with WF-EE.

Conclusions: The present study demonstrated the protective effects of the water fraction of Fructus Ligustri Lucidi ethanol extract against hypercalciuria and trabecular bone deterioration in experimentally type 1 diabetic mice, and the underlying mechanism may be attributed to its regulations on duodenal calcium transporting proteins and renal CaSR.

1. Introduction

Skeletal involvement in patients with diabetes mellitus was initially described over 60 years ago as a retardation of bone development and 'bone atrophy' in children with long-standing disease (Carnevale et al., 2004). Low bone mineral density (BMD) is consistently observed in type 1 diabetes (T1DM), and recent meta-analyses and cohort studies confirmed that T1DM and T2DM are associated with higher fracture risk (Isidro and Ruano, 2010; Drake et al., 2012). It was well reported that the enhancement of urinary calcium excretion is one of the multiple pathways affecting bone metabolism during diabetes (Isidro and Ruano, 2010; Montagnani et al., 2011). Hypercalciuria has been traced back both to the osmotic diuresis promoted by glycosuria and to renal hemodynamic changes induced by prostaglandin excess (Raskin et al., 1978), furthermore, our previous study demonstrated that the decrease of transcellular calcium transporters abundance in kidney contributed to hypercalciuria in type 1 diabetic mice (Zhang et al., 2011).

Fructus Ligustri Lucidi (FLL), the fruit of *Ligustrum lucidum* Ait, is a commonly prescribed herb to nourish the endocrine and renal systems and to strengthen the bones in Traditional Chinese

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Medicine (TCM) (Zhang et al., 2008b; Siu et al., 2013). In the clinical TCM practice, FLL is a component among kidney-tonifying herbal formulae for the treatment of osteoporosis (Sun et al., 2008; Zhang et al., 2008a; Siu et al., 2013). Our previous study revealed that the crude extract of FLL could improve calcium balance by regulating transcellular calcium absorption in intestine and reabsorption in kidney (Zhang et al., 2006, 2008b, 2008c), consequently exerting bone-sparing function in aged female rats and aged osteoporotic rats with estrogen deficiency (Zhang et al., 2008d; Dong et al., 2014). In addition, the in vitro results demonstrated that FLL extract was able to directly promote osteogenesis (Li et al., 2010), and that the serum from rats administered with FLL could inhibit the proliferation and the differentiation of osteoclasts (Zhang et al., 2008a).

When exploring the active component to account for the beneficial effects of FLL on mediating calcium homeostasis, it was found that the water-soluble fraction of FLL ethanol extract was responsible for the positive action of FLL in mature female rats (Dong et al., 2012). Thus, the present study was aimed to determine the effects of the water fraction of FLL ethanol extract on urinary calcium excretion and trabecular bone properties in streptozotocin-induced type 1 diabetic mice. It is hoped that this study will further increase our understanding on the molecular actions of the active fraction in FLL, which might be useful in managing calcium balance and bone health for diabetic patients.

2. Materials and methods

2.1. Water-soluble fraction of FLL ethanol extract (WF-EE)

It was prepared as previously described (Dong et al., 2012). Briefly, Fructus Ligustri Lucidi (FLL) was obtained from Jiangsu province of China. A voucher specimen was deposited in The Hong Kong Polytechnic University. The crude plant was extracted with 70% ethanol, the mixture was filtered and lyophilized to dryness to obtain FLL ethanol extract, which was then suspended in hot water and made into dried powders by a process of evaporation and lyophilisation. The two most prominent peaks in WF-EE were identified to be nuezhenide and salidroside as demonstrated in our previous report (Dong et al., 2012).

2.2. Animal treatment

10-week-old male DBA/2J mice (Slac Laboratory Animal, Shanghai, China) were allowed to acclimate to their environment for 1 week before inducing diabetes. Mice were fasted for 4 h and then given an intraperitoneal injection of freshly prepared streptozotocin (STZ) at 40 mg/kg dissolved in 10 mM citrate buffer (pH 4.2, $n=25$) or vehicle (non-diabetic control mice, $n=10$) daily for 5 consecutive days. One week post-STZ injection (week 0), the fasting blood glucose (FBG) levels were measured with blood glucose monitoring system (Roche). The mice with FBG more than 9 mmol/L were randomly divided into diabetic osteoporosis group (DOP, $n=10$) and WF-EE-treated group (574 mg/kg, i.g, $n=10$). The dose of WF-EE referred as our previous reports (Dong et al., 2012).

FBG was monitored and body weight was measured at 3 and 6 weeks post-drug treatment. Six weeks after drug administration, spot urine of each mouse was collected. Serum, tibias and kidneys were immediately harvested for a variety of biochemical and molecular analyses. The animal study protocol was reviewed and approved by the institution's Animal Ethics Committee at the University of Shanghai for Science and Technology.

2.3. Serum and urine chemistries

Calcium (Ca) and creatinine (Cr) concentrations of serum and urine were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). The level of urine Ca was corrected by the concentration of urine Cr. The serum levels of intact parathyroid hormone (PTH 1-84) and fibroblast growth factor-23 (FGF-23) were detected using mouse bioactive PTH and FGF-23 (C-Term) ELISA assay (Immutopics, Inc., San Clemente, CA, USA).

2.4. Micro-CT analysis

The operation and detection parameters were as described previously (Zhang et al., 2014). Morphologic three-dimensional parameters for trabecular bone at tibial proximal metaphysis were obtained as the following: (1) the mean mineral density of bone volume (BMD/BV); (2) trabecular bone number (Tb.N); (3) trabecular bone separation (Tb.Sp); (4) connectivity density (Conn. D).

2.5. RT-PCR

The RNA extraction from kidney was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, California, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of cDNAs was performed by reverse transcription reactions with 4 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, California, USA) with oligo $dT_{(15)}$ primers (Fermentas) as described by the manufacturer. The first strand cDNAs served as the template for the regular PCR performed using a DNA Engine (ABI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The PCR primers used in this study are shown in Table 1.

2.6. Western blotting

The kidneys were homogenized and extracted in Laemmli buffer (Boston Bioproducts, Worcester, MA, USA), followed by 5 min boiling and centrifugation to obtain the supernatant. Samples containing 40 μg of protein were separated on 8% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) nonfat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with goat anti-calcium-sensing receptor (CaSR) polyclonal antibody (Santa Cruz Biotechnology, USA) at 4 °C overnight. After three washes with TBST,

Table 1
Primer sequences.

Gene	Sequence (5′→3′)	Gene ID	Product size (bp)
TRPV5	For: TCTCAATTGGTGGGTCAGAGA Rev: GCATTATAGCAGCATCCAGGT	194352	236
TRPV6	For: ACATAGCTCCTGCTCACTCC Rev: TCTCTCTGTAGAGGTCCCGT	64177	183
CaBP-9k	For: CAAAAATATGCAGCCAAGGA Rev: AGCGTGCGTTCAATCAGTAG	12309	249
CaBP-28k	For: TGGTTACCTGGAAGGAAAGG Rev: GGGTAAGACGTGAGCCAACT	12307	166
PMCA1b	For: GAATGGTGGTCACTGCTGTA Rev: CCCTCCTTCTTCACTCTTCA	435740	212
CaSR	For: ATTTTCCTGACCGCCTTTGT Rev: GGACTCGATTGGTCTTCACC	12374	238
GAPDH	For: CAGAACATCATCCCTGCATC Rev: CTGCTTCACCACCTTCTTGA	14433	183

TRPV, transient receptor potential vanilloid; CaBP, calcium binding protein; PMCA, plasma membrane calcium-ATPase; CaSR, calcium-sensing receptor; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.