

Liuwei Dihuang Wan, A Traditional Chinese Medicinal Formula, Protects Against Osteoporosis

Hui Sun, Li - jing Li, Ai - hua Zhang, Ning Zhang, Wen - jun Sun and Xi-jun Wang*

National TCM Key Lab of Serum Pharmacochimistry, Heilongjiang University of Chinese Medicine, and Key Pharmacometabolomics Platform of Chinese Medicines, Heping Road 24, Harbin 150040, China

Abstract

The treatment and prevention of osteoporosis involve great challenges. Liuwei Dihuang Wan (LW), a classic Chinese medicinal formula, has been widely used for clinically treating 'kidney deficiency' in Asia, however, its potency in osteogenesis remains unknown. The purpose of this study is to systemically investigate the effects of LW on ovariectomy-induced osteoporosis in rats. A total of forty-eight 7-month-old female Wistar rats were used and randomly divided into sham-operated group and three ovariectomized (OVX, model) groups: OVX with vehicle; OVX with LW; OVX with nilestriol. Daily oral administration began 4 weeks after the surgery and lasted for 24 weeks. The bone mineral density (BMD), bone mineral content (BMC), uterine index, blood mineral levels and biochemical markers were examined in LW orally treated rats. The trabecular bone micro architecture was also evaluated by MicroCT. Results showed that LW treatment significantly enhanced BMD, BMC, and blood antioxidant enzymes activities, decreased blood Ca and P levels compared to the model group. LW prevented total BMD decrease in the femur induced by OVX, which was accompanied by a significant decrease in skeletal remodeling, as was evidenced by the decreased levels of the bone turnover markers, such as alkaline phosphatase (ALP). Treated rats showed significantly improved mineral content in ashed femurs compared to untreated animals. The treatment could also enhance the bone strength and prevent the deterioration of trabecular micro architecture. This experiment demonstrates that the administration of LW to ovariectomized rats reverses bone loss and prevents osteoporosis. The present study indicated that LW might be a potential alternative medicine for the prevention and treatment of osteoporosis.

Keywords: Liuwei Dihuang Wan; Osteoporosis; Traditional chinese medicine; Natural products

Introduction

Osteoporotic can lead to high mortality and disability; reduce the flexibility of the body and quality of life [1]. Current treatment of osteoporosis is largely dependent on drugs. Accumulating data have indicated that the loss of estrogen at menopause is a major contributor to pathogenesis of the disease [2]. Estrogen replacement therapy (ERT) is the main treatment postmenopausal osteoporosis [3]. However, recent findings indicated ERT increased the risk of postmenopausal women to develop breast cancer, stroke, thrombosis, Alzheimer's disease and cardiovascular disease [4,5]. These findings have led to the advice that ERT should not be considered first-line therapy for the prevention of osteoporosis. Alternative approaches for prevention or treatment of osteoporosis are worth exploring. Traditional Chinese medicine (TCM) therapy offers a possible alternative [6]. Potential efficacy of traditional medicines has stimulated the interest of scientists and doctors to turn on to TCM for treatment of osteoporosis [7].

TCM is characterized by the wide use of herbal formulae, which are capable of systematically treating diseases determined by interactions among various herbs. Liuwei Dihuang Wan (LW) is a typical TCM prescription that has been used for more than a thousand years in China and consists of six herbs including *Rehmannia glutinosa* Libosch. (Family: Scrophulariaceae), *Cornus officinalis* Sieb. (Family: Cornaceae), *Dioscorea opposita* Thunb. (Family: Dioscoreaceae), *Alisma orientale* (G. Samuelsson) Juz (Family: Alismataceae), *Poria cocos* (Schw.) Wolf (family: Polyporaceae) and *Paeonia suffruticosa* Andrews (family: Paeoniaceae) [8]. It has long been used clinically in treatment of many kinds of diseases with the sign of Yin insufficiency of kidney [9]. According to the theory of TCM, LW has the properties of tonifying the "Yin" of kidney, the fundamental

system to support reproduction, development and performance over life time [10]. Modern pharmacology shows that LW performs a lot of pharmacological and biological activities, which has been used to improve or restore declined functions related to osteoporosis diseases [11].

Given the significant health and economic impact of osteoporosis on individuals and society, safe, effective, and readily available preventative measures are needed for early intervention [12]. The use of LW could be a valuable approach and its public use has increased steadily in Asia within the last decade, particularly among women between the ages of 40-69 [13]. Despite the popularity of LW, rigorous scientific evidence supporting their use for therapeutic potential is lacking. Ovariectomized rats have been used extensively in osteoporosis models [14]. Rats are widely accepted animal model for studying osteoporosis, since the progressive loss of bone matrix is similar to that in postmenopausal women with osteoporosis [15]. Recently, a number of studies have revealed the beneficial effects and some possible mechanisms of action of LW [10,11]. No study, to present date, has been done on the protective effect of LW against anti-

*Corresponding author: Xi-jun Wang, National TCM Key Lab of Serum Pharmacochimistry, Heilongjiang University of Chinese Medicine, Key Pharmacometabolomics Platform of Chinese Medicines, Heping Road 24 Harbin 150040, China, Tel: 86-451-821- 108 - 18; E-mail: xijunwagnls@126.com

Received April 09, 2013; Accepted April 23, 2013; Published April 25, 2013

Citation: Sun H, Li L, Zhang A, Zhang N, Sun W, et al. (2013) Liuwei Dihuang Wan, A Traditional Chinese Medicinal Formula, Protects Against Osteoporosis. Pharmaceut Anal Acta 4: 226. doi:10.4172/2153-2435.1000226

Copyright: © 2013 Sun H, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

osteoporosis in OVX model. In the present study, the *in vivo* effects of LW on osteoporosis were evaluated using 7-month-old OVX or sham-operated female Wistar rats orally administered with LW for 24 weeks.

Materials and Methods

Reagents

LW was prepared from the commercial and standardized product LW manufactured by Tongtengtang Pharmaceutical Co., Ltd. (Beijing, China). According to the Pharmacopeia of P. R. China, the product is made from Radix Rehmanniae Praeparata (prepared root of *Rehmannia glutinosa*, 160 g), Fructus Corni processed, fruit of *Cornus officinalis*, 80 g), Cortex Moutan (root bark of *Paeonia suffruticosa*, 60 g), Rhizoma Dioscoreae (rhizome of *Dioscorea oppositifolia*, 80 g), Poria (sclerotia of *Poria cocos*, 60 g), and Rhizoma Alismatis (rhizome of *Alisma plantago-aquatica*, 60 g). Nilestriol was purchased from Shanghai Hualian Pharmaceutical Co., Ltd. ALP kits were purchased from Nanjing Jiancheng Bioengineering Institute. Serum radioimmunoassay kits were purchased from Weifang Sunway Biotech Engineering Group Co., Ltd. Osteocalcin kit was purchased from Beijing Furui Biological Engineering Company.

Preparation of LW extract

LW was extracted twice with 75% methanol, followed by evaporation of the solvent under reduced pressure at 50°C. The methanol extract yield was 30.8%. The dosage used in the experiments was based on the dry weight of the extract. The methanol extract was suspended in distilled water and administered orally to each rat with a volume of 1 ml/100 g body weight (2 g/mL).

Animals and treatment

Forty-eight young adult (7 months) female Wistar rats, weighing 350 ± 20g, were obtained from the Laboratory Animal Center, Heilongjiang University of Chinese Medicine. The animals were acclimatized for 1 week to our laboratory conditions prior to experimental manipulation and were exposed to a 12-h light and 12-h dark cycle at a room temperature of 22°C. They had free access to standard laboratory chow and water *ad libitum*. The animals were randomly assigned into four groups: control (sham operated rats, received saline), OVX (model), OVX with LW, and OVX with nilestriol, each containing 7 animals. The detailed experimental protocol was shown in figure 1. All animal care and experimental procedures were performed in compliance with the policies on the care and use of animals of the Ethical Committee of Heilongjiang University of Chinese Medicine. All efforts were made to ameliorate suffering of animals.

Ovariectomy

Before the surgery, the rats were anesthetized with pentobarbital. A vertical incision was made approximately 15 cm in the abdomen using a sterilized sharp knife. The right and left ovaries were cut and removed. Before the ovaries were cut, the fallopian tubes were tied to

prevent bleeding. The muscle layer under the skin was stitched up by sterile and soluble suture. Then, the outer layer of skin was sewn with nonwater soluble suture. The procedure for sham operated rats was just the same with ovariectomized rats, but both of the ovaries were not removed. Rats were left recuperating for 1 month before commencing the treatment.

Histomorphometric analysis

Upon sacrifice, the proximal tibia were dehydrated in 4% paraformaldehyde/0.1 M phosphate buffer, at 4°C for 24 h, then add into 10% EDTA decalcification solution for 2 weeks, and then were used for HE staining test. Frontal sections of the tibiae were prepared. Measurements were performed on the entire marrow region within the cortical shell of the proximal tibia metaphysis from 1-4 mm distal to the growth plate-metaphyseal junction using an Image Analysis System. The parameters measured were trabecular bone volume, trabecular thickness, trabecular number. All measurements were performed randomly at the metaphyseal region, which was located 3-7 mm from the lowest point of the growth plate and 1mm from the lateral cortex. The selected area is the secondary spongiosa area, which is rich in bone trabecular. The micro architecture sections of lumbar L4 trabecular were examined using μ CT (Germany).

Biochemical analyses

The rats were killed after blood collection, the uterus was removed immediately, and calculated uterus index= the wet weight of uterus / body weight. The blood chemistry parameters evaluated was ALP using an auto analyzer. Serum ALP and E2 were assayed using clinical test kits. Blood Ca and P level were measured by atomic absorption spectrophotometry. Serum levels of bone glaprotein (BGP) and osteoprotegerin (OPG) were assessed by specific immunoradiometric assay.

Measurement of bone ash and Ca content

In order to determine the amount of mineralized bone, after the breaking test, the right femurs were mineralized at 800°C for 12 h and weighed to the nearest 0.00001 g. Femurs was weighed before and after ashing. The ash was then solubilized in 6 N HNO₃ and Ca and P assay were analyzed by atomic absorption spectrophotometer. Values were expressed as milligram of Ca and P per cubic centimeter of bone volume.

Bone densitometry

Femoral bone mineral content (BMC) and bone mineral density (BMD) of the left femur (proximal, middle, and distal portions) was measured by dual energy x-ray absorptiometry (DCS-600; ALOKA CO., Ltd., Tokyo, Japan).

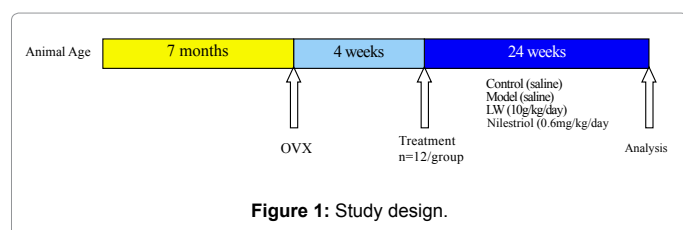
Statistical analysis

Results were presented as mean ± standard error. All data were analysed using the Statistical Package for Social Sciences software (SPSS 17; SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used for normality. ANOVA followed by Tukey's tests were used for normally distributed data while Kruskal-Wallis and Mann-Whitney tests were used for not normally distributed data.

Results

HE staining

After 24 weeks of treatment, HE staining indicated that the fractured femora of the rats in the LW group (Figures 2A and 2B)



had significantly higher callus volumes than that of the model group (Figure 2C). The abundance of callus tissue in the LW group showed that most of the callus should have been resorbed and replaced with lamellar bone through the bone remodeling process. The callus volume of the nilestriol group (Figure 2D) was similar to that of the LW group. Therefore, LW appeared to be able to improve the fracture healing of osteoporotic bone.

Histomorphometric analysis

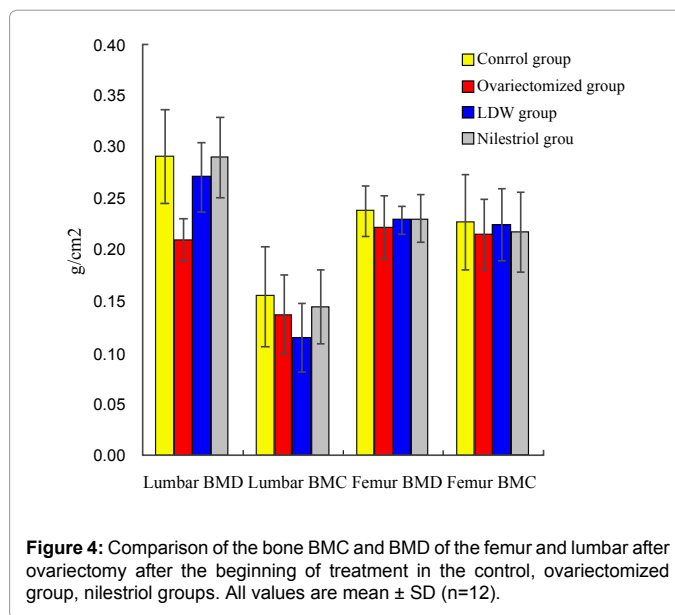
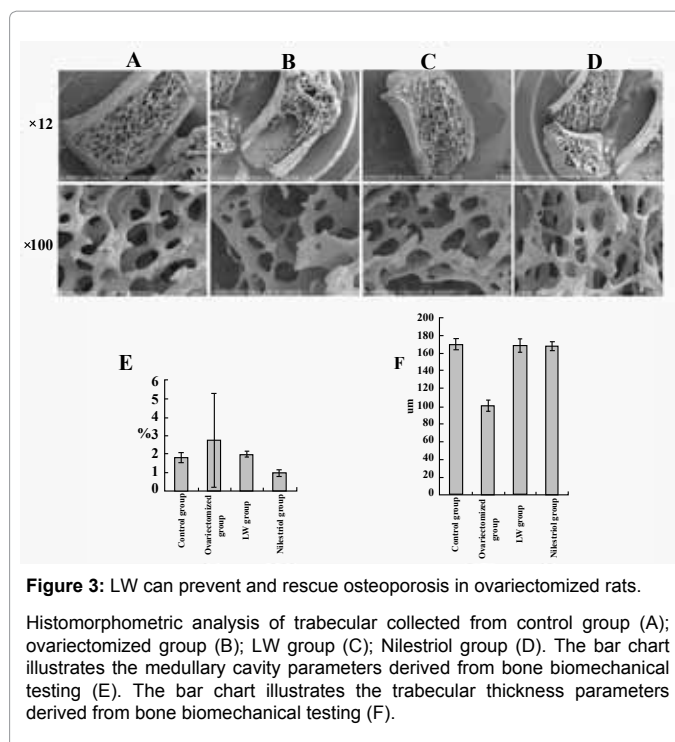
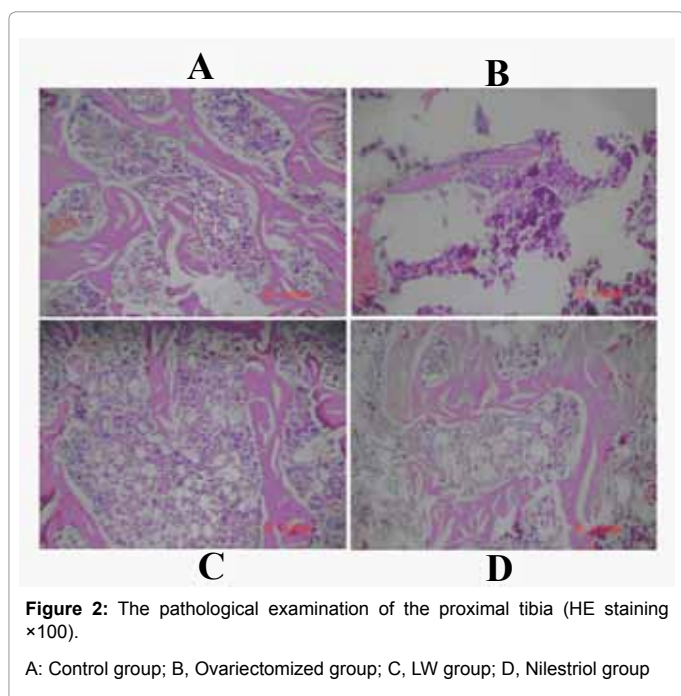
Trabecular thickness in the control group was moderate, and bone surface is covered with collagen filaments arranged in neat rows, rules, in close proximity to the surface of the filaments in a grid pattern (Figure 3A). Bone trabecular micro-structure of model group was completely thinning and, arranged in a haphazard and even compaction, trabecular fracture phenomenon was everywhere, absorption lacuna was also visible, and filaments remains significant degenerative changes (Figure 3B). LW substantially restores normal state from disorder phenomenon in model group (Figure 3C). The surface fibrils thickness of bone trabecular in the nilestriol group appear little uniform (Figure 3D). LW could prevent OVX-induced bone loss by increasing trabecular bone area and decreasing trabecular separation in OVX rats. After 24 weeks administration, the area percentage of the bone marrow cavity of LW and nilestriol group was significantly reduced (Figure 3E, $P < 0.05$) and trabecular thickness significantly increased (Figure 3F).

BMC and BMD

The femur and lumbar BMC and BMD were lower in the OVX group than in the control group ($P < 0.05$), respectively. Treatment with LW or nilestriol significantly prevented reductions in BMC and BMD ($P < 0.05$, Figure 4). These results indicated that the effects of LW on bone were similar to those of nilestriol and thus LW could restore the bone loss in OVX rats.

Bone ash ratio and Ca content

The ash weight ratio and Ca content of the fifth lumbar vertebra



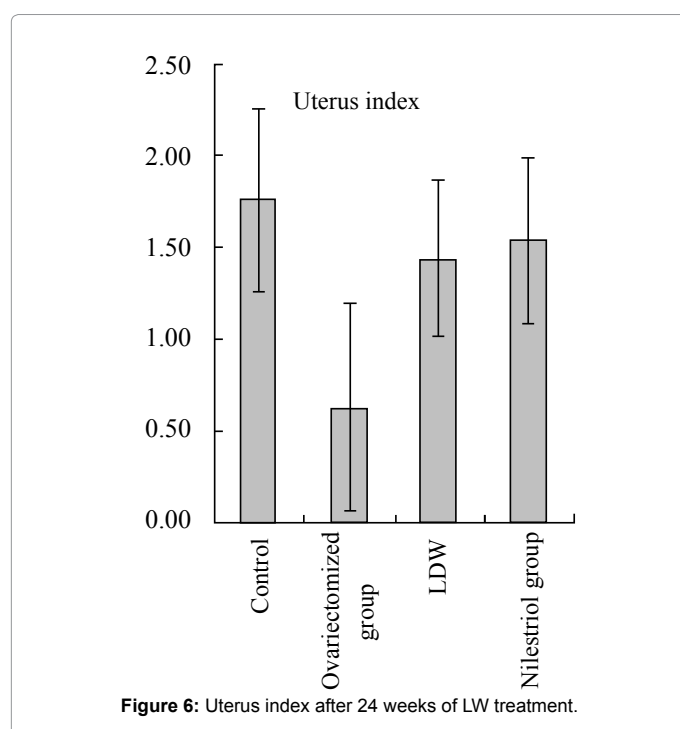
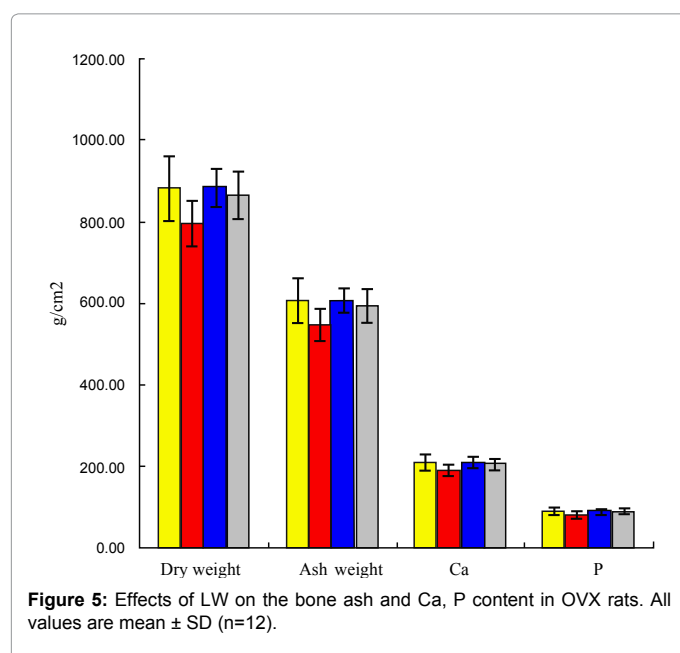
were lower in the OVX group than in the control group, respectively. Administration of LW or nilestriol caused significant increase in the ash weight ratio, and Ca content of the fifth lumbar vertebra compared with the control group (Figure 5).

Uterus index

Following 24 weeks of treatment, marked uterine atrophy was observed in the model group when compared with the LW group ($P < 0.05$). The uterine weight of the rats treated with nilestriol was unchanged when compared with the LW group. Finally, the uterine weight in the LW group was considerably increased when compared with the VC group ($P < 0.05$; Figure 6).

Effects of LW on biochemical parameters in ovariectomized rat

The present data (Table 1) indicated that blood ALP activity, BGP and OPG levels in the model group were significantly ($p < 0.05$) increased compared to the sham control. Table 1 shows that only treatment with the LW had a significant ($p < 0.01$) decreasing effect on all 3 parameters. The model group had a significant ($p < 0.05$) decrease only on E2. The presence of LW and nilestriol normalized the levels of blood E2 to the normal values of control.



Discussion

Osteoporosis, an important systemic disorder, affecting mainly Caucasian women, with a diverse and multifactorial etiology, is of great importance in health care [16]. Plenty of studies into this disease have been carried out in recent years [17]. A large variety of animal species, including rodents, rabbits, dogs, and primates, have been used as animal models in osteoporosis research. Among these, the laboratory rat is the preferred animal for most researchers [18]. Estrogen deficiency is considered as the major determinant of bone loss in postmenopausal women. Due to the numerous side effects of ERT, alternative antiosteoporotic agents that are comparable in effectiveness to estrogen but with minimal side effect are being investigated [19]. Herbal drugs are fairly preferred due to their effectiveness, fewer side effects, and relatively low cost. Traditional medicine could be considered as a natural heritage from Mother Nature as a source of medicine [20]. Thus, more extensive studies should be conducted to explore the healing properties of different types of medicinal plants to produce an alternative and effective treatment for the osteoporotic patient. Accumulating data have indicated that the traditional medicine has been widely used for thousands of years to treat bone disorders [21].

TCM has been widely used for thousands of years to treat fractures and joint diseases [22]. Many herbs shown to have kidney-tonifying activities are used in TCM formulas for prevention and treatment of osteoporosis [23]. LW is one of the most frequently used herbs prescribed for treatment of osteoporosis in Asia [11]. However, the influence of LW on osteoporosis in animals is relatively unknown. Therefore, we undertook this study to evaluate the efficacy of LW in preventing osteoporosis using the OVX model, and provided the basic data to further study for the molecular mechanism for LW in treatment of osteoporosis diseases. To evaluate the efficacy of LW for the treatment of postmenopausal osteoporosis, a rat OVX model was used in this study. The BMD, BMC, uterine index, blood mineral levels and biochemical markers were examined in LW orally treated rats.

HE staining indicated that the fractured femora of the rats in the LW group had significantly higher callus volumes compared with that of rats in the model group. Histomorphometric analysis indicated that LW could prevent OVX-induced bone loss by increasing tibial trabecular bone area and decreasing trabecular separation in OVX rats. Micro architectural properties are a newly emerged marker for the evaluation of the true impact of a treatment on the quality of trabecular bone. LW substantially restores normal state from disorder phenomenon in bone trabecular micro-structure of model group. After 24 weeks administration, the area percentage of the bone marrow cavity of LW and nilestriol group was significantly reduced and trabecular thickness significantly increased. Although low bone mass is a major risk factor for fracture, the preservation of trabecular bone architecture significantly contributes to bone strength and may reduce fracture risk beyond BMD and BMC, as demonstrated by a number of studies that have reported close correlations between microstructural properties and the biomechanical strength of bones. In this work, BMD and BMC were found to be significantly different between the sham and ovariectomized groups. Treatment with LW significantly enhanced BMD and BMC in OVX rats. The ash weight ratio and Ca content of the fifth lumbar vertebra were lower in the OVX group than in the control group, respectively. Administration of LW or nilestriol caused significant increase in the ash weight ratio, Ca and P content of the fifth lumbar vertebra compared with the OVX control group.

	ALP	BGP	E2	OPG	Ca	P
Control group	79.57 ± 37.51	1.90 ± 0.24	17.51 ± 1.56	1.50 ± 0.24	2.38 ± 0.08	1.55 ± 0.15
Ovariectomized group	113.8 ± 21.60 [#]	2.87 ± 0.479 [#]	10.84 ± 3.13 [#]	2.87 ± 0.47 [#]	2.56 ± 0.24	1.86 ± 0.93
LW group	90.6 ± 66.34 [*]	2.27 ± 0.537 [*]	13.61 ± 3.05 [*]	2.27 ± 0.53 [*]	2.35 ± 0.15	1.45 ± 0.28
Nilestriol group	81.98 ± 23.12 [*]	1.96 ± 0.259 [*]	12.12 ± 2.34 [*]	2.73 ± 0.55	2.43 ± 0.12	1.65 ± 0.23

Note: [#]Significant difference compared with the control group (P<0.05).
^{*}Significant difference compared with the Ovariectomized group (P <0.05).

Table 1: Effects of LW on biochemical parameters in ovariectomized mice (mean ± SD, n=10).

The uterine weight was significantly decreased in the OVC when compared with the LW group. Additionally, ovariectomy resulted in marked uterine atrophy in the ovariectomized rats when compared with the control group, indicating the success of the ovariectomy procedure. The uterine weight in the LW group was significantly greater than that of the model group. The treatment with LW was able to increase the uterine weight of ovariectomized rats, suggesting that the dose of the therapy used in the current study was adequate. The present data indicated that blood ALP activity, BGP and OPG levels in the model group were significantly increased compared to the sham control. ALP is an enzyme present in many tissue cells like liver, kidney, intestine, placenta, and germ cells and in osteoblasts. ALP has become the clinically most relevant enzyme in the diagnosis of bone diseases. The ALP activity on sections was seen more in the OVX group, due to lack of inhibiting activity of estrogen on osteoclasts causing the increase in bone resorption. The serum activity of alkaline phosphatase, an index of bone formation, has been reported to be significantly greater in an OVX group than in a sham-operated group. A similar change was observed in this study. LW treatment also improved the loss of Ca content in the OVX rats. These results clearly show that LW treatment in rats can ameliorate osteopenia induced by ovariectomy. In summary, LW is effective in preventing bone loss caused by ovarian hormone deficiency as seen from femur BMD, BMC values, and blood Ca, and P levels. We suppose that LW improves bone quality possibly partly through enhancing OVX rats' antioxidant enzymes activities and reducing bone mineral elements.

Conclusions

The increasing incidence of postmenopausal osteoporosis and its related fractures have become global health issues in the recent days. Herbal medicine has been widely used for the treatment of bone disease. LW, a classic prescription of TCM, has been used in treating osteoporosis. In this study, we investigated the anti-osteoporosis effects of LW in OVX rats. Several methods are assessed, such as biochemical markers, and histomorphometry, that are used for monitoring and evaluation of this animal model in preventive or therapeutic strategies for osteoporosis. Results showed that LW treatment significantly enhanced BMD, BMC, and blood antioxidant enzymes activities, E2, decreased BGP, OPG, blood Ca and P levels compared to the OVX group. The LW treatment could also enhance the bone strength and prevent the deterioration of trabecular micro architecture. Treated rats showed significantly improved mineral content in ashed femurs compared to untreated animals. Therefore, it is still valuable to develop safer preventive medicine to suppress osteoporosis. Our study had shown that LW exhibits antiosteoporotic effects, suggesting that it may play a promising role in the treatment of osteoporosis.

Acknowledgements

This work was supported by grants from the Key Program of the Natural Science Foundation of the State (Grant No. 90709019), the National Key Program on the Subject of Drug Innovation (Grant No. 2009ZX09502-005), the National Specific Program on the Subject of Public Welfare (Grant No. 200807014), and the National Program for Key Basic Research Projects in China (Grant No. 2005CB523406).

References

- Deng FY, Lei SF, Zhang Y, Zhang YL, Zheng YP, et al. (2011) Peripheral blood monocyte-expressed ANXA2 gene is involved in pathogenesis of osteoporosis in humans. *Mol Cell Proteomics* 10: M111.
- Chen RQ, Wong CM, Lam TH (2012) Construction of a traditional Chinese medicine syndrome-specific outcome measure: the Kidney Deficiency Syndrome questionnaire (KDSQ). *BMC Complement Altern Med* 12: 73.
- Hussan F, Ibraheem NG, Kamarudin TA, Shuid AN, Soelaiman IN, et al. (2012) Curcumin Protects against Ovariectomy-Induced Bone Changes in Rat Model. *Evid Based Complement Alternat Med* 2012: 174916.
- Badescu L, Badulescu O, Badescu M, Ciocoiu M (2012) Mechanism by *Sambucus nigra* Extract Improves Bone Mineral Density in Experimental Diabetes. *Evid Based Complement Alternat Med* 2012: 848269.
- Liu C, Wang G, Chen G, Mu Y, Zhang L, et al. (2012) Huangqi decoction inhibits apoptosis and fibrosis, but promotes Kupffer cell activation in dimethylnitrosamine-induced rat liver fibrosis. *BMC Complement Altern Med* 12: 51.
- Sun H, Zhang A, Wang X (2012) Potential role of metabolomic approaches for Chinese medicine syndromes and herbal medicine. *Phytother Res* 26: 1466-1471.
- Zhang A, Sun H, Wang P, Han Y, Wang X (2012) Future perspectives of personalized medicine in traditional Chinese medicine: a systems biology approach. *Complement Ther Med* 20: 93-99.
- Fu B, Lun X, Gong Y (2005) Effects of the combined therapy of acupuncture with herbal drugs on male immune infertility—a clinical report of 50 cases. *J Tradit Chin Med* 25: 186-189.
- Wu CR, Lin LW, Wang WH, Hsieh MT (2007) The ameliorating effects of LiuWei Dihuang Wan on cycloheximide-induced impairment of passive avoidance performance in rats. *J Ethnopharmacol* 113: 79-84.
- Wang P, Sun H, Lv H, Sun W, Yuan Y, et al. (2010) Thyroxine and reserpine-induced changes in metabolic profiles of rat urine and the therapeutic effect of Liu Wei Di Huang Wan detected by UPLC-HDMS. *J Pharm Biomed Anal* 53: 631-645.
- Perry B, Zhang J, Sun C, Saleh T, Wang Y (2012) Liuwei dihuang lowers body weight and improves insulin and leptin sensitivity in obese rats. *Evid Based Complement Alternat Med* 2012: 847167.
- Wang X, Zhang A, Sun H (2012) Future perspectives of Chinese medical formulae: chinmedomics as an effector. *OMICS* 16: 414-421.
- Zhang A, Sun H, Yang B, Wang X (2012) Predicting new molecular targets for rhein using network pharmacology. *BMC Syst Biol* 6: 20.
- Shin IS, Lee MY, Ha HK, Seo CS, Shin HK (2012) Inhibitory effect of Yukmijihwang-tang, a traditional herbal formula against testosterone-induced benign prostatic hyperplasia in rats. *BMC Complement Altern Med* 12: 48.
- Li SY, Li JS, Wang MH, Xie Y, Yu XQ, et al. (2012) Effects of comprehensive therapy based on traditional Chinese medicine patterns in stable chronic obstructive pulmonary disease: a four-center, open-label, randomized, controlled study. *BMC Complement Altern Med* 12: 197.
- Deng ZQ, Zheng H, Zhao L, Zhou SY, Li Y, et al. (2012) Health economic evaluation of acupuncture along meridians for treating migraine in China: results from a randomized controlled trial. *BMC Complement Altern Med* 12: 75.
- Arai M, Katai S, Muramatsu SI, Namiki T, Hanawa T, et al. (2012) Current status of Kampo medicine curricula in all Japanese medical schools. *BMC Complement Altern Med* 12: 207.

18. Yang J, Zeng F, Feng Y, Fang L, Qin W, et al. (2012) A PET-CT study on the specificity of acupoints through acupuncture treatment in migraine patients. *BMC Complement Altern Med* 12: 123.
19. Liao YH, Lin CC, Li TC, Lin JG (2012) Utilization pattern of traditional Chinese medicine for liver cancer patients in Taiwan. *BMC Complement Altern Med* 12: 146.
20. Wang X, Sun H, Zhang A, Jiao G, Sun W, et al. (2011) Pharmacokinetics screening for multi-components absorbed in the rat plasma after oral administration traditional Chinese medicine formula Yin-Chen-Hao-Tang by ultra performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry combined with pattern recognition methods. *Analyst* 136: 5068-5076.
21. Zhang A, Sun H, Yuan Y, Sun W, Jiao G, et al. (2011) An in vivo analysis of the therapeutic and synergistic properties of Chinese medicinal formula Yin-Chen-Hao-Tang based on its active constituents. *Fitoterapia* 82: 1160-1168.
22. Wang X, Sun H, Zhang A, Sun W, Wang P, et al. (2011) Potential role of metabolomics approaches in the area of traditional Chinese medicine: as pillars of the bridge between Chinese and Western medicine. *J Pharm Biomed Anal* 55: 859-868.
23. Li M, Wang W, Wang P, Yang K, Sun H, et al. (2010) The pharmacological effects of morroniside and loganin isolated from Liuweidihuang Wan, on MC3T3-E1 cells. *Molecules* 15: 7403-7414.