ORIGINAL ARTICLE

Gushukang exerts osteopreserve effects by regulating vitamin D and calcium metabolism in ovariectomized mice

Xiao-Li Li[1] · Liang Wang[2] · Xiao-Lei Bi[3] · Bing-Bing Chen[1] · Yan Zhang[4]

Abstract

Calcium homeostasis plays vital roles in the management of bone health. Traditional herbal formula Gushukang (GSK) was clinically applied to treat primary osteoporosis. This study aimed to explore the osteoprotective effects of GSK and its roles in maintaining calcium homeostasis in ovariectomized (OVX) mice. The OVX mice were orally treated with low (0.38 g/kg), middle (0.76 g/kg) and high (1.52 g/kg) dose of GSK for 8 weeks. GSK treatment dramatically increased serum calcium level and decreased urinary calcium excretion as well as enhanced calcium content in bone of OVX mice. Serum level of 25-hydroxyvitamin D was significantly increased in OVX mice with exposure to GSK. Treatment with GSK improved bone mass and micro-structure of trabecular bone at distal metaphysis of femur and proximal metaphysis of tibia in OVX mice shown by safranin O staining and micro-CT measurement. GSK treatment at all doses up-regulated mRNA expression of calcium-binding protein-28k and vitamin D receptor in kidney of OVX mice, and dose-dependently decreased mRNA expression of claudin-14 and elevated mRNA expression of claudin-16 in duodenum of OVX mice. Taken together, GSK exerted beneficial effects on trabecular bone of OVX mice by improving calcium homeostasis via regulating paracellular calcium absorption in duodenum and transcellular calcium reabsorption in kidney.

Keywords Gushukang · Calcium transport · Kidney · Duodenum · Osteoporosis

Introduction

Over the past decade, the number of individuals taking calcium supplementation worldwide has been on the rise [1]. This is mostly because of the established role of calcium in the prevention and treatment of osteoporosis and, to a lesser extent, its role in the prevention of fractures. Recently, a rising body of evidence on the adverse effect of calcium supplementation on nonskeletal, especially cardiovascular, health has been a cause for concern [2, 3]. With the advance in general interest in alternative medicine and natural products, traditional herbal medicine can serve as a viable source to offer benefits for maintenance of calcium balance, consequently improving bone health [4].

The disorders of calcium metabolism, such as in vivo negative calcium balance, results in development of osteoporosis; thus, the fine-tuning on circulating calcium concentration plays key role in management of bone health [5]. This challenging task is accomplished by a combination of transcellular and paracellular calcium transport [6]. The paracellular pathway is a route for passive transport that passes between tubule cells with the tight junction constituting the primary permeability barrier. Claudins are members of a family of tight-junction membrane proteins that act simultaneously as paracellular pores and barriers and determine the selectivity to small ions and neutral solutes [7]. Earlier studies found that the herbal medicine *Ligustrum lucidum* Ait. and its active component could maintain bone mass and

Xiao-Li Li and Liang Wang contributed equally to this work.

✉ Yan Zhang
  medicineyan@163.com

[1] School of Medical Instrument and Food Engineering, University of Shanghai for Science and Technology, Shanghai 200093, People's Republic of China

[2] Department of Orthopaedics, The 309th Hospital of Chinese People's Liberation Army, Beijing 100091, People's Republic of China

[3] Liaoning Konruns Pharmaceutical Co., Ltd, Dandong 118301, People's Republic of China

[4] Spine Disease Research Institute, Longhua Hospital, Affiliated to Shanghai University of Traditional Chinese Medicine, Room 908, Building #12, South Wanping Road 725, Shanghai 200032, People's Republic of China

increase bone mechanics by increasing transcellular calcium transport via facilitating intestinal calcium absorption and/or renal calcium reabsorption in aged female rats [8] and ovariectomized (OVX) rats [9] as well as in growing male rats [10] and female rats [11]. However, so far there is no report demonstrating the effect of traditional herbal medicine on paracellular calcium transport.

An herbal medicine formula, Gushukang (GSK), consists of seven traditional Chinese herbs, such as *Herba Epimedii*, *Rhizoma Drynariae*, *Rehmannia glutinosa* (Gaertn.), and *Radix Astragali*, and is recorded in Chinese Pharmacopeia. Previous clinical study showed that GSK could slow down loss of bone mass and elevate bone mineral density (BMD) in patients with primary osteoporosis [12]. Pharmacological studies indicated that GSK was capable of increasing BMD, restoring bone microstructure, strengthening bone biomechanical properties and promoting bone fracture healing [13]. It remains unclear whether GSK could protect against osteoporosis via improving calcium homeostasis.

In this study, the female mice with estrogen deficiency by ovariectomy were used for mimicking postmenopausal osteoporosis. The osteoprotective effects of GSK and its role in maintaining calcium homeostasis in OVX mice were studied; furthermore, the potential molecular mechanism involved in the regulation of GSK on calcium metabolism was investigated from the view of transcellular and paracellular calcium transport.

Materials and methods

Animals study design

Five-month-old female C57BL/6J mice (Slac Laboratory, Shanghai, China) were housed in a specific pathogen-free room with alternating 12 h periods of light and darkness, a constant temperature of 23 ± 1 °C, and humidity of $55 \pm 5\%$. The mice were either dorsal ovariectomized (OVX) or sham-operated (Sham) under light ether anesthesia. Starting from 1 week post-surgery, the mice were divided into five groups with ten in each group: sham-operated mice (Sham), OVX mice orally administered by intragastric gavage with vehicle (OVX), low (GSK-L, 0.38 g/kg body weight), middle (GSK-L, 0.76 g/kg body weight) or high dose (GSK-L, 1.52 g/kg body weight) of Gushukang (Konruns Pharmaceutical Co., LTD., Liaoning, China). The low dose of GSK in mice was equivalent to that used in patients. The phytoestrogen-free diet (D00031602, Research Diet Inc, New Brunswick, NJ, USA) was used during experiment. It was prepared according to the AIN-93M formulation where corn oil was used instead of soybean oil.

After 8 weeks of treatment, blood was taken by cardiac puncture exsanguination. Serum was collected and stored at −80 °C for further biochemical analyses. The uterus and bilateral tibias and femurs were aseptically removed. The uterus was weighed to confirm the success of ovariectomy. Duodenal mucosa and kidney were collected rapidly and stored at −80 °C. All procedures were reviewed and approved for consideration of animal welfare by the Animal Ethics Committee of University of Shanghai for Science and Technology.

Chemistries in serum and urine

Calcium (Ca) concentration in serum and urine was measured by standard colorimetric methods as previously described [14]. Serum levels of phosphorus (Wako Pure Chemical Industries Ltd., Osaka, Japan), intact parathyroid hormone (Immutopics, Inc., San Clemente, CA, USA), 25-hydroxyvitamin D (IDS, Boldon, UK) and calcitonin (LifeSpan BioSciences, Inc., Seattle, WA, USA) were determined. Urinary creatinine level was measured by Creatinine Colorimetric Detection Kit (ALPCO, Salem, NH, USA).

Calcium content in bone

The tibias were incinerated at 800 °C for 12 h and the ash weighed. About 10 mg of bone ash was then dissolved in 2 ml of 37% HCl and diluted with Milli-Q water. The calcium content was determined by the kit used for calcium assay in serum and urine.

Histological staining on bone

The tibias and femurs were fixed in 4% formaldehyde/PBS (pH 7.2), decalcified in 0.5 M EDTA (pH 8.0), and embedded in paraffin by standard histological procedures. Serial sections of 3 μm were cut. Safranin O (Sigma-Aldrich) staining was performed together with fast green and counter stain by hematoxylin. Stained slides were visualized under microscope. Trabecular bone mass expressed as bone area over total area (BA/TA) was measured at the femoral end and the tibial head using an OsteoMeasure system (OsteoMetrics Inc., Decatur, GA, USA).

Micro-CT analysis

The tibia and femur of each animal were scanned to obtain three-dimensional (3D) images and quantitative parameters of trabecular bone at proximal metaphysis of tibia and distal metaphysis of femur. The scanning parameters used were 70 kVp, 111 μA, and 1000 projections per 180°, resulting in a 10.5 μm isotropic voxel size and a total scan time of 13.2 min with a high-resolution micro-viva-CT40 system (Scanco Medical, Bassersdorf, Switzerland). Trabecular bone micro-architecture was assessed using the

μCT Evaluation Program (Image Processing Language v. 5.0A, Scanco). The 3D parameters for trabecular bone were obtained as follows: (1) mean mineral density of total volume (BMD/TV); (2) trabecular bone number (Tb.N); (3) trabecular bone thickness (Tb.Th); (4) trabecular bone separation (Tb.Sp).

RT-PCR

Total tissue RNA was isolated according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 3 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo $dT_{(15)}$ primers (Fermentas). The first-strand cDNAs served as the template for the regular PCR performed using a DNA Engine (ABI). β_2-Microglobulin (β2M) 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 were as previously described [15]. The primer sequences for claudins were as following: claudin-2, forward, TCCAGAGCTCTTCGAAAGGA, reverse, TAGGATGTAGCCCACCAGTT; claudin-14, forward, CACTGGCCTTAGAGCTTCCATT, reverse, CTC CACTCACATACAGAACAGC; claudin-16, forward, GCG ACAGAGACCAATAGCAA, reverse, AGTCATCAGCGT TCACCATC.

Statistical analysis

The data from these experiments were reported as mean ± standard error of mean (SEM) for each group. All statistical analyses were performed using PRISM version 5.0 (GraphPad). If variances associated with each experimental mean were unequal (Bartlett's test for homogeneity of variances), the data were log-transformed before analysis. Inter-group differences were analyzed by one-way ANOVA, and only followed by Tukey's multiple comparison test as a post test to compare the group means if overall $P < 0.05$. Differences with P value of less than 0.05 were considered statistically significant.

Results

Body weight and uterus index

Ovariectomy induced the increase in body weight of mice (Fig. 1a, $P < 0.05$, vs. Sham) during experimental period, and treatment with Gushukang (GSK) at all doses for 4, 6 and 8 weeks could significantly ($P < 0.05$) suppress the
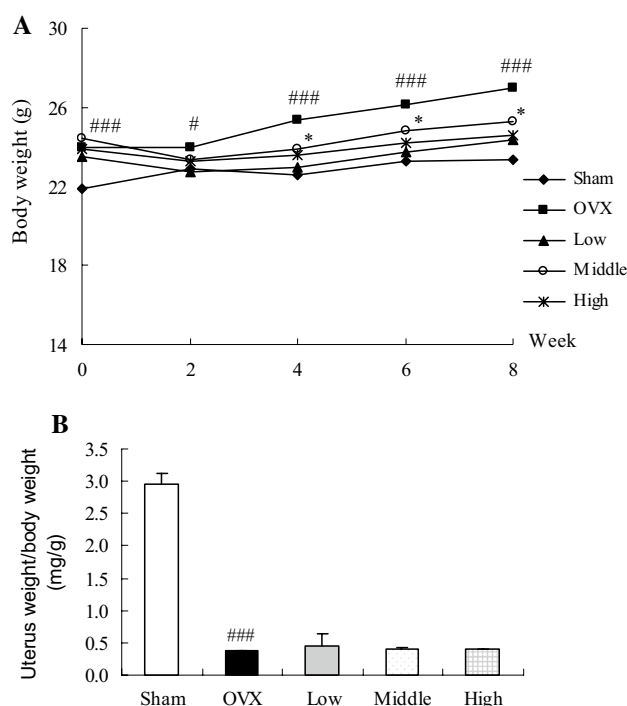
Fig. 1 Effects of Gushukang on the changes of body weight during experimental period and uterus index in ovariectomized mice. **a** Body weight. **b** Uterus weight over body weight at killing. Values were expressed as mean ± SEM, $n = 10$. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, vs. Sham; $^{*}P < 0.05$, vs. OVX

elevation in body weight as compared to those of vehicle-treated ovariectomized (OVX) mice. As expected, estrogen deficiency induced by ovariectomy resulted in the atrophy of uterus, as reflected by the significant decrease in uterus index of OVX mice (Fig. 1b, $P < 0.001$, vs. Sham). Treatment of OVX mice with GSK did not produce any changes in uterus index.

Calcium content in serum, urine and bone

As compared to Sham control, the calcium level in serum and urine of OVX mice was significantly reduced (Table 1, $P < 0.05$) and enhanced ($P < 0.05$), respectively; moreover, the ratio of calcium content over ash weight (Ca/ash, $P < 0.05$) and the calcium content in tibia ($P < 0.01$) were both decreased in OVX mice, reflecting that unbalanced calcium metabolism occurred in estrogen-deficient mice. GSK treatment dramatically inhibited ovariectomy-induced changes of serum and urinary calcium level ($P < 0.05$) as well as increased calcium content in bone of OVX mice ($P < 0.05$). In addition, serum phosphorus level was significantly decreased in OVX mice with GSK treatment at high dose as compared to vehicle-treated OVX mice ($P < 0.05$).

Table 1 Calcium level in serum, urine and bone

	Sham	OVX	GSK-L	GSK-M	GSK-H
Serum Ca (mg/dl)	10.46 ± 0.21	$9.67 \pm 0.19^{\#}$	$10.32 \pm 0.15^{*}$	$10.35 \pm 0.20^{*}$	$10.39 \pm 0.28^{*}$
Serum P (mg/dl)	10.17 ± 0.51	9.45 ± 0.49	9.42 ± 0.48	9.72 ± 0.47	$7.53 \pm 0.31^{*}$
Urine Ca/Cr (mg/mg)	0.27 ± 0.05	$0.49 \pm 0.11^{\#}$	$0.30 \pm 0.06^{*}$	$0.29 \pm 0.06^{*}$	$0.28 \pm 0.03^{*}$
Ca/ash (mg/mg)	0.468 ± 0.009	$0.415 \pm 0.007^{\#}$	$0.451 \pm 0.013^{*}$	$0.459 \pm 0.011^{*}$	$0.461 \pm 0.010^{*}$
Tibial Ca (mg)	13.4 ± 0.7	$7.9 \pm 0.5^{\#\#}$	$11.8 \pm 0.6^{*}$	$11.6 \pm 0.5^{*}$	$12.5 \pm 0.3^{**}$

The ovariectomized (OVX) mice were treated with vehicle, or Gushukang with low (GSK-L, 0.38 g/kg), middle (GSK-M, 0.76 g/kg) and high (GSK-H, 1.52 g/kg) dose for 8 weeks. Values were expressed as mean $\pm$ SEM, $n = 10$ ($n = 7$ for Ca/ash)

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. Sham; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. OVX
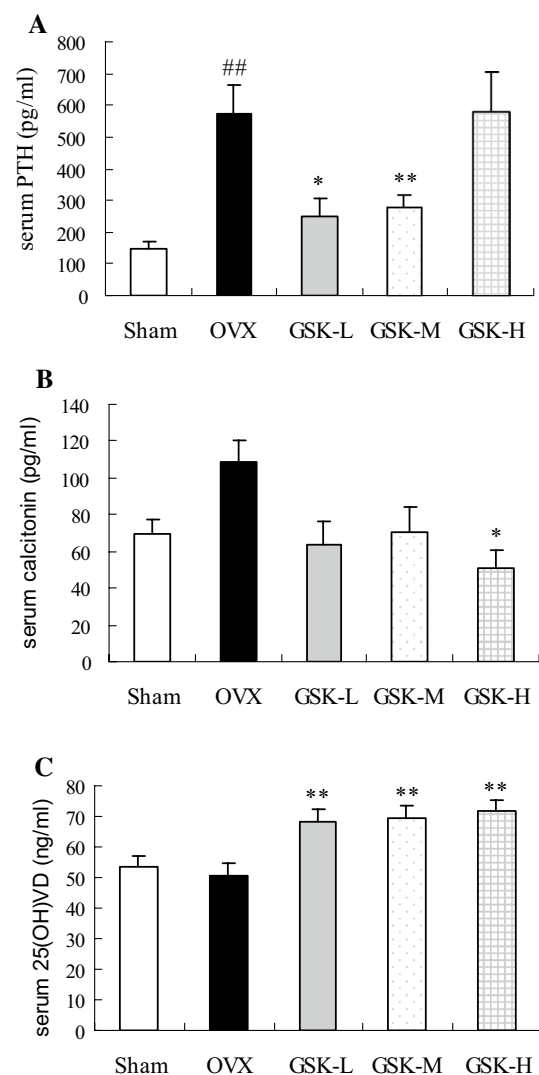
Calciotropic hormone level in serum

Parathyroid hormone (PTH), calcitonin and 25-hydroxy-vitamin D [25(OH)VD] are all calciotropic hormones, the levels of which were measured in serum in this study. Ovariectomy elevated serum PTH level by almost threefold (Fig. 2a, $P < 0.01$), but did not statistically alter serum level of calcitonin (Fig. 2b) and 25(OH)VD (Fig. 2c) as compared to those in Sham group. The OVX mice treated with GSK showed the decrease in serum PTH level in low-dose ($P < 0.05$) and middle-dose ($P < 0.01$) groups, and the reduction in serum calcitonin level ($P < 0.05$) in high-dose group, as compared to those in vehicle-treated OVX group. Intriguingly, the serum 25(OH)VD level in GSK-treated OVX mice was consistently increased with comparison to that in vehicle-treated OVX mice ($P < 0.01$).

Safranin O staining on femur and tibia

The study on local histomorphology (Fig. 3) was performed on distal femoral end (Fig. 3a, top panel) and proximal tibial metaphysis (Fig. 3a, bottom panel) by safranin O staining. The images showed the loss and destruction of woven trabecular bone in OVX mice (Fig. 3a). Treatment with GSK moderately attenuated the ovariectomy-induced pathological changes of trabecular bone at femur and tibia. The quantitative data shown by bone area over total area (BA/TA) demonstrated that the trabecular bone area at the femoral end ($P < 0.05$) and the tibial head ($P < 0.01$) was significantly decreased in OVX mice as compared to those in Sham mice (Fig. 3b). The treatment with GSK could markedly enhance BA/TA at both bone sites in OVX mice.

Micro-CT analysis on femur and tibia

The profiles of three-dimensional (3D) images (Fig. 4a) clearly demonstrated the loss of trabecular bone mass and the breakage of cancellous bone at distal metaphysis of femur (top panel) and proximal metaphysis of tibia (bottom panel) of OVX mice, which were in agreement with

Fig. 2 Effects of Gushukang on the level of calciotropic hormones in serum of ovariectomized mice. **a** Parathyroid hormone (PTH). **b** Calcitonin. **c** 25-Hydroxyvitamin D [25(OH)VD]. Values were expressed as mean $\pm$ SEM, $n = 10$. $^{\#\#}P < 0.01$, vs. Sham; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. OVX

A

Sham   OVX   GSK-L   GSK-M   GSK-H

B
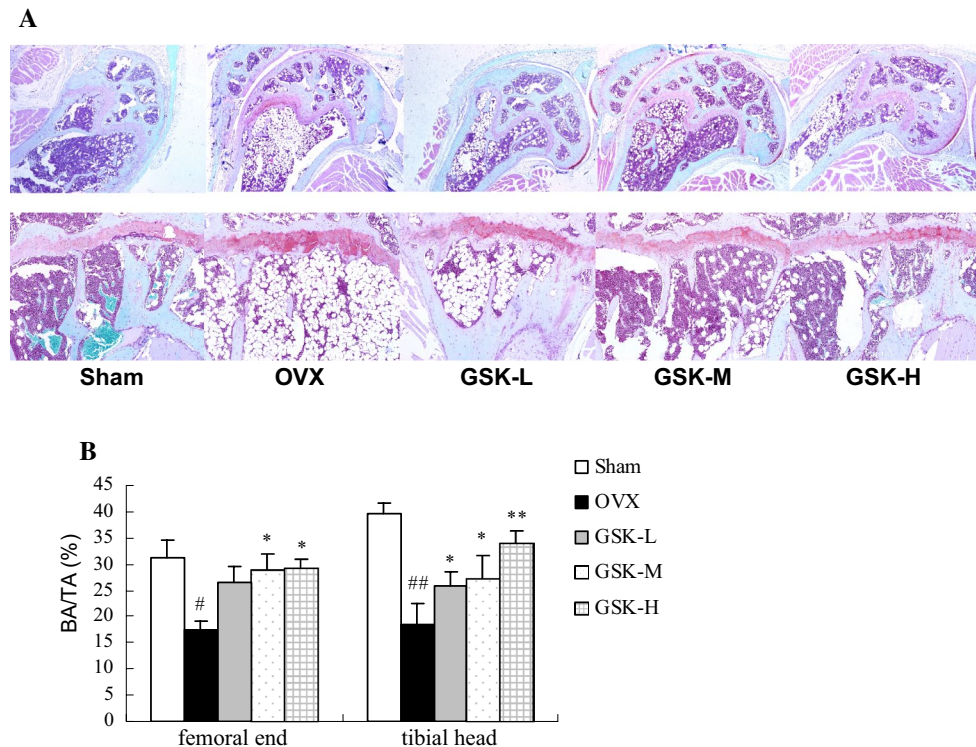
Fig. 3 Effects of Gushukang on histology of femur and tibia in ovariectomized mice. **a** Top panel, distal femoral metaphysis (magnification ×40). Bottom panel, proximal tibial metaphysis (magnification ×100). **b** Trabecular bone area over total area (BA/TA). Values were expressed as mean ± SEM, $n = 8$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. Sham; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. OVX

the results from safranin O staining. The 3D bone biological parameters for proximal tibia head (Fig. 4b) quantitatively reflected these changes. Ovariectomy triggered the decrease in trabecular bone mineral density (BMD) by 25.4% ($P < 0.05$), trabecular bone number (Tb.N) by 25.3% ($P < 0.05$), trabecular bone thickness (Tb.Th) by 25.5% ($P < 0.01$), and the increase in trabecular bone separation (Tb.Sp) by 33.1% ($P < 0.05$), as compared to those parameters in Sham group. Notably, these changes of biological parameters for bone micro-architecture and bone mass at proximal metaphysis of tibia were statistically improved in OVX mice upon to GSK treatment for 8 weeks ($P < 0.05$).

mRNA expression of regulators for vitamin D and calcium metabolism in kidney of OVX mice

Gene expression of regulators for calcium reabsorption including calcium-binding protein-9k (CaBP-9k), CaBP-28k, transient receptor potential vanilloid 5 (TRPV5) and plasma membrane calcium-ATPase 1b (PMCA1b), and for vitamin D metabolism including 25-hydroxyvitamin D 24-hydroxylase (24-OHase), 25-hydroxyvitamin D 1-hydroxylase (1-OHase) and vitamin D receptor (VDR), was determined in mice kidney by RT-PCR (Fig. 5a). Ovariectomy alone increased mRNA expression of 24-OHase

(Fig. 5b, $P < 0.01$) and decreased mRNA expression of CaBP-28k ($P < 0.05$) and VDR ($P < 0.01$). Treatment of OVX mice with GSK at all three doses could significantly up-regulate mRNA expression of CaBP-28k ($P < 0.05$) and VDR ($P < 0.01$), and high dose of GSK could down-regulate mRNA expression of 24-OHase ($P < 0.001$). However, the expression level of CaBP-9k, TRPV5, PMCA1b and 1-OHase was not different among experimental groups (Fig. 5).

mRNA expression of claudins (CLD) in kidney

Gene expression of CLD-2, CLD-14 and CLD-16 was not altered in kidney of OVX mice as compared to those of Sham-operated mice (Fig. 6a, b). Moreover, the expression level of these three regulators was comparable between GSK treatment group and OVX group, suggesting GSK did not act on CLD-2, CLD-14 and CLD-16 mRNA expression in kidney of OVX mice.

mRNA expression of regulators for duodenal calcium absorption

Gene expression of regulators for transcellular calcium absorption including CaBP-9k, TRPV6, PMCA1b and VDR
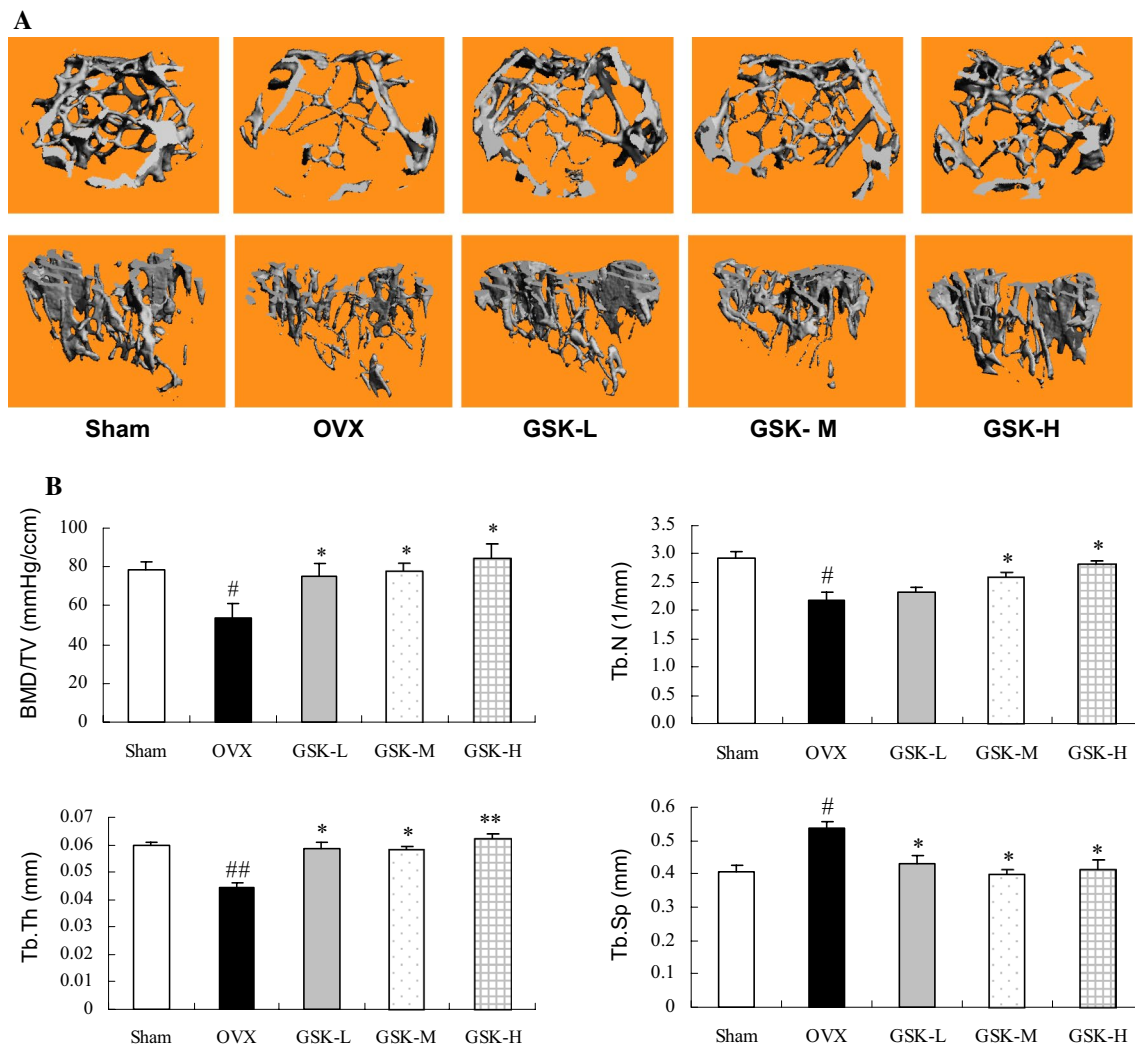
Fig. 4 Effects of Gushukang on bone mass and micro-architecture of trabecular bone in ovariectomized mice. The quantitative biological parameters were measured by micro-CT. **a** The reconstructed three-dimensional images at the distal end of femur (top panel) and the proximal metaphysis of tibia (bottom panel). **b** Bone biological parameters of proximal tibial head. *BMD/TV* bone mineral density over total volume, *Tb.N* trabecular bone number, *Tb.Th* trabecular bone thickness, *Tb.Sp* trabecular bone separation. Values were expressed as mean ± SEM, $n = 8$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. Sham; $*P < 0.05$, $**P < 0.01$, vs. OVX

was not changed in duodenum of OVX mice in response to GSK administration (data not shown). GSK treatment markedly reversed the ovariectomy-induced up-regulation of CLD-14 ($P < 0.05$) and the down-regulation of CLD-16 ($P < 0.01$) in mice duodenum, whereas mRNA expression of CLD-2 in duodenum was not different among groups (Fig. 7).

Discussion

The cumulative evidences showed that calcium is one of main components in the prevention and treatment of osteoporosis [16]. However, the close relationships of calcium with myocardial contraction, nerve conduction, hormonal modulation and blood clotting may result in increased cardiovascular risk [17]. Concerns for safety of using medicinal calcium supplementation and its role in the treatment of osteoporosis amongst medical scientists are increasing [18]. Thus, traditional herbal medicines have received considerable attention because of their multiple biological effects in certain tissues and low toxicity, especially their potential actions on modulating calcium metabolism, as compared to western drugs [19].

Gushukang (GSK), a traditional Chinese medicine formula, has been clinically applied in the treatment of primary osteoporosis, and animal study also showed its beneficial effect on bone of laying hens [20]. The present study demonstrated that GSK effectively improve micro-structure of trabecular bone and increase bone mineral density as well

Fig. 5 Effects of Gushukang on mRNA expression of regulators for calcium reabsorption and vitamin D metabolism in kidney of ovariectomized mice. **a** RT-PCR bands. **b** The quantitative result for PCR. *CaBP-9k* calcium-binding protein-9k, *CaBP-28k* calcium-binding protein-28k, *24-OHase* 25-hydroxyvitamin D 24-hydroxylase, *VDR* vitamin D receptor, *1-OHase* 25-hydroxyvitamin D 1-hydroxylase, *TRPV5* transient receptor potential vanilloid 5, *PMCA1b* plasma membrane calcium-ATPase 1b. Values were expressed as mean ± SEM, $n = 10$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. Sham; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. OVX
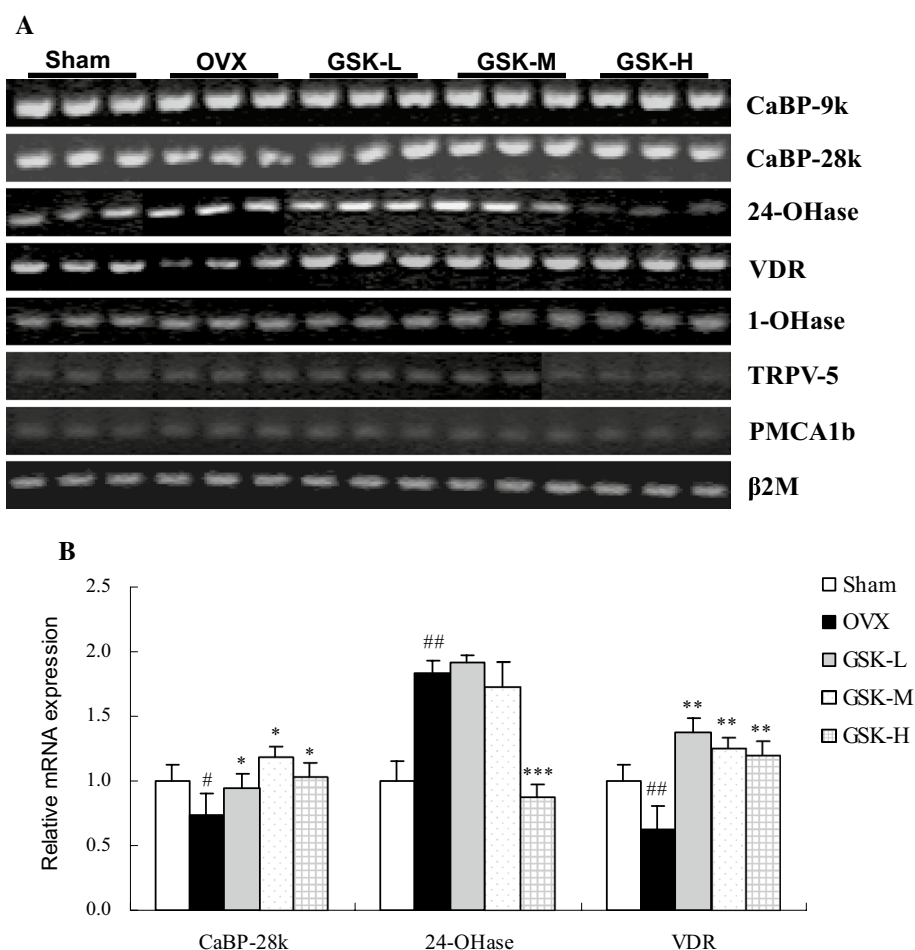
as recover trabecular bone histomorphology at distal femoral metaphysis and proximal tibial metaphysis in ovariectomized (OVX) mice, indicating the bone-sparing function of GSK in mice with estrogen deficiency mimicking postmenopausal osteoporosis. Importantly, treatment with GSK for 8 weeks did not increase uterus index of OVX mice, which demonstrated that GSK did not lead to the undesirable effect (uterotrophic responses) in uterus of estrogen-deficient mice.

Calcium content in serum, urine and bone was measured to determine if in vivo regulation of GSK on calcium homeostasis was involved in its osteoprotective effects in OVX mice. Notably, GSK was capable of enhancing calcium level in serum and bone tissue and suppressing calcium excretion in urine of OVX mice, suggesting a positive regulation of GSK on calcium balance. This study was the first to found a traditional herbal formula showing beneficial effects on calcium metabolism by reducing calcium loss and maintaining calcium balance, and our earlier studies were the first reporting that single traditional herb Fructus Ligustri Lucidi improved calcium balance in aged female rats [8], aged OVX rats [9] and diabetic mice [21], consequently attenuating bone deteriorations in rats [22] and mice [21]. Our research studies suggested that the regulation on in vivo calcium

homeostasis by alternative and complementary medicine with traditional herbal receipt or even single herb could be a potential strategy for preventing and treating osteoporosis.

Vitamin D deficiency occurred in osteoporotic postmenopausal women [23, 24], consistently this study showed the reduced level of serum 25-hydroxyvitamin D in OVX mice. Insufficient vitamin D level decreases calcium fixation in bone [23] and calcium transport in duodenum and kidney [25, 26], thus contributing to increased bone resorption and osteoporosis [27]. Our study showed that serum 25-hydroxyvitamin D level was significantly increased in OVX mice by exposure to GSK treatment with all doses, fully suggesting that GSK was able to rectify in vivo status of vitamin D deficiency, which, at least partially, explained the positive effects of GSK on in vivo calcium metabolism. The formation of 25-hydroxyvitamin D is traditionally recognized as occurring by the action of hydroxylation in liver cells, and novel pathways involved in forming 25-hydroxyvitamin D are currently found in skin and adrenal gland [28]. Therefore, how GSK affects the tissue in stimulating the production of 25-hydroxyvitamin D requires further exploration.

Given that vitamin D has a pivotal role in calcium and bone metabolism and that kidney is a major tissue for

Fig. 6 Effects of Gushukang on mRNA expression of claudins (CLD) in kidney of ovariectomized mice. **a** RT-PCR bands. **b** The quantitative result for PCR. Values were expressed as mean ± SEM, $n = 10$
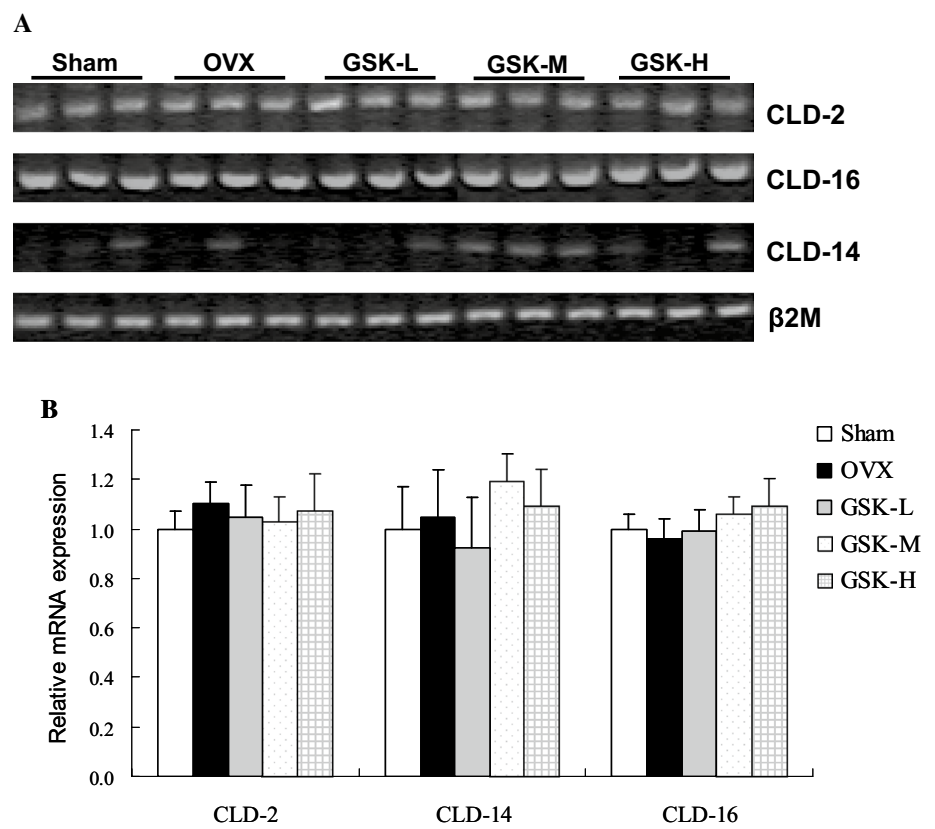
Fig. 7 Effects of Gushukang on mRNA expression of claudins (CLD) in duodenum of ovariectomized mice. **a** RT-PCR bands. **b** The quantitative result for PCR. Values were expressed as mean ± SEM, $n = 10$. $^{##}P < 0.01$, vs. Sham; $^{*}P < 0.05$, $^{**}P < 0.01$, vs. OVX
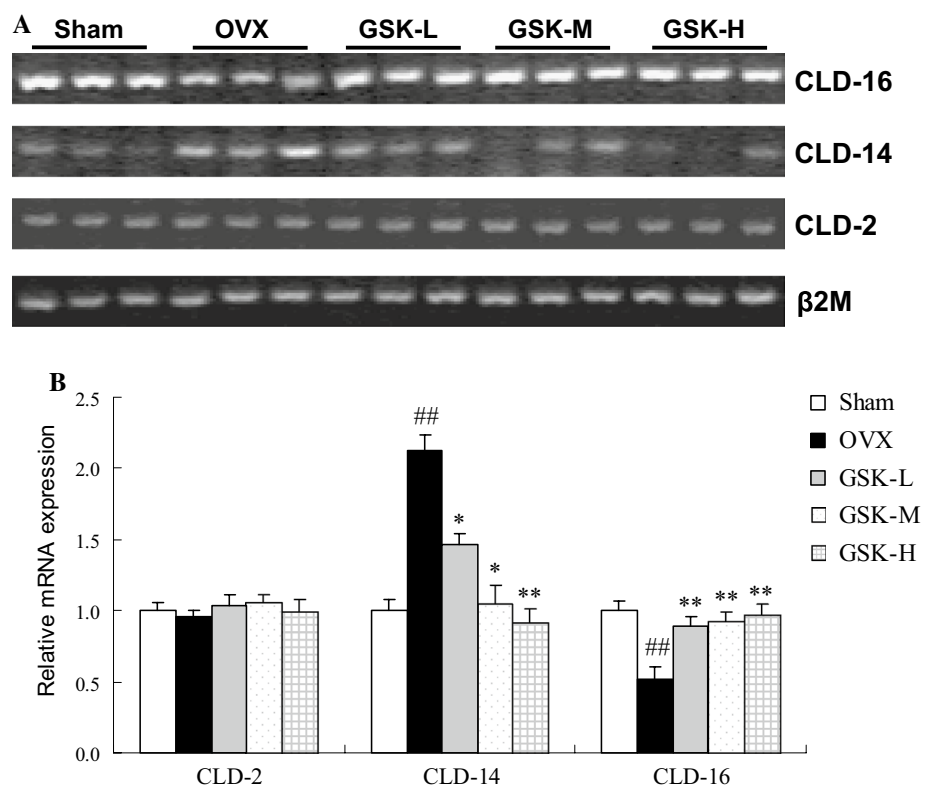
vitamin D metabolism [29], the modulation of GSK on vitamin D enzymes, 25-hydroxyvitamin D-1 hydroxylase (1-OHase) and 25-hydroxyvitamin D-24 hydroxylase (24-OHase), and vitamin D receptor (VDR) in kidney was determined. Our study showed that mRNA expression of 24-OHase and VDR in kidney of OVX mice was markedly enhanced and decreased, respectively, which could aggregate the inactivation of vitamin D and the weakening of vitamin D signaling. Treatment of OVX mice with GSK could significantly reversed the changes in the expression of renal 24-OHase and VDR, indicating that GSK potentially displayed beneficial effects on vitamin D metabolism.

Besides vitamin D, other calciotropic hormones like parathyroid hormone (PTH) and calcitonin act in a well-defined negative feedback loop to maintain calcium homeostasis [30]. Serum PTH level was significantly higher in OVX mice than that of Sham mice, which was in agreement with previous results in OVX rats [31, 32]. Significant decrease in serum level of PTH was shown in OVX mice with treatment of GSK at low and middle dose in this study, which could be attributed to GSK-induced elevation in serum level of vitamin D, since parathyroid gland is extremely sensitive to the effect of vitamin D that could decrease PTH mRNA and serum PTH level in rats and humans [33]. It needs to be further investigated to see whether GSK could be used clinically to treat patients with hyperparathyroidism. While, this study showed unexpected results that high dose of GSK reduced calcitonin level and did not influence PTH level in OVX mice, thus, the in vivo safety of GSK at high dose will be a concern in further study and the potential interaction and crosstalk among calciotropic hormones in response to GSK need to be further explored.

The circulating calcium concentration is accurately fine-tuned by the regulators in duodenum for calcium absorption and in renal tubule for calcium reabsorption [34], which is accomplished by a combination of transcellular and paracellular calcium transport [35]. Transcellular calcium transporting process is closely regulated by calcium-binding protein-28k (CaBP-28k) and -9k (CaBP-9k) in a vitamin D-dependent manner [36]. GSK-induced elevation of serum vitamin D level might account for the up-regulation of CaBP-28k in kidney of OVX mice, thereby decreasing urinary level of calcium, which might be caused by the action of *Herba epimedii* showing suppressive effect on renal calcium wasting in OVX mice [37]. However, expression of CaBP-9k was not altered in duodenum or kidney of OVX mice with exposure to GSK treatment, suggesting that other pathways like glucocorticoid receptor (GR) and PTH receptor (PTHR) might also be involved in the regulation of GSK on CaBP-9k level since GR and PTHR jointly modulate CaBP-9k in mice [38]. In addition, GSK did not act on the expression of components for active transcellular calcium absorption

in duodenum of OVX mice (data not shown), revealing that transcellular calcium absorption was not promoted by GSK treatment in OVX mice.

Many studies have focused on the highly regulated active transcellular transport pathways for calcium from the duodenum of the intestine and the tubule of the kidney [39]. However, comparatively little work has examined the molecular constituents creating the paracellular shunt across intestinal and renal epithelium [35]. Genetic mutations and gene expression changes in claudins may lead to alteration of the paracellular permeability to calcium, ultimately affecting in vivo calcium metabolism [40, 41]. Previous studies showed that claudin-16 (CLD-16) overexpression increased calcium permeability [42] and that CLD-16 knockout mice had reduced calcium permeability [26]. When co-expressed with CLD-16, CLD-14 inhibited the permeability of CLD-16 and reduced paracellular permeability to calcium [43]. Consistent with these results, this study clearly demonstrated that the OVX mice in response to GSK treatment had decreased expression of CLD-14 and increased expression of CLD-16 in duodenum, indicating GSK might stimulate duodenal paracellular calcium transport via suppressing CLD-14-inhibited CLD-16 expression. While no changes in claudins involved in calcium transport (claudin-2, claudin-14, or claudin-16) were found in kidney of GSK-treated OVX mice.

In conclusion, the present study demonstrated that GSK exerted osteoprotective effects in ovariectomized mice as shown by the improvement of trabecular bone microstructure and the enhancement of trabecular bone mass. The beneficial effects of GSK on bone tissue might be attributed to its positive regulation on calcium homeostasis by regulating vitamin D metabolism and claudins-involved passive calcium absorption in duodenum and CaBP-28k-involved active calcium uptake in kidney. Importantly, this study is the first to suggest the potential effect of traditional herbal formula on paracellular calcium absorption. The contribution of each single herb to the protective effects of GSK against postmenopausal osteoporosis needs to be further identified.

Compliance with ethical standards

Conflict of interest All authors have no conflicts of interest.

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