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ORIGINAL ARTICLE

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Phytoestrogen-rich herb formula "XLGB" prevents OVX-induced deterioration of musculoskeletal tissues at the hip in old rats

Abstract This study investigated a phytoestrogen-rich herb formula, Xianlinggubao (XLGB) (including genistein 510μg/g and daidzein 2500μg/g), concerning prevention of OVX-induced deterioration of musculoskeletal tissues in 11-month-old female Wistar rats, which were randomized into Sham, OVX, and XLGB groups. Daily oral administration of XLGB (250mg/kg/day) started after OVX for 3 months. mRNA of MHC-I IIa IIb of abductor muscle was determined by RT-PCR. The proximal femoral BMD and geometry, microarchitecture, and mechanical strength were evaluated by pQCT, micro-CT, and compressive testing, respectively. The bone turnover biochemical markers serum osteocalcin (OC) and urinary deoxypyridinoline (DPD) were evaluated. The results showed that (1) XLGB-treated OVX rats showed no difference compared to the Sham group, whereas OVX induced significant deterioration in variables related to bone density, microarchitecture, and mechanical strength ($P < 0.05$); (2) biochemical markers showed no difference between sham and XLGB groups as compared with higher bone turnover in OVX rats ($P < 0.05$); (3) mRNA expression of MHC-I IIa IIb was downregulated in OVX rats but upregulated after XLGB treatment ($P < 0.05$); and (4) as compared with the OVX group, no uterine hypertrophy was found in XLGB-treated rats. In conclusion, findings of this study suggested that the herbal preparation XLGB was able to prevent OVX-induced deterioration of musculoskeletal tissues at the hip without causing uterine stimulation.

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Introduction

Osteoporosis, which is an ever-increasing problem as the population ages, is closely associated with an increase in osteoporotic fractures, especially at the spine and hip. Women experience more osteoporotic fractures as compared with men because of both menopause and longer life expectancy [1,2]. Hormone replacement therapy (HRT) is one of the effective approaches for relieving menopausal symptoms and for prevention and treatment of osteoporosis and osteoporotic fractures, in spite of side effects-related increased incidence of breast cancer [3,4]. Our recent review showed that increase in bone mineral density (BMD) under drug treatment was not parallel to fracture risk reduction [5]. This was explained by the fact that the osteoporotic or fragility fractures, especially at the hip, were attributed to both skeletal and nonskeletal factors related to recurrent falls, and that skeletal muscle also underwent age-related atrophy (sarcopenia) parallel to bone loss (osteoporosis) [6–10].

The incidence of fragility fractures may differ among various ethnic groups [1,2,10,11]. Studies have suggested that, as compared with the rest of the industrialized world, the lower incidence of menopausal symptoms, osteoporosis, fragility fractures, and breast cancer in Oriental countries might be associated with high consumption of dietary phytoestrogens classified as isoflavones, lignans, and coumestans, a diverse group of compounds found in many edible plants or herbs with a structural similarity to estrogen and which bind to estrogen receptors [11–15]. Yin Yang Huo (YYH) (*Epimedium leptorhizum*) is one of such plants or herbs traditionally used to tone the "kidney system" and nourish bones in Oriental herbal medicine, and it has been claimed to have multitarget effects in prevention and treatment of conditions such as joint diseases, gonadal

dysfunctions, muscle weakness, fractures, and relief of menopausal-related symptoms over thousands of years [16–25]. Herbal preparations with YYH as the main component have therefore been recently developed for the prevention and treatment of osteoporosis in Asian countries such as China [16,21–24] and Japan [17–20], and are now commercially available and accepted by the health systems of many Asian countries for prevention of menopausal-related medical conditions [23,26–28]. One of such herbal preparations is the commercially available Xianlinggubao (XLGB), which is composed of 70% YYH and 30% *Fructus psoraleae* [22,23]. This preparation was reported to be phytoestrogen rich and has been used as an alternative to HRT to strengthen bone and muscle in postmenopausal women without adverse estrogenic effects on reproductive tissues [21–23]. Because traditional medicine and related research is basically an efficacy-driven approach [27–29], the scientific basis for such claims was still lacking.

This experimental study was designed to use state-of-the art evaluation biotechnologies and methods to characterize the biologically active phytoestrogen of herbal preparation XLGB and to investigate its claimed protective effects on musculoskeletal tissues in old and ovariectomized rats.

Materials and methods

Animals, grouping, and treatment

Twenty-seven 11-month-old female Wistar rats (SIPPR-BK Experimental Animal, Shanghai, China) (body weight, 440.1 ± 16.0 g) were housed three per cage in the university animal house at 22°C and with daily 12-h light and 12-h dark cycles. Standard chow for rats (SIPPR-BK Experimental Animal) was provided with water ad libitum. After acclimation for 1 month, rats were divided into three body weight-matched groups, including a sham-operated group (Sham), bilateral ovariectomized group (OVX), and herbal XLGB group (n = 9, in each). Herbal XLGB extract, which is commercially available (Guizhou Xianling Pharmaceutical, Guiyang City, Guizhou, China), was administrated orally at 250 mg/kg body weight/day, which was started on day 4 after OVX, for 3 months. The dose used for this experimental study was based on body weight-based conversion of a reported effective dose of XLGB used for prevention of postmenopausal osteoporosis [22,23]. Biological active components of XLGB were quantified by high performance liquid chromatography (HPLC; Beckman System Gold, Beckman, Fullerton, CA, USA) (Fig. 1). Body weight was measured at both baseline and the end of the experiment. Urine was collected at the time of specimen collection, for which the rats were kept individually in metabolic cages for 24 h without providing food and water. Blood samples were collected for serum preparation by abdominal aorta puncture under anesthesia intraperitoneally using ketamine and xylazine (100 mg/kg and 5 mg/kg, respectively). Both urine and serum samples were then stored at −20°C before biochemical assay.

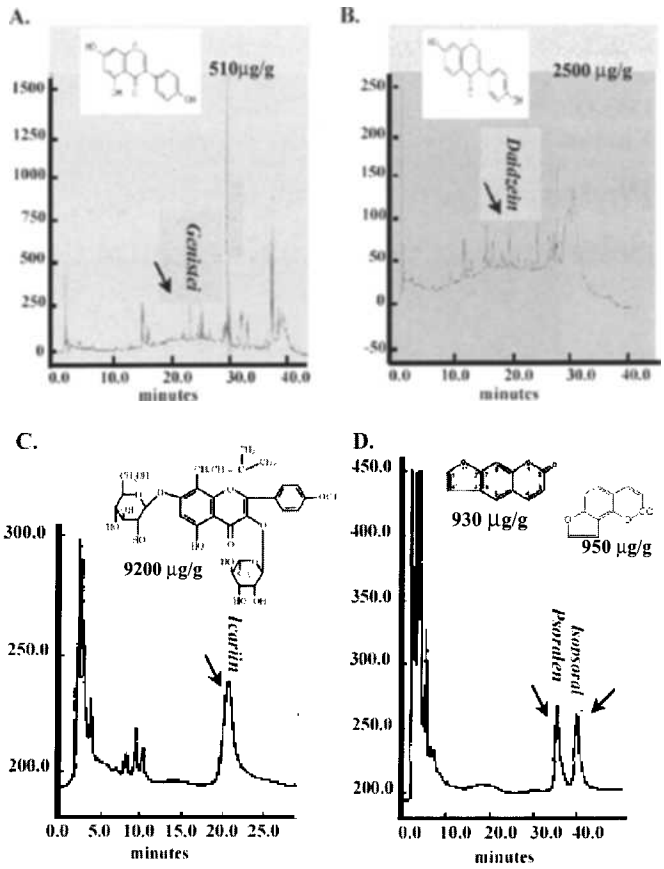

Fig. 1. High performance liquid chromatography (HPLC) chromatograms for quantification of phytoestrogen molecules of the herbal preparation Xianlinggubao (XLGB), including *genistein* 510 μg/g (**A**), *daidzein* 2500 μg/g (**B**), *icariin* 9200 μg/g (**C**), *psoralen* 930 μg/g, and *isopsoralen* 950 μg/g (**D**)

Evaluations

Rats were killed with an overdose of sodium pentobarbital at the end of 3 months after operation and treatment for the following evaluations.

Peripheral quantitative computed tomography (pQCT)

The left femur was isolated, wrapped with gauze immersed with saline, and stored at −20°C before use. A custom-made plastic rat femur holder was designed for multilayer pQCT scanning at the base of the femoral neck (Densiscan 2000; Scanco Medical, Bassersdorf, Switzerland) (Fig. 2), where fractures occurred during compressive testing as reported recently [30]. Both integral and trabecular bone mineral density (BMD) (iBMD, tBMD) of the femoral neck were measured. Neck geometry was also evaluated, including the cross-sectional area of both bony tissue and marrow cavity (CSTA and CSCA), as well as the cross-sectional moment of inertia (CSMI). The pQCT precision error of this region in rats was reported previously as ranging from 1.96% to 2.25% [31].

Micro-computed tomography (micro-CT)

Micro-CT (μCT-40; Scanco Medical) was used to evaluate the microarchitecture of one representative femur, which was selected based on the average pQCT tBMD values and scanned at a resolution of 12 μm (Fig. 3). The trabecular compartment within the greater trochanter was quantified using the provided protocol, including trabecular bone tissue volume density (BV/TV), trabecular connectivity density (Conn.Dens.), and the structure model index (SMI), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular plate separation (Tb.Sp), and degree of anisotrophy (DA) [32].

Mechanical test

A hip compressive loading jig for rats was custom-made based on a similar design to simulate site-fall configuration (Fig. 4). An angle of 10° between the femoral shaft and the horizontal and an angle of 15° internal rotation in the neck were used, mimicking the testing conditions for a human cadaver [33]. Compressive testing was performed at a speed of 2 mm/min using a material testing machine with a 2.5 KN load cell (H25KS Hounsfield Test Equipment, Redhill, Surrey, UK).

Bone turnover biochemical markers

Serum osteocalcin (OC) was assayed by radioimmunoassay using rat ^{125}I-labeled OC, goat antirat OC antibody, and donkey antigoat second antibody (Biochemical Technologies, Stoughton, MA, USA) that had 6.8% intraassay and 8.9% interassay precision error. Urinary deoxypyridinoline (DPD) was assayed after acid hydrolysis using HPLC, which showed 3.6% intraassay and 8.5% interassay precision error. Creatinine (Cr) in urine was assayed by the modified Jaffés's method, and urinary excretion of DPD was expressed as a ratio to Cr excretion [34].

mRNA expression in hip muscle

A muscle sample of about 1.5 mm^3 was obtained from the left abductor muscle, frozen in liquid nitrogen, and subsequently stored at −80°C before quantifying the mRNA expression level of the myosin heavy chain (MHC) isoform by reverse transcription-polymerase chain reaction (RT-PCR) (Light Cycler; Roche Diagnostics, Penzberg, Germany). Briefly, total RNA was isolated from the frozen muscle sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated according to the standard protocol [35]. The RNA concentration was determined by optical density (OD) at 260 nm (by using an OD_{260} unit equivalent to 40 μg/ml), and the final concentration was adjusted to

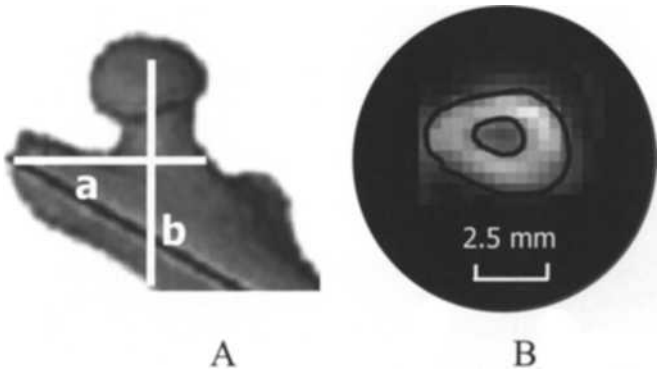

Fig. 2. Peripheral quantitative computed tomography (pQCT) scanning at base of femoral neck in rat. **A** *Line a*, the region at the base of the femoral neck; *line b*, the axis of the femoral neck. **B** Scanning was performed perpendicular to the axis of the femoral neck with a slice thickness of 1 mm at a resolution of 220 μm (pixel size). Both periosteal contour and endosteal contour were drawn for calculation of cross-sectional area of marrow cavity (*area within inner circle*) area and cross-sectional area of cortical bone (*area between two circles*)

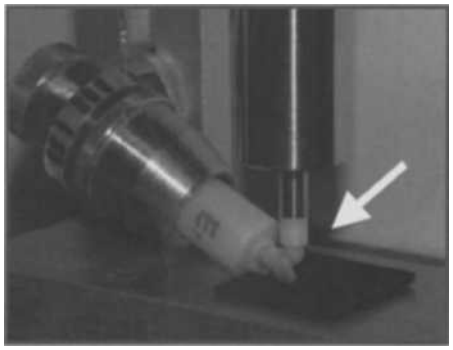

Fig. 4. The custom-made compressive testing jig for rat proximal femur in a "side-fall" configuration. The compressive load was applied through a metal indenter coated with polyethylene (*white arrow*), which enabled homogeneous stress distribution onto the femoral head during compression

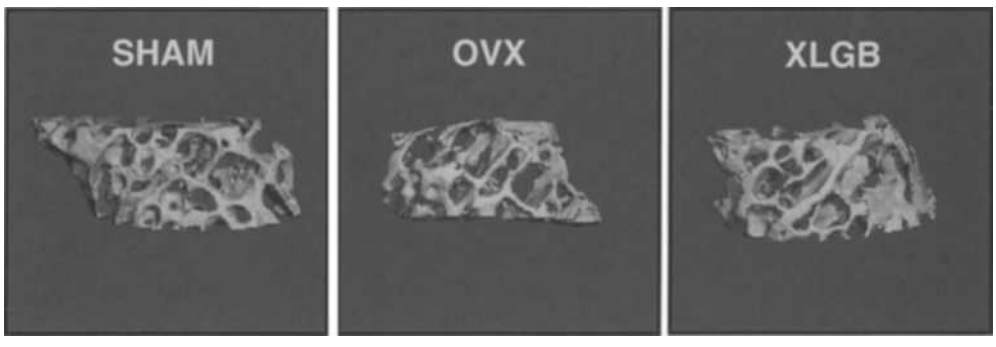

Fig. 3. Representative micro-CT 3-D microarchitecture of trabecular bone from sham-operated rats (*SHAM*), ovariectomized rats (*OVX*), and OVX rats treated with herbal preparation XLGB (*XLGB*)

Table 1. Comparison of body weight and uterine weight

Group	Body weight (g)			Uterine weight (mg)
	Baseline	Follow-up	Difference (%)	
Sham	440.0 ± 17.3	497.2 ± 19.5	13.0%*	1100.0 ± 43.2
OVX	439.9 ± 17.3	571.9 ± 22.5**	29.9%*	219.9 ± 8.7**
XLGB	440.5 ± 16.1	563.8 ± 20.6**	28.1%*	222.4 ± 8.1**

Data are mean ± SD; $n = 9$ for each group
Sham, sham operated; OVX, ovariectomized; XLGB, Xianlinggubao
* $P < 0.05$, compared with baseline; ** $P < 0.05$, compared with Sham

1 μg/μl. Total RNA was reverse-transcribed and amplified for each muscle sample before electrophoresis [36]. The primer for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as normalization control was as follows: sense primer, 5′-CATCACCATCTTCCAGGAGCG-3′, and antisense primer, 5′-TCACCTTGCCCACAGCCT TG-3′. The molecular size of the PCR products was estimated by a comparison with a DNA molecular weight marker (Smart Ladder MW; Eurogentec, Seraing, Belgium).

Statistical analysis

One-way analysis of variance (ANOVA) with post hoc test was used to evaluate the differences of skeletal and nonskeletal variables among the three groups. The results are presented as mean ± SD; statistical significance was set for $P < 0.05$. All statistical evaluations were performed using SPSS version 10.0 (SPSS, Chicago, IL, USA).

Results

Quantification of bioactive components of herbal XLGB

HPLC measurements showed that the herbal formula XLGB was composed of isoflavone, flavone, and coumarin, with quantifiable phytoestrogens including genistein 510 μg/g, daidzein 2500 μg/g, icariin 9200 μg/g, psoralen 930 μg/g, and isopsoralen 950 μg/g.

Body weight and uterine weight

Both OVX and XLGB-treated rats showed significantly higher body weight (15.0% and 13.4%) and lower uterine weight (79.8% and 80.0%) as compared with those of sham-operated rats, respectively ($P < 0.05$) (Table 1).

pQCT measurements

XLGB-treated OVX rats showed no difference in BMD to the Sham group, but significantly 12.9% and 33.3% higher iBMD and tBMD, respectively, as compared with those of OVX rats ($P < 0.05$). However, the modest increase in CSTA of OVX rats compared to the Sham group was significantly enhanced by XLGB ($P < 0.05$). OVX rats revealed on average significantly higher CSCA (40.8% and 38.5%) as compared with Sham and XLGB-treated rats, respectively ($P < 0.05$). In addition, the CSMI of the OVX group was also correspondingly lower (21.9% and 24.2%) than that of both Sham and XLGB-treated groups, respectively ($P < 0.05$) (Table 2).

Micro-CT analysis

Representative samples showed no differences in all measured structural parameters between XLGB-treated OVX rats and the Sham group. As compared to OVX alone, XLGB-treated OVX rats showed 66.7%, 14.1%, 43.3%, and 22.2% higher values in BV/TV, Tb.N, Conn.D, and Tb.Th, respectively, whereas values were 7.8% and 26.0% lower in Tb.Sp and SMI, respectively. No obvious difference was found in structural variables between the SHAM and XLGB groups (Table 2).

Mechanical test

The XLGB treatment group showed no difference to the Sham group, but significantly 22.0% higher maximal compressive load as compared with that of OVX rats ($P < 0.05$) (see Table 2).

Biochemical markers

Significantly high bone turnover was shown in OVX rats, with 15.0% higher serum OC and 36.0% higher urinary DPD as compared with Sham rats ($P < 0.05$). Herbal XLGB revealed biphasic effects in inhibiting OVX-induced increase in DPD level by 21.7% ($P < 0.05$ vs. OVX group) and maintaining OVX-increased bone formation with 13.3% increase in serum OC level ($P < 0.05$ vs. Sham group) (see Table 2).

RT-PCR of muscle gene expression

OVX rats showed significant downregulation of mRNA expression level with 65.6%, 47.4%, and 33.3% lower MHC isoform gene for MHC-I, MHC-IIa, and MHC-IIb, respectively ($P < 0.05$, all) as compared with sham-operated controls. The herbal XLGB-treated rats even revealed

Table 2. Comparison of bone mineral, structure, strength, and turnover markers

Methods	Variables	Sham	OVX	XLGB
pQCT	iBMD (g/cm^3)	1.30 ± 0.13	1.16 ± 0.10*	1.31 ± 0.13**
	tBMD (g/cm^3)	1.21 ± 0.27	0.90 ± 0.21*	1.20 ± 0.27**
	CSTA (mm^2)	14.91 ± 1.37	15.97 ± 1.55	16.71 ± 1.58*
	CSCA (mm^2)	4.80 ± 1.43	6.76 ± 1.61*	4.88 ± 1.27**
	CSMI (mm^4)	22.82 ± 4.19	17.82 ± 2.95*	23.51 ± 4.31**
Micro-CT[a]	BV/TV (%)	0.24	0.12	0.20
	Conn.D (1/mm^2)	18.43	15.43	22.11
	SMI	0.26	1.04	0.77
	Tb.Th (mm)	0.12	0.09	0.11
	Tb.Sp (mm)	0.44	0.51	0.47
	Tb.N (1/mm)	2.29	1.92	2.19
Mechanical test	Max-Load (N)	83.57 ± 11.26	69.56 ± 10.74*	84.89 ± 8.04**
Biochemical marker	OC (nmol/l)	5.47 ± 0.22	6.29 ± 0.24*	6.20 ± 0.23*
	DPD (pg/ml)	63.20 ± 25.38	85.94 ± 9.31*	67.25 ± 11.09**

Data are mean ± SD; $n = 9$ for each group

[a]Only one representative sample with average pQCT BMD value was available for microCT evaluation

pQCT, peripheral quantitative computed tomography; micro-CT, μ-computed tomography; iBMD, integral bone mineral density; tBMD, trabecular BMD; CSTA, cross-sectional area of bony tissue; CSCA, cross-sectional area of marrow cavity; CSMI, cross-sectional moment of inertia; BV/TV, trabecular bone tissues volume density; Conn.D, trabecular connectivity density; SMI, structure model index; Tb.Th, trabecular thichness; Tb.Sp, trabecular plate separation; Tb.N, trabecular number; OC, osteocalcin; DPD,deoxypyridinoline

*$P < 0.05$, compared with Sham; **$P < 0.05$, compared with OVX

Table 3. Comparison of mRNA expression level of MHC-I/IIa /Ib gene of adductor muscles by RT-PCR

Gene expression	Sham	OVX	XLGB
MHC-I mRNA	0.61 ± 0.02	0.21 ± 0.02*	0.71 ± 0.02*,**
MHC-IIa mRNA	0.19 ± 0.02	0.10 ± 0.01*	0.58 ± 0.03*,**
MHC-IIb mRNA	0.42 ± 0.02	0.28 ± 0.02*	1.20 ± 0.03*,**

Data are mean ± SD; $n = 9$ for each group

MHC, myosin heavy chain; RT-PCR, reverse transcription-polymerase chain reaction

*$P < 0.05$, compared with Sham; **$P < 0.05$, compared with Sham

significant upregulation of mRNA expression of the MHC isoform gene, with 16.4%, 205.3%, and 185.7% for MHC-I, MHC-IIa, and MHC-IIb genes, respectively, as compared to the Sham group ($P < 0.05$, all) (Table 3).

Discussion

Traditional herbal medicine has been widely used in clinical practice in many Oriental countries over thousands of years [24,27,28]. This study used a standard OVX rat model and systematically evaluated a commercially available "kidney toning" herbal preparation, XLGB, which was claimed to be effective in the prevention and treatment of osteoporosis clinically.

The rat hip was used as the site for studying the potential therapeutic effects of XLGB on OVX-induced deterioration or fragility of both skeletal (femoral neck base) and nonskeletal (adductor muscle) compartments. pQCT evaluations suggested that the herbal treatment was able to inhibit OVX-induced bone resorption on the endocortical envelope and slightly enhance bone formation on the periosteal envelope, based on evaluation of cross-sectional area of marrow cavity (CSCA), cross-sectional area of cortical bone (CSTA), as well as the bone distribution interpreted by CSMI. Such phenomena were also seen in studies using growth hormone- or parathyroid hormone (PTH)-treated OVX rats [37–39]. Micro-CT evaluation of the current study also suggested protective effects of XLGB on trabecular microarchitecture. Mechanical strength of proximal femur was therefore maintained in OVX rats treated with XLGB. Such antiosteoporotic effects of XLGB were explained by evaluation of biochemical bone turnover markers of the current study, which showed suppression of bone resorption with lower urinary DPD and moderate increased bone formation with higher serum OC as compared with Sham rats. The pharmacological estrogenic effects of herbal XLGB may partially be attributed to its biologically active phytoestrogens, including the most potent genistein and daidzein [13,15,21,40].

The importance of phytoestrogen in regulation of bone homeostasis has been recognized mainly by their direct action on osteoblasts via genomic mechanisms involving the

activation or inhibition of the nuclear estrogen receptor (ER-β) [40–43]. The inhibition of osteoclastic bone resorption resulting from direct action of phytoestrogens has also been reported, but presumably via nongenomic mechanisms because mammalian osteoclasts lack estrogen receptors or from an indirect action mediated by inhibitory cytokines released by osteoblasts in response to the actions of phytoestrogens [42–44]. Several previous experimental studies using an OVX rat model also showed similar protective effects of extracts composed of four or more medicinal herbs on estrogen deficiency bone loss, evaluated biochemically and mechanically and by histomorphometry and bone densitometry [18,19,45]. In vitro bioassay also suggested that the phytoestrogen-containing herbal extracts inhibited osteoclastic activities and moderately stimulated osteoblastic activity [17,46]. A synthetic phytoestrogen (iproflavone) also revealed a similar cellular mechanism [47]. A direct comparison for treatment among studies done by others and the current study is, however, not possible because of differences in herbal formulae and concentration of phytoestrogens (not specified in some other studies), and in age of rats, duration of the intervention, measurement site, and evaluation techniques used. However, in accordance with findings of others related to potential stimulating effects on the uterus [19,45], the current study also did not show that XLGB induced uterine hypertrophy.

It is well known that nonskeletal factors play an important role in prevention of fragility fractures, especially fall-related hip fractures [2,8,10,48,49]. The present study was the first one to examine the claim of beneficial effects of this herbal preparation on nonskeletal effects by studying herbal intervention effects on the gene expression of the rat abductor muscle. Results showed that the OVX-induced downregulation of MHC isoform mRNA was upregulated after herbal treatment, especially for type II muscle fibers. The underlying mechanism might also be related to the estrogen receptor ER-β recently reported in skeletal muscle fibers [50,51]. The age-related impairment in balance and postural control was reported to be attributed to sarcopenia and deterioration of type IIa muscle fibers [52]. Whether more ER-β is distributed in type IIa muscle fibers remains a subject for further investigation. Clinical evidence to confirm findings generated from animal experiments is highly desirable, although it was speculated that as compared with Western women, the lower incidence of hip fractures in Oriental countries was associated with high consumption of dietary phytoestrogen-rich food [11–15].

A few limitations related to design of the study and data interpretation are noted for the present study. First, there was only one representative sample with average pQCT measurement value for micro-CT evaluation, although our recent study showed consistent or parallel OVX-induced microarchitecture deterioration of trabecular bone in goats measured by the same pQCT and micro-CT methods [32]. Second, muscle morphology at tissue level and function or even gait pattern were not determined, which are definitive parameters showing better association with body balance, posture, and fall incidence and fragility fractures [53–55]. Third, the study was not able to delineate whether the preventive effect of the herbal preparation on OVX-induced bone loss was also partially attributed to better muscle–bone interaction [49] associated with the finding of higher mRNA expression of MHC isoforms, which are regarded as molecules responsible for converting chemical energy into mechanical force during muscle contraction [56], or the higher body weight found in OVX rats with or without herbal treatment.

Finally, it should be emphasized that based on the recommendation made by the practice of traditional herbal medicine [27,28,57], the approach for quantifying the phytoestrogen profile in the current study may serve the basis of quality control of the herbal preparation XLGB apart from the defined herbal combination with 70% *Epimedium leptorhizum* and 30% *Fructus psoraleae*.

In conclusion, this was the first experimental study to show a clinically used herbal preparation XLGB in prevention of OVX-induced deterioration of muscuoloskeletal tissues at the hip without inducing uterine hypertrophy. This finding implies the potential of such herbal preparations in reduction of fragility fractures to result from both skeletal and nonskeletal pathways.

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