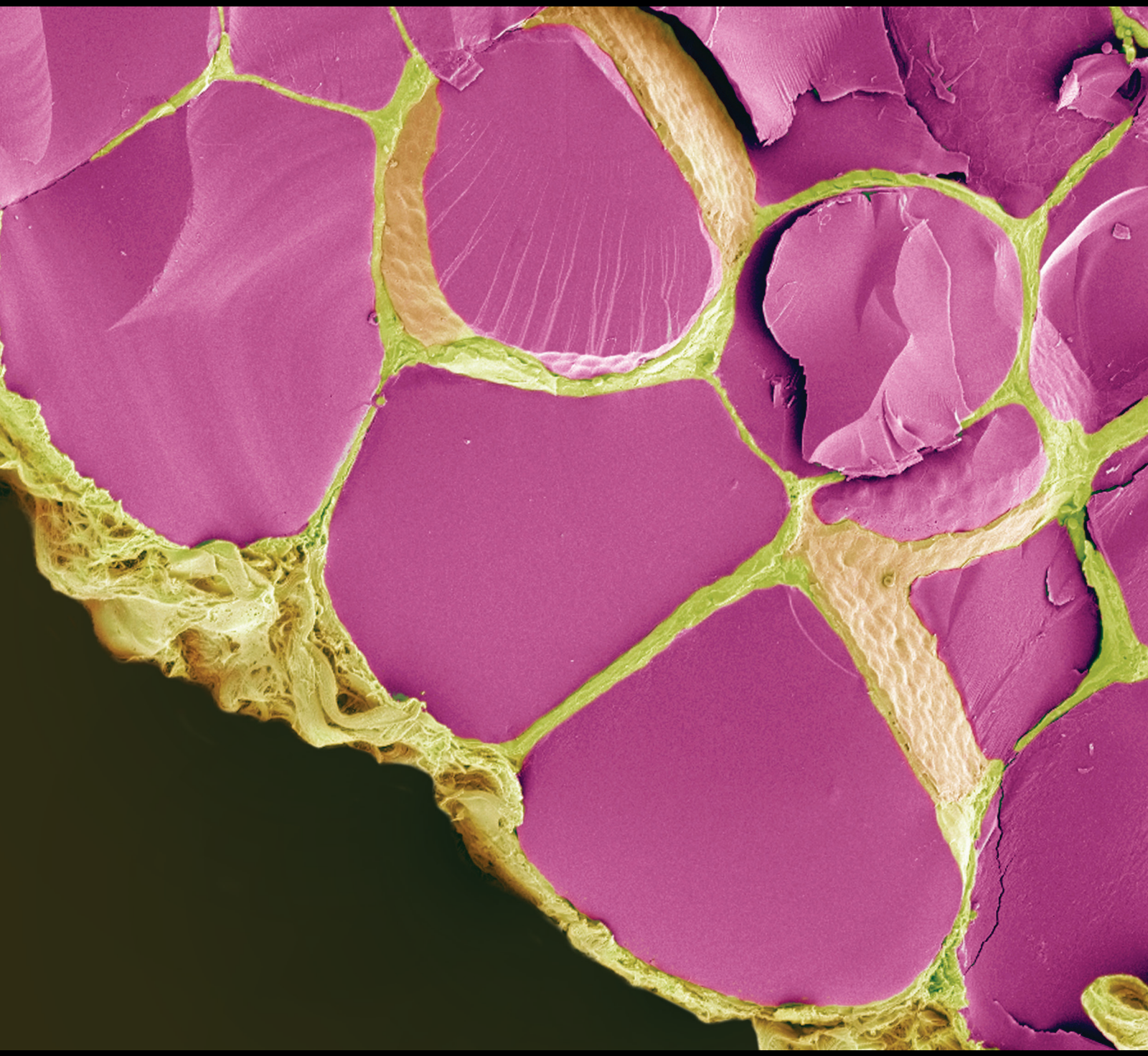
International Journal of Endocrinology

Osteoporosis 2014

Guest Editors: Ling-Qing Yuan, Hong W. Ouyang, Thomas Levin Andersen, Yebin Jiang, Francesco Pantano, and Peng-Fei Shan



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Editorial

Osteoporosis 2014

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After successfully launching the special issue of "Osteoporosis" in 2013, we are pleased to publish this updated special novel issue. The current issue focuses on the various aspects of advances in osteoporosis, including original articles with both clinical and basic research, as well as reviews. Collectively, the current issue reflects the enormous effort done worldwide to improve the understanding, identification, and treatment of osteoporosis.

Clinical Studies. Sarcopenia and osteoporosis are highly prevalent among elderly patients with frailty, which increase the risk of fracture, disability, or even death. Y. Wang et al. performed a cross-sectional analysis in 316 participants aged 65 years and older from Changsha, China, investigating the prevalence of sarcoosteoporosis. Their results show that the prevalence of sarcoosteoporosis is more likely to increase with age and even more so in the elderly with higher levels of comorbidities and with frailty/prefrailty, especially in women. Compared with DXA, quantitative computed tomography (QCT) could examine the true volumetric BMD in three dimensions at any skeletal site. Y. Ma et al. determined lumbar spine and hip volumetric bone mineral density (vBMD) in 826 Chinese adults using QCT. Their results demonstrate that, after achieving peak bone mass (30 to 39 years old in females and 20 to 29 years old in males), the vBMD is decreased with aging. Moreover, their result showed that there is a positive correlation between QCT vBMD and DXA projectional areal BMD (aBMD). Regarding detecting osteoporosis, QCT spine vBMD is more sensitive than CTXA Hip aBMD. J.-H. Huang et al. determined the effect of serum Mg on bone mineral metabolism in CKD patients with or without diabetes. Their study shows that lower serum Mg level results in deficiency in PTH action and exacerbation of osteoporosis in CKD patients, especially those with diabetes. L. Song et al. review the potential mechanisms involved in osteoporosis after organ transplant and demonstrate that combination of vitamin D with bisphosphonates and appropriate dose of glucocorticoids is the most effective protocol to increase BMD of patients with organ transplant, while signaling pathway regulator or BMSC implantation could be a novel direction for the treatment of osteoporosis in patients with organ transplantation. K. Sanders et al. investigated whether vitamin D could decrease fall and fractures. Their study enrolled 2096 females at high risk of fall and/or fracture. The participants completed a prospective 12-month daily fall calendar, which was compared with a 12-month falls recall questionnaire. The conclusion of their study is that "intensive ascertainment of falls is not feasible, 12-month falls recall questions with fewer responses may be an acceptable alternative." P. Di Carlo et al. reviewed the prevalence of

vitamin D deficiency in HIV/HCV coinfected patients. They found lower serum vitamin D in HIV/HCV coinfected patients, which might be associated with progression of live diseases.

Basic Studies. Alendronate is a commonly used medication to prevent aseptic loosening with arthroplasty. However, as an oral medication, it has low bioavailability with a long time of administration. D. Song et al. used alendronate in bone cement powder to investigate whether the content of alendronate regulated shear strength of bone-bone cement and metal-bone cement interfaces. Their results reveal that mixed alendronate and bone cement powder could reduce the shear strength at the bone-bone cement interface but not at metal-bone cement interface. K.-J. Kim et al. investigated the effect of Alisol A 24-acetate, a biologically active compound from a traditional Korean herb medicine *Alisma canaliculatum,* on the osteoclastogenesis. Their results demonstrate that Alisol A 24-acetate could decrease the osteoclastogenesis through downregulating NFATc1 and inhibit the expression of DC-STAMP and cathepsin K. They suggested the Alisol A 24-acetate might be a potential scaffold in development of new antiosteoporosis agents. S. Hu et al. used nanoindentation assessment and atomic force microscopy to evaluate the material and structural characteristics of bone in estrogen deficient rat. Their results reveal that estrogen deprivation results in deterioration of structural characteristics but not the nanomechanical properties of the trabecular bone. J.-M. Hou et al.'s study found that lactoferrin promoted osteoblast proliferation while it inhibited apoptosis through IGF-1R.

There are ongoing progresses in osteoporosis research, and the present special issue covers only some areas of new developments.

Acknowledgments

We would like to express our appreciation to the editorial board members and external reviewers, who have contributed a great deal to the high quality of this special issue. Meanwhile, we want to express our appreciation to the editor broad, who offers us great suggestions and supports in this special issue.

Ling-Qing Yuan
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Research Article

Lactoferrin Induces Osteoblast Growth through IGF-1R

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Objectives. To investigate the role of the IGF-1R by which lactoferrin induces osteoblast growth. *Methods.* Osteoblast received 5 d lactoferrin intervention at a concentration of 0.1, 1, 10, 100, and 1000 μg/mL, and the IGF-1 and IGF-1R were detected using RT-PCR and western blot. The osteoblast into the control, 100 μg/mL lactoferrin, Neo-scramble (NS, empty vector), NS + 100 μg/mL lactoferrin, shIGF-1R and shIGF-1R + 100 μg/mL lactoferrin group. We test the apoptosis and proliferation and the level of PI3K and RAS in osteoblasts after 5 d intervention. *Results.* (1) 1, 10, 100, and 1000 μg/mL lactoferrin induced the expression of IGF-1 mRNA and protein. 10 μg/mL and 100 μg/mL lactoferrin induced the expression of IGF-1R mRNA and protein. (2) Lactoferrin (100 μg/mL) induced osteoblast proliferation while inhibiting apoptosis. Osteoblasts with silenced IGF-1R exhibited decreased proliferation but increased apoptosis. MMT staining and flow cytometry both indicated that there was no significant difference between the shIGF-1R group and the shIGF-1R + 100 μg/mL lactoferrin group. (3) Lactoferrin (100 μg/mL) induced PI3K and RAS phosphorylation and silence of IGF-1R resulted in decreased p-PI3K and p-RAS expression. Lactoferrin-treated shIGF-1R cells showed significantly higher level of p-PI3K and p-RAS when compared with shIGF-1R. *Conclusion.* Lactoferrin induced IGF-1/IGF-1R in a concentration-dependent manner. Lactoferrin promoted osteoblast proliferation while inhibiting apoptosis through IGF-1R. Lactoferrin activated PI3K and RAS phosphorylation via an IGF-1R independent pathway.

1. Introduction

Lactoferrin, the transferrin family member, is an iron-binding glycoprotein that displays anti-inflammatory, antibacterial, and immunomodulatory activities [1]. Lactoferrin can be secreted by exocrine epithelial cells, and its concentration in normal human serum varies within the range of $2–7 \times 10^{-6}$ g/mL [2]. Serum lactoferrin mainly derives from neutrophils, and its local concentration may increase during inflammation. Our previous studies demonstrated that injection of lactoferrin at a concentration of $1\,g{\cdot}kg^{-1}{\cdot}d^{-1}$ and $2\,g{\cdot}kg^{-1}{\cdot}d^{-1}$ significantly increased bone mass and improved bone microstructure in ovariectomized rats [3]. *In vitro* studies also revealed that lactoferrin could in one aspect induce osteoblast proliferation and differentiation, while in another aspect inhibit osteoblast apoptosis. However, the molecular mechanisms by which lactoferrin regulates osteoblast growth are still unclear [4]. A specific receptor for lactoferrin has been cloned from human intestine [5], but the expression of receptor mRNA could not be detected in osteoblast, and the receptor was not expressed on the surface of any lactoferrin target cells. Present study suggested that osteoblast expressed low-density lipoprotein receptor-related protein LRP1 and LRP2 on its surface, and lactoferrin could activate the P42/44/MAPK signaling pathway through interaction with LRP1, indicating that LRP1 at least partially participated in the proliferation of osteoblast [6]. Lactoferrin could also induce osteoblast proliferation by activating PI3K, but the mechanisms were yet to be clarified. Our early study has found there was no statistical difference between the group in the presence of 5 μM OSI906 (the selective inhibitor of IGF-1 receptor and insulin receptor) and LF and the group only exposed to 5 μM OSI906. It indicated that OSI906 could block the mitogenic effect of LF in osteoblasts [7]. So in this study, we design the shIGF-1R to verify that lactoferrin promotes osteoblast growth by IGF-1R receptor;

Table 1: Primer sequences.

Gene	Genbank numbers	Primer sequence	Product size (bp)
β-Actin	NM-031144.2	Forward 5′-GGAGATTACTGCCCTGGCTCTA-3′ Reverse 5′-GACTCATCGTACTCCTGCTTGCTG-3′	150
IGF-1	NM 001082478.1	Forward 5′-GCACTCTGCTTGCTCACCTTTA-3′ Reverse 5′-TCCGAATGCTGGAGCCATA-3′	148
IGF-1R	NM 052807.2	Forward 5′-GGTCTCTAAGGCCAGAGGTGGA-3′ Reverse 5′-GACGAACTTGTTGGCATTGAGGTA-3′	122

Table 2: IGF-1R shRNA sequences.

Name	Sequence
S1	Forward: CCGGGCGGTGTCCAATAACTACATTCTCGAGAATGTAGTTATTGGACACCGCTTTTTG Reverse: AATTCAAAAAGCGGTGTCCAATAACTACATTCTCGAGAATGTAGTTATTGGACACCGC
S2	Forward: CGGCCAACGAGCAAGTTCTTCGTTCTCGAGAACGAAGAACTTGCTCGTTGGTTTTTG Reverse: ATTCAAAAACCAACGAGCAAGTTCTTCGTTCTCGAGAACGAAGAACTTGCTCGTTGG
S3	Forward: CCGGAGCAGGTTGTAACAATCTATTCTCGAGAATAGATTGTTACAACCTGCTTTTTTG Reverse: AATTCAAAAAAGCAGGTTGTAACAATCTATTCTCGAGAATAGATTGTTACAACCTGCT

we silenced the insulin-like growth factor-1 receptor (IGF-1R) in osteoblast and detected the level of proteins involved in downstream signaling pathways and thereby investigated the role of the IGF-1R by which lactoferrin induces osteoblast growth.

2. Materials and Methods

2.1. Cell Culture. Eight Sprague-Dawley male rats aged 24 h were killed by cervical dislocation. Rats' heads were obtained under sterilized condition, and the skulls were sampled in a PBS-filled petri dish. After removal of connective tissues by PBS washing, skulls were cut into a volume of approximately 1 mm^3, digested subsequently by 0.25% trypsin (Hyclone, USA) and 0.1% type I collagenase (Invitrogen, USA) and then inoculated in a 25 cm^2 flask for cell culture and passage in a 37°C incubator containing 5% CO_2. Afterwards, osteoblast cells (passage 3) were seeded on a 6-well plate at a concentration of 1×10^5 cells/well for adherent growth, and were synchronized by culturing in serum-free DMEM media for 24 h. Cells were randomly assigned into a control group and 5 experimental groups, which, respectively, received lactoferrin (New Zealand, purity > 90%) intervention at a concentration of 0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1000 μg/mL for 5 consecutive days.

2.2. Real-Time PCR. Total cellular RNA was extracted by Trizol reagent (Invitrogen, USA), and reverse transcription (20 μL system) was performed according to the instructions provided by the kit. RT-PCR was performed using the SYBR Premix Ex Taq TM II kit (DRR081A, Takara, Japan) on a Thermal Cycler Dice TM Real Time system (TP800, Takara, Japan). Primers for β-actin and IGF-1 were designed and synthesized by Takara (Table 1). Quantitative gene expression analysis was carried out by using the $2^{-\Delta\Delta Ct}$ method.

2.3. Western Blot. Total cellular protein was extracted by a protein extraction kit (Newgene Bio, Shanghai, China) and quantified by a BCA protein assay kit (Newgene Bio, Shanghai, China). After that, a total of 60 μg protein was loaded and electrophoresed through a 10% SDS-PAGE gel. Separated proteins were subsequently transferred onto a PVDF membrane and incubated with primary antibody (Abcam, America) at 4°C overnight, then with rabbit anti-mouse secondary antibody (Zhongshan Biotech, Beijing, China) at room temperature for 2 h. Western blots were developed using the SuperSignal West Dura Extended Duration Substrate, and image analysis was carried out after X-ray scanning. The experiment was repeated 3 times.

2.4. Lentiviral Transfection. Based on the principle of shRNA design and according to the sequence of IGF-1R, 3 shRNA sequences were designed and synthesized by Shanghai Newgenebio Company (Table 2). The shRNA vectors were cotransfected into HEK293T cells with the lentiviral packaging plasmids, and the recombinant lentiviral particles were used to infect primary rat osteoblasts. RT-PCR was performed to verify the efficiency of gene silencing by detecting IGF-1R expression, and the shRNA vector exhibiting the highest silencing efficiency (S1, silencing efficiency > 80%) was selected for future experiment. Noninfected and infected osteoblasts were digested in logarithmic growth phase, and were, respectively, inoculated in a 6-well plate at a concentration of 1×10^5 cells/well. Both noninfected and infected cells were then randomly assigned into a control group and 5 experimental groups, including 100 μg/mL lactoferrin group, Neo-scramble (NS, empty vector) group, NS + 100 μg/mL lactoferrin group, shIGF-1R transinfection group, and shIGF-1R + 100 μg/mL transinfection group. Cells were then cultured in a 37°C incubator containing 5% CO_2.

2.5. Determination of Cell Proliferation. Cells obtained from the transfection experiment were inoculated in a 96-well plate

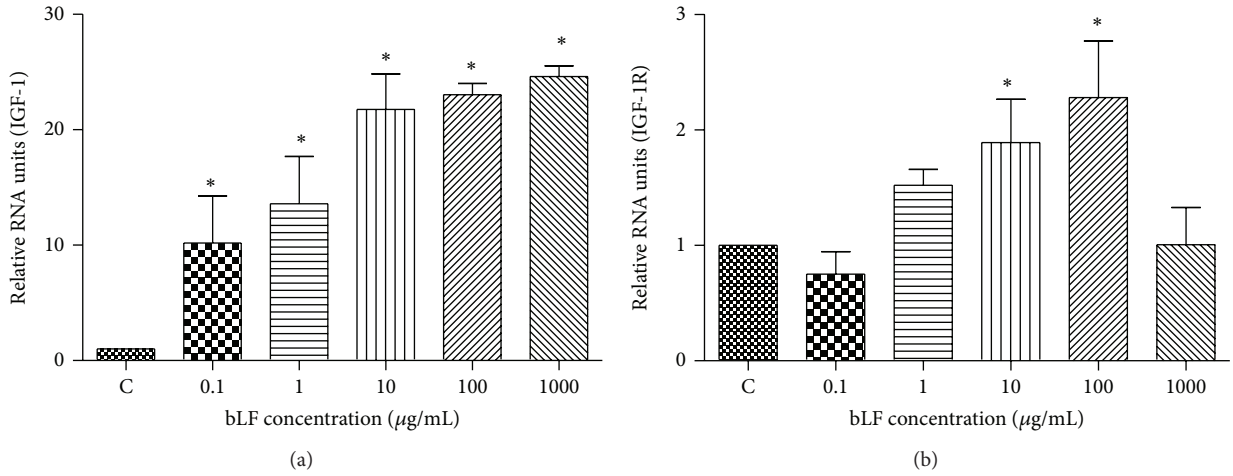

Figure 1: The effects of lactoferrin on IGF-1/IGF-1R mRNA expression in osteoblast. (a) IGF-1, (b) IGF-1R; $^{*}P < 0.01$ compared with control. The experiment was repeated 3 times.

at a concentration of 3×10^3cells/well. Each group had 6 repeats. After 5 d of lactoferrin intervention, cells were added with 20 μL MTT (5 mg/mL, Gibco) and cultured for 4 h. After that, 150 μL of dimethyl sulfoxide (DMSO, Sigma) was added to each well, and the plate was oscillated for 10 min until the crystals were fully dissolved. Optical density (OD) at a wavelength of 490 nm was determined by a microplate reader, and a blank control was introduced. The experiment was repeated 3 times.

2.6. Determination of Cell Apoptosis. Cells obtained from the transfection experiment were cultured in an incubator (5% CO_2, 37°C) for 24 h and then digested by trypsin. Each group had 3 repeats. After washing with ice-cold PBS, cells were resuspended in 1x Binding buffer to a concentration of 1×10^6 cells/mL. For detection of apoptosis, 5 μL of 7-AAD and 5 μL of annexin V-APC (Nanjing KGI) were added to 500 μL of cell suspension and incubated for 10 minutes at 4°C in the dark. Cell apoptosis was tested by a flow cytometry within 1 h.

2.7. Detection of IGF-1R Downstream Signaling in Osteoblast. Cells obtained from the transfection experiment were cultured in a 37°C incubator containing 5% CO_2. Each group had 3 repeats. After 5 d of lactoferrin intervention, osteoblast were digested with trypsin and collected for western blot detections of PI3K (CST #4292), p-PI3K (CST #4228), RAS (Santa Cruz SC-863), and p-RAS (CST #3321).

2.8. Statistical Analysis. Experimental data were presented as mean ± SD. Software SPSS16.0 was used for one-way analysis of variance (ANOVA). $P < 0.05$ was considered as significant difference.

3. Results

3.1. The Effects of Lactoferrin on IGF-1/IGF-1R mRNA Expression in Osteoblast. Lactoferrin intervention significantly increased ($P < 0.01$) IGF-1 mRNA expression in a concentration dependent manner, and osteoblasts added with 1000 μg/mL lactoferrin showed the highest level of IGF-1 mRNA expression. Lactoferrin intervention at a concentration of 10 μg/mL and 100 μg/mL significantly ($P < 0.01$) induced IGF-1R mRNA expression, but lactoferrin with other concentrations (0.1 μg/mL, 1 μg/mL, and 1000 μg/mL) had no significant influence on IGF-1R mRNA expression (Figure 1).

3.2. The Effects of Lactoferrin on IGF-1/IGF-1R Expression in Osteoblast. Lactoferrin intervention significantly induced ($P < 0.01$) IGF1 expression in all osteoblasts except those added with 0.1 μg/mL lactoferrin ($P < 0.05$). Lactoferrin intervention at a concentration of 10 μg/mL ($P < 0.05$), 100 μg/mL ($P < 0.01$), and 1000 μg/mL ($P < 0.01$) significantly increased IGF1R expression, while cells in other groups showed no significant variations in IGF1R expression (Figure 2).

3.3. Lentiviral Vector Construction and Selection

(1) Determination of Lentiviral Infection Efficiency. Lentiviral infection efficiency was determined by calculating the percentage of fluorescent cells in 10 randomly selected high power fields. The NS and shRNA-1 group had infection efficiency at approximately 70%, shRNA-2 group had an infection efficiency of 45%, and the shRNA-3 group had an infection efficiency of 20% (Figure 3).

(2) Selection of the Optimal Interference Sequence. Cells were, respectively, transfected with shRNA-1 (S1), shRNA-2 (S2), shRNA-3 (S3), and NS vector (NS), and RT-PCR was performed to detect IGF-1R expression after 72 h of cell culturing. Compared with the control group, cells in S1, S2, and S3 all showed significantly silenced IGF-1R expression,

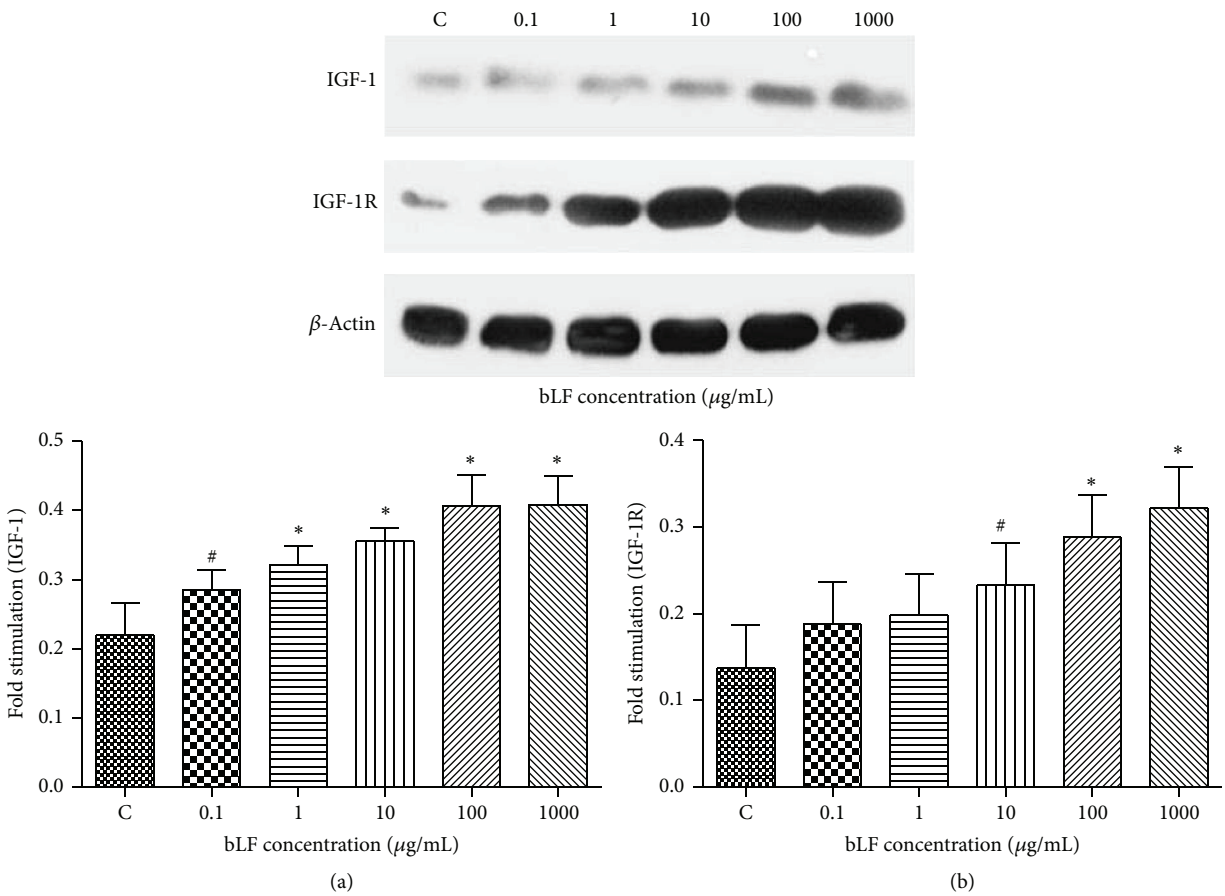

Figure 2: The effects of lactoferrin on IGF-1/IGF-1R protein expression in osteoblast. (a) IGF-1, (b) IGF-1R; $^{\#}P < 0.05$ and $^{*}P < 0.01$ compared with control. The experiment was repeated 3 times.

and the S1 group exhibited the highest level of IGF-1R silencing (Figure 4).

3.4. Detection of Cell Proliferation. MTT-staining revealed that lactoferrin concentrated at 100 μg/mL significantly promoted osteoblast proliferation, while shIGF-1R silencing significantly suppressed osteoblast proliferation. No significant difference in cell proliferation was detected between shIGF-1R cells treated with and without 100 μg/mL lactoferrin (Figure 5).

3.5. Detection of Cell Apoptosis. Flow cytometric analysis indicated that lactoferrin concentrated at 100 μg/mL significantly inhibited osteoblast apoptosis, while shIGF-1R silencing significantly promoted osteoblast apoptosis. No significant difference in cell apoptosis was detected between shIGF-1R cells treated with and without 100 μg/mL lactoferrin (Figure 6).

3.6. Detection of IGF-1R Downstream Signaling in Osteoblast. Western blot suggested that when compared with the control, lactoferrin intervention at a concentration of 100 μg/mL significantly induced the expression of p-PI3K and p-RAS, while shIGF-1R silencing significantly decreased the expression level of p-PI3K and p-RAS. Lactoferrin-treated shIGF-1R cells exhibited significantly increased level of p-PI3K and p-RAS when compared with shIGF-1R cells (Figure 7).

4. Discussion

Our previous study confirmed that after different days of intervention (1 d, 3 d, 5 d, and 7 d), differently concentrated lactoferrin (0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1000 μg/mL) could induce osteoblast proliferation and differentiation and could also inhibit osteoblast apoptosis and death. We have identified the optical concentration (100 μg/mL) and time (5 d) for lactoferrin intervention [7]. However, the molecular mechanism by which lactoferrin induced osteoblast proliferation was yet to be clear. This study suggested that lactoferrin induced the expression of IGF-1 mRNA and protein in a concentration-dependent manner. Furthermore, as the osteoblasts expressed IGF-1R, a receptor involved in regulating cell proliferation, differentiation, and apoptosis, the lactoferrin could exert a concentration-dependent effect in promoting IGF-1R transcription and expression. By silencing the expression of IGF-1R and by

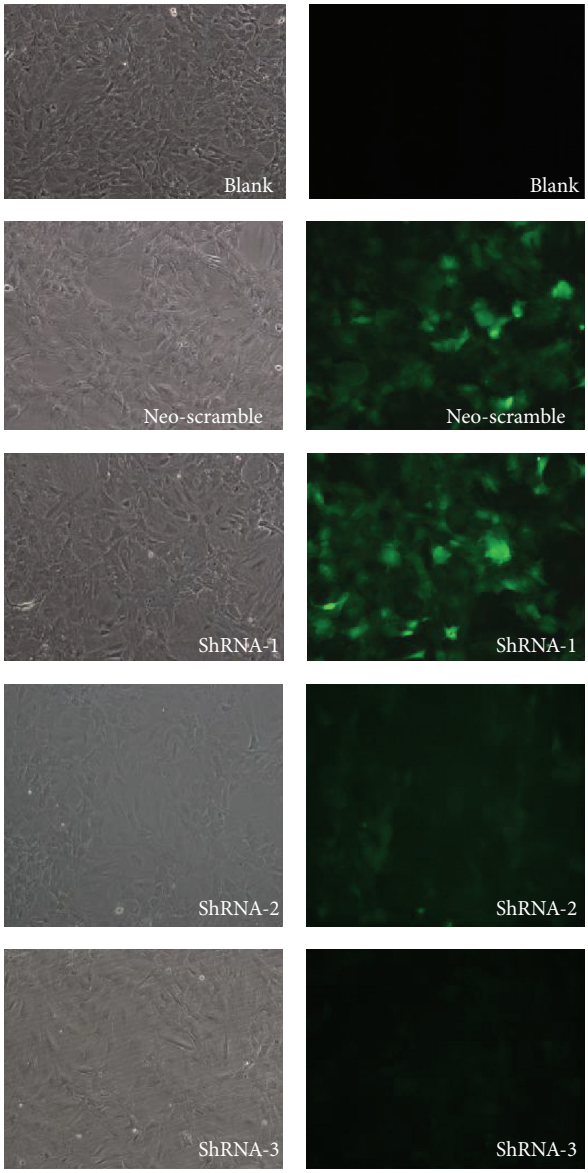

Figure 3: Infection efficiency in cells transfected with different lentiviral vectors. The NS and ShRNA-1 group had infection efficiency at approximately 70%, shRNA-2 group had an infection efficiency of 45%, and the shRNA-3 group had an infection efficiency of 20%.

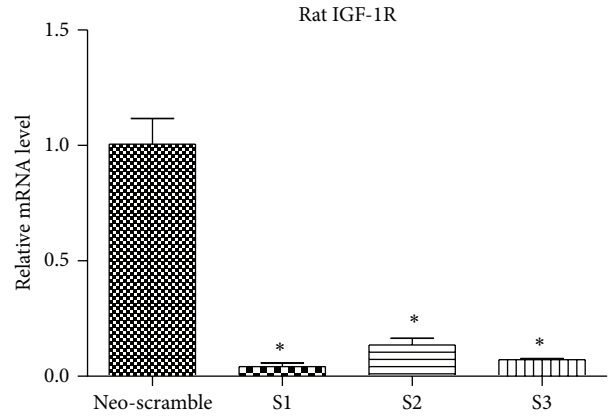

Figure 4: Effects of shIGF-1R transfection on IGF-1R expression in osteoblasts. $^{*}P < 0.01$ compared with NS group. The experiment was repeated 3 times.

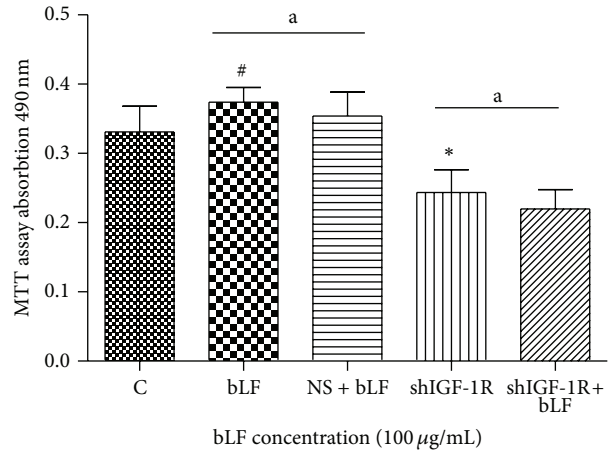

Figure 5: Effects of lactoferrin in shIGF-1R transfected osteoblast proliferation. $^{*}P < 0.01$ and $^{\#}P < 0.05$ compared with the control. $^{a}P > 0.05$ compared between bLF and NS + bLF group and compared between shIGF-1R and shIGF-1R+bLF group. NS: Neo-scramble (empty vector).

detecting the level of IGF-1R downstream signaling pathway, we investigated the relationship between lactoferrin, IGF-1R, and osteoblast proliferation and apoptosis and explained the molecular mechanism of lactoferrin in inducing osteoblast proliferation.

Lactoferrin (80 kDa) is an iron-binding glycoprotein belonging to the transferrin family, and it mainly exists in breast milk, epithelial secretion, and neutrophil secretory vesicles. Lactoferrin is a multieffect factor involved in antibacteria and immunomodulation, and more importantly, it can induce osteoblast proliferation and differentiation, while inhibiting osteoblast apoptosis and osteoclastogenesis [8, 9]. In our previous study, we found that lactoferrin increased bone mineral density, improved bone microstructure, promoted bone formation, and inhibited bone resorption in ovariectomized rats [3]. *In vitro* study also showed that lactoferrin intervention (100 μg/mL) for consecutive 5 d significantly induced osteoblast proliferation [7]. When compared with the control, osteoblast received 24 h lactoferrin intervention displayed significantly suppressed serum deprivation-induced apoptosis [10].

Grey et al. [9] detected the express of LRP1 and LRP2 on osteoblast cell surface. Since lactoferrin could activate the P42/44/MAPK pathway via LRP1, their finding indicated that LRP1 at least partially participated in the regulation of osteoblast proliferation. Lactoferrin could also induce cell proliferation by activating PI3K, but receptors for such signaling transduction were yet to be identified. On the other hand, Grey et al. [9] demonstrated that lactoferrin

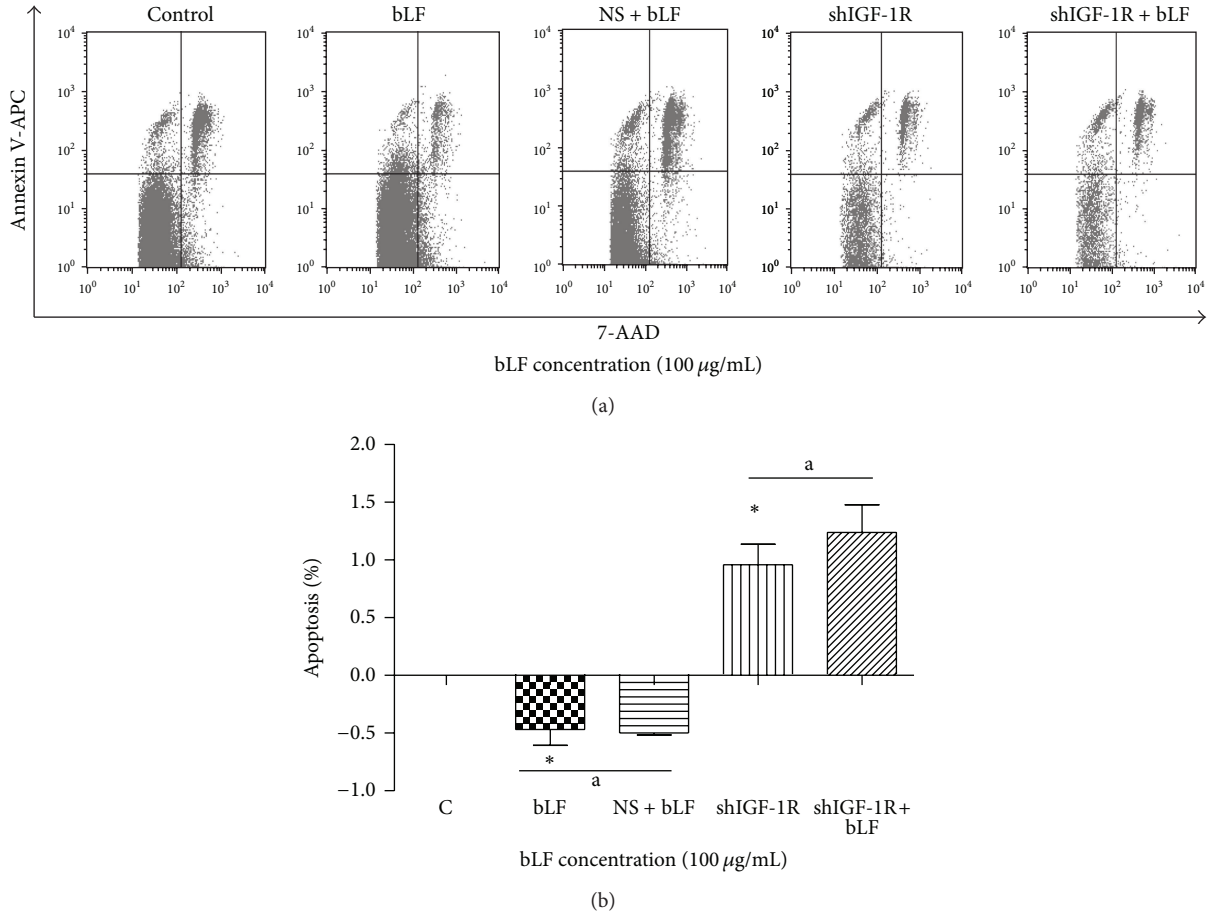

Figure 6: Effects of lactoferrin in shIGF-1R transfected osteoblast apoptosis. $^{*}P < 0.01$ compared with the control. $^{a}P > 0.05$ compared between bLF and NS + bLF group, and compared between shIGF-1R and shIGF-1R+bLF group. NS: Neo-scramble (empty vector).

inhibited osteoblast apoptosis through a LRP1-independent pathway, but the molecules involved in this pathway, and the mechanisms by which lactoferrin inhibited cell apoptosis were still unclear.

The IGF-1R is a tetramer consisting of 2 α subunits and 2 β subunits. After binding to its ligand (IGFl/IGF2), IGF-1R could, via the mediation of IGFBPs, significantly induce mitosis, enhance DNA synthesis, and promote cell proliferation and differentiation of the target cells [11, 12]. IGF-1R is an important member of the tyrosine kinase receptor family. Binding of IGF-1 to IGF-1R would induce the phosphorylation of insulin receptor substrate-1 (IRS-1), thereby activating the downstream signaling pathways [13]. The PI3K-dependent AKT pathway and the RAS-mediated MAPK pathway are 2 pathways that are more intensively studied regarding the IGF-1R-regulated downstream signaling pathways [14]. In this study, we designed 3 interfering sequences for IGF-1R (S1, S2, and S3), constructed IGF-1R lentiviral vectors, selected the optimal interfering sequence S1 using the RT-PCR technique, and silenced the expression of IGF-1R by transfecting the recombinant vector into osteoblasts. Compared with the control, osteoblasts with silenced IGF-1R exhibited significantly decreased proliferation while increasing apoptosis, indicating that IGF-1R is an essential receptor enabling osteoblasts to maintain normal mitosis and avoid apoptosis. In order to clarify whether the lactoferrin-induced osteoblast proliferation and apoptosis inhibition were mediated by IGF-1R, we performed lactoferrin intervention (100 μg/mL) in IGF-1R-silenced osteoblasts. Lactoferrin significantly induced osteoblast proliferation and suppressed cell apoptosis, indicating that the function of lactoferrin was IGF-1R-dependent.

Western blot revealed that lactoferrin could activate the phosphorylation of both PI3K and RAS; thus, the lactoferrin-triggered IGF-1R downstream pathway mainly involved the PI3K-dependent AKT pathway and the RAS-dependent MAPK pathway. When compared with the control, IGF-1R-silenced cells displayed significantly decreased level of PI3K and RAS phosphorylation, while the intervention of lactoferrin (5 d, 100 μg/mL) elevated PI3K and RAS phosphorylation, and the difference was statistically significant. This result suggested that, in IGF-1R knockout osteoblasts, lactoferrin could still activate PI3K and RAS phosphorylation, and such activation might be mediated through an IGF-1R-independent pathway (Figure 8).

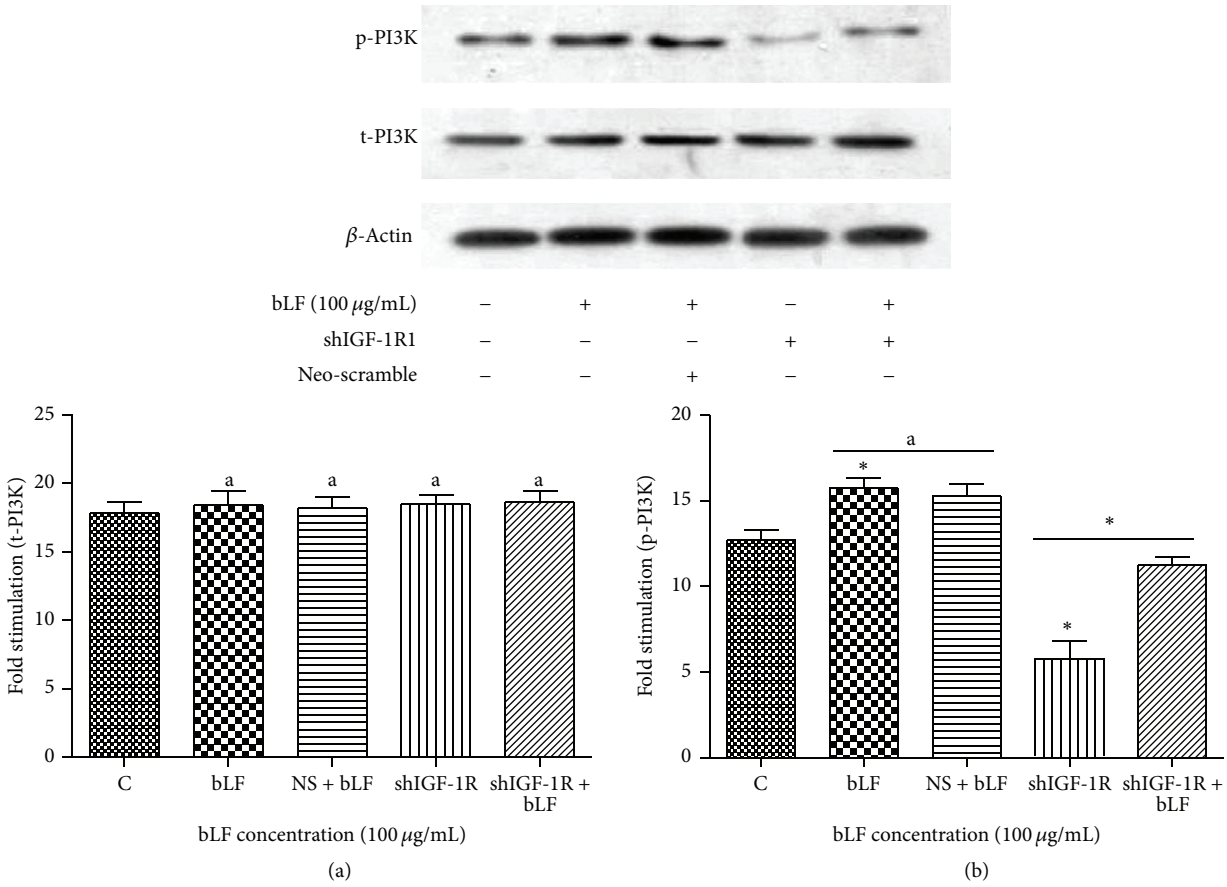

Figure 7: Effects of lactoferrin on PI3K phosphorylation in shIGF-1R transfected cells. $^{*}P < 0.01$ compared with the control. $^{a}P > 0.05$ compared between bLF and NS + bLF group. $^{*}P < 0.01$ compared between shIGF-1R and shIGF-1R+bLF group. NS: Neo-scramble (empty vector).

Among the various lactoferrin receptors, LRP1 and LRP2 are 2 receptors expressed on osteoblast cell surface and are multiple-ligand members of the LRP family [6]. In osteoblast, functional LRP1 could mediate the endocytosis of lactoferrin and induce the formation of cytoplasmic membrane-bound vesicles. As a receptor involved in mitogenic signaling, LRP1 could also activate the p42/44 mitogen-activated protein kinase (MAPK) pathway, thereby inducing the mitosis of osteoblasts. These findings suggested that LRP1 at least partially participated in the lactoferrin-induced mitosis in osteoblasts. In addition, lactoferrin could also activate the PI3K-dependent Akt pathway in a LRP1-independent manner [15], but the mechanism requires further studies.

Grey et al. [9] have confirmed that LRP1 could induce osteoblast mitosis by phosphorylating MAPK, and we observed that the MAPK pathway was activated, via LRP1, in osteoblast with silenced IGF-1R expression. However, the receptors involved in the lactoferrin-induced PI3K-dependent Akt activation were yet to be identified. The findings of our study demonstrated that IGF-1R was not a key receptor mediating the lactoferrin-dependent PI3K and RAS-dependent MAPK pathway activation, and whether lactoferrin could activate PI3K signaling through other receptors would need more investigation. Fulzele et al. [11] found that silence of IGF-1R induced insulin receptor (IR) upregulation. Since the PI3K/AKT and RAS/MAPK are the major pathways regulating IR downstream signaling transduction; whether lactoferrin activates PI3K via IR requires further studies.

In conclusion, we found that lactoferrin induced IGF-1/IGF-1R expression in a concentration dependent manner, and it induced proliferation while inhibiting apoptosis of osteoblasts through the mediation of IGF-1R. We also identified that lactoferrin activated the PI3K/RAS signaling pathway through an IGF-1R-independent mechanism.

Conflict of Interests

None of the authors have any financial interest in relation to the submission.

Authors' Contribution

The academic affiliations of the authors were that Jian-Ming Hou, En-Yu Chen, and Fan Lin designed the research project. Jian-Ming Hou supervised the project. En-Yu Chen performed the *in vitro* experiments, analyzed the data, and

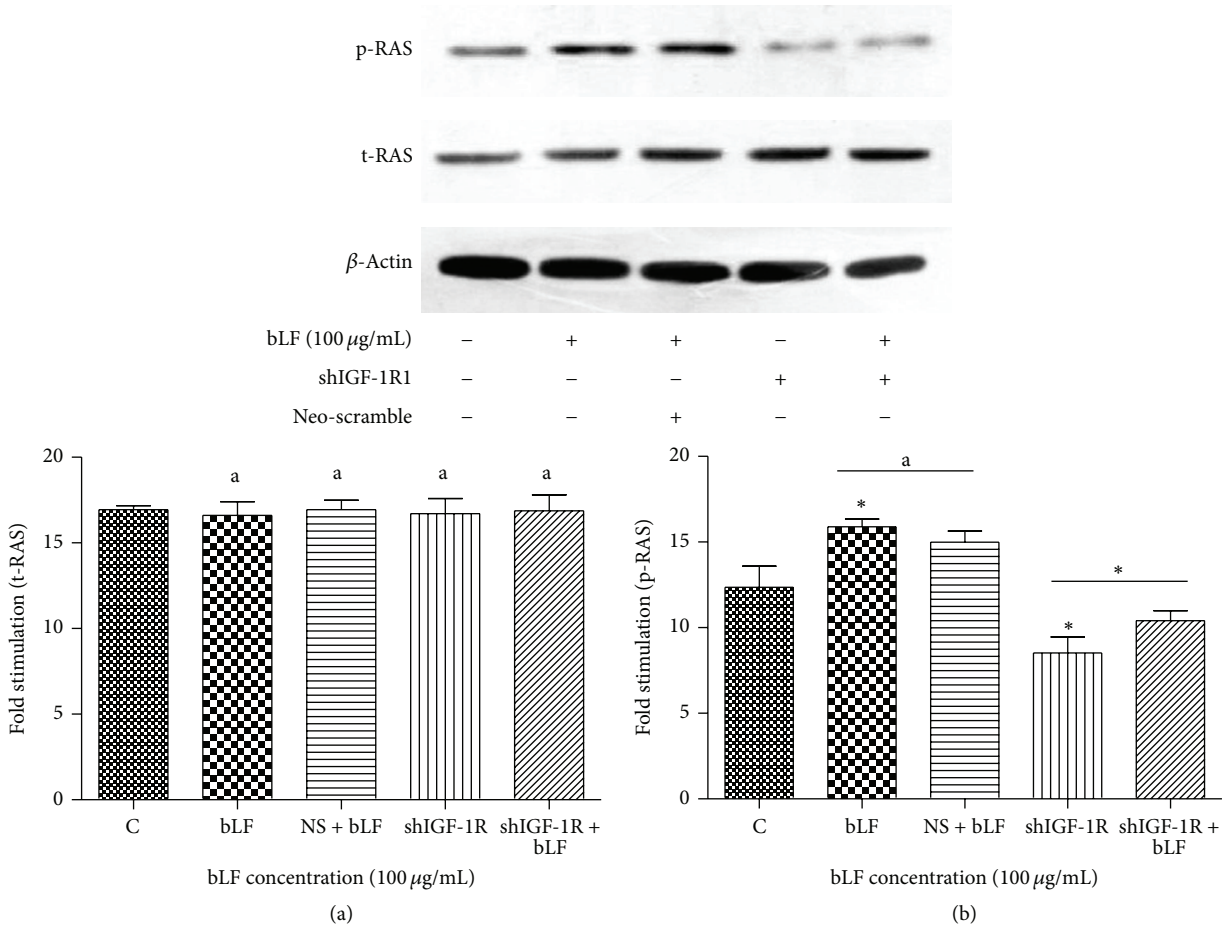

FIGURE 8: Effects of lactoferrin on RAS phosphorylation in shIGF-1R transfected cells. $^{*}P < 0.01$ compared with the control. $^{a}P > 0.05$ compared between bLF and NS + bLF group. $^{*}P < 0.01$ compared between shIGF-1R and shIGF-1R+bLF group. NS: Neo-scramble (empty vector).

wrote the paper. Qing-Ming Lin, Xu-Hua Lan, Ying Xue, and Man Wu revised the paper. Jian-Ming Hou, En-Yu Chen, and Fan Lin contributed equally to this work, as co-first author.

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Research Article

Micro/Nanostructures and Mechanical Properties of Trabecular Bone in Ovariectomized Rats

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Bone mechanical properties encompass both geometric and material factors, while the effects of estrogen deficiency on the material and structural characteristics of bone at micro- to nanoscales are still obscure. We performed a series of combined methodological experiments, including nanoindentation assessment of intrinsic material properties, atomic force microscopy (AFM) characterization of trabecular (Tb) nanostructure, and Tb microarchitecture and 2D BMD. At 15 weeks after surgery, we found significantly less Tb bone mineral density (BMD) at organ (−27%) and at tissue level (−12%), Tb bone volume fraction (−29%), Tb thickness (−14%), and Tb number (−17%) in ovariectomy (OVX) rats than in sham operated (SHAM) rats, while the structure model index (+91%) and Tb separation (+19%) became significantly greater. AFM images showed lower roughness Tb surfaces with loosely packed large nodular structures and less compacted interfibrillar space in OVX than in SHAM. However, no statistically significant changes were in the Tb intrinsic material properties—nanoindentation hardness, elastic modulus, and plastic deformation—nanoindentation depths, and residual areas. Therefore, estrogen deprivation results in a dramatic deterioration in Tb micro/nanoarchitectures, 3D volumetric BMD at both organ and tissue levels, and 2D BMD, but not in the nanomechanical properties of the trabeculae per se.

1. Introduction

Bone strength encompasses architectural, geometric, and material factors that contribute to bone fragility [1–5]. The contributions of bone mass and bone mineral density (BMD) and architecture to bone strength are well recognized. However, these variables cannot explain all the variance of bone strength [2]. Bone is a natural material displaying a remarkable hierarchical organization, where mechanical integrity of bone is dictated by its structures and materials at different length scales, from nanoscale, for example, mineral crystals and collagen fibrils, and microscale, for example, microarchitecture and osteocyte lacunar network, to macroscale, for example, bone size and geometry [6–8]. Therefore, evaluation of the structure-function relationship between bone composition and mechanical properties at various length-scales is important to understand the bone fragility in osteoporosis.

Estrogen-deficiency attributable to menopause is considered to be a culprit of postmenopausal osteoporosis and can result in low bone mass and microarchitectural deterioration with a consequent susceptibility to fracture [9, 10]. These changes are believed to result in bone tissue loss and decrease in BMD, mainly through alterations in bone remodeling rates. The enhanced remodeling activity [11] affects bone mineralization, which in turn contributes to the degradation of microarchitecture and affects the bone strength at the macroscale [12]. Bone matrix, especially collagen cross-links, is deteriorated in postmenopausal osteoporotic individuals [13, 14]. Because mineralization significantly affects Young's

modulus and hardness [15], newly formed bone would be undermineralized and less resistant to bend [16]. It might be assumed that bone material properties at the tissue level should also be impaired in estrogen deprivation that increases bone turnover. However, the material properties of bone at the micro- to nanoscales and their effects on mechanical performance are still elusive.

Nanoindentation is a widely used technique to determine the mechanical properties of bone at nanoscale [17–19]. In nanoindentation, one can extract the hardness and elastic modulus from the load-displacement curves of the measured materials, for example, osteons, lamellae and individual trabeculae, collagen fibrils, and individual mineral crystals [20–23]. The nanoindentation technique has been used to investigate the mechanical properties of various microstructures in bone with individual, age-related structural and mechanical alteration at the bone material level and the mechanical properties of bone in individuals with a particular bone disease [18, 19, 24]. Nevertheless, there is still a lack of experimental studies on the changes in bone tissue properties with osteoporosis in postmenopausal women, and the influence of menopause on the mechanical properties of bone remains obscure [22, 25–27]. Guo and Goldstein [22] measured the nanomechanical properties of trabecular bone in ovariectomy (OVX) rat using nanoindentation. They did not found distinct changes of the hardness or elastic modulus of trabeculae in the longitudinal section after OVX. The same conclusion was recently made for tibial trabecula by Lane et al. [28] and for transilial biopsies in healthy pre- and postmenopausal women by Polly et al. [29]. However, the hierarchically organized structure of bone has an irregular, yet optimized and elegant, arrangement and orientation of components, making the material heterogeneous and anisotropic [30]. The mechanical properties may vary with the cortical versus trabecular compartments, orientations, and types of bones [31]. Turner et al. [32] compared the elastic moduli between cortical and trabecular bone from the femoral midshaft and distal femur and found a pronounced difference in the elastic modulus of cortical bone in the transverse and longitudinal directions. Therefore, observations should be interpreted with cautions to compare the tissue-level mechanical properties of different types of bones. In these previous studies [22, 28, 29], the vertebral trabecular bone specimens were sectioned in only the longitudinal direction while the elastic modulus was measured in the transverse direction. Little data is available for bone sectioned in the cross-sectional direction.

Atomic force microscopy (AFM) provides a powerful tool to the mechanical properties of bone tissues at nanoscale [33]. It operates in the near field with a sharp probe by scanning the surface of the sample in a distance of a few angstroms. Compared with traditional optical microscope (TEM) and scanning electronic microscope (SEM), it enables characterization of three-dimensional (3D) surface morphology with minimal sample preparation and high resolution [28, 34, 35]. AFM has been widely used to visualize the bone matrix and to determine the spatial relationship between mineral and collagen and their morphology/topology as well [36, 37]. Furthermore, the combination of AFM and nanoindentation can characterize the structures and properties of both natural tissues and synthetic materials at the nanometer resolution [38, 39]. Nevertheless, the fragility of the nanosized components in bone has not been well understood.

The purpose of this study was to evaluate the effect of estrogen deprivation on the nano/microscale structural and mechanical properties of vertebral trabeculae in rats. We performed a series of experiments, including nanoindentation assessment of intrinsic material properties of trabeculae in the longitudinal direction, AFM characterization of trabecular nanostructures, and micro-computed tomography (μCT) evaluation of trabecular microarchitecture.

2. Materials and Methods

2.1. Animals. Twenty 10-month-old Sprague-Dawley female rats, with body weight of 305 ± 10 g (mean ± SD), were provided by the Laboratory Animal Center of the Second Xiangya Hospital, Central South University, China. They were randomly assigned to two groups: ovariectomy (OVX) and sham-ovariectomy (SHAM), with 10 rats in each group. They were pair-fed, housed at 25°C with an alternating 12 h light/dark cycle, and allowed free access to water. At 15 weeks after operation, the rats were sacrificed. Their lumbar spine was dissected, wrapped in gauze soaked in normal saline, sealed in plastic bags, and stored at −70°C until measurements. All animal procedures were approved by our institutional animal care and use committee.

We chose 10-month-old rats because our previous study has shown that 10-month-old SD rats represent a good model of osteoporosis from estrogen deficiency [40, 41]. This study as well as our previous experiments [40, 41] shows no ovary atrophy in SHAM rats 10–15 months of age. The OVX rat models, especially young growing rat 3 months old, have been intensively investigated and widely used in the study of postmenopausal osteoporosis [42]. The young growing rats have main advantages, such as being relatively inexpensive to obtain and to maintain. However, the young growing rats have the great biologic disadvantages, such as continuously growing, not only in the primary spongiosa with open growth plate, but also with active periosteal bone apposition [43]. We previously have shown that cortical bone is not yet matured yet in rats until they become 7.5 months old [41]. In rats older than 10 months, the bone growth rate for the proximal tibial epiphysis is less than 3 μm/day and stops after the age of 15 months. A female rat around 10 months of age has reached peak bone mass and can be manipulated to simulate clinical findings of postmenopausal osteoporosis [44].

2.2. Measurement of 2D Projectional DXA BMD. BMD was measured using a Hologic QDR 4500A dual-energy X-ray absorptiometry bone densitometer (Hologic, Bedford, MA, USA) under the conditions of 40 kVp and 100 kVp, with a scanning width of 18 cm and a velocity of 4.8 s/cm [5, 45]. The scans were analyzed with specific software for small animals. Quality-control scans were performed daily, using the manufacturer-supplied phantom, with a long term (2 years) root mean square coefficient of variation of 0.52%. The total body and vertebral BMD were measured in vivo, under

general anesthesia, at baseline and again just before all rats were sacrificed. BMD of the dissected sixth lumbar vertebrae (L6) was determined after the animals were sacrificed.

2.3. Nanoindentation. A diamond blade saw (Buehler, Lake Bluff, IL, USA) was used to cut the center of the L6 vertebral body along the cross-sectional direction. The specimens were dehydrated in ascending grades of ethanol, with six changes from 70% to 100% and embedded in polymethyl methacrylate. The cutting surfaces of the embedded specimens were metallographically polished with silicon carbide abrasive papers with (1200, 800, and 600 grits). Then they were polished by using microcloths (Buehler, Lake Bluff, IL, USA) with fine grades of alumina powders. Finally, the specimens were cleaned in a distilled water ultrasonic bath for 10 min to remove surface debris [23]. The samples were tested dry immediately after polishing.

Nanoindentation is a testing technique commonly used to study the mechanical properties (e.g., elastic modulus, yield strength, and hardness) of materials at nanoscale [23, 24]. This technique can reduce the depth of indentation to the submicron range and extend the spatial resolution to about 1 nm [17, 23]. Oliver and Pharr proposed the theoretical basis of nanoindentation in 1992 [17]. The relationship between the contact stiffness and the elastic properties is

$$\frac{dP}{dh} = \beta \frac{2}{\sqrt{\pi}} \sqrt{A_c} E_r, \tag{1}$$

where β is an empirical indenter shape factor and A_c is the projected area of the contact area. For the triangular pyramidal indenter, $\beta = 1.034$. The effective modulus E_r is given by

$$\frac{1}{E_r} = \frac{\left(1 - \nu_s^2\right)}{E_s} + \frac{\left(1 - \nu_i^2\right)}{E_i}, \tag{2}$$

where ν is Poisson's ratio and the subscripts s and i refer to the sample and indenter, respectively. For bone, Poisson's ratio was assumed to be 0.3. The hardness is defined as the maximum load $P_{\max}$, divided by the contact area A_c:

$$H = \frac{P_{\max}}{A_c}. \tag{3}$$

A fused silica sample, which exhibits elastic isotropy and has a small elastic modulus-hardness ratio, was used to calibrate the indenter tip shape function.

All experiments were performed at room temperature (20°C). The Nanoindenter XP (MTS Systems Co., Oak Ridge, TN) was used, with force of 50 nN and displacement resolution of 0.01 nm [46]. Figure 1 presents a representative load-displacement curve of indentation. A permanent hardness impression was made by pressing the indenter into the specimen to a depth of 1000 nm at a constant indentation rate of 10 nm/s. There were two constant load holding periods in the whole process. First, at the peak load, the indenter was held for 100 s to minimize the effects of viscoelasticity and creep. The second constant load holding period, near the end of the test at 90% of the peak load, was used to establish the thermal drift rate in the machine and specimen for appropriate correction of the data [47, 48]. For each sample, four different trabeculae were measured to reduce random errors, as shown in Figure 2(a). The sites and directions of indentation were also randomly selected. Five indentations were made for each site within a 30~50 μm region (Figure 2(b)) in the longitudinal direction of vertebral trabeculae, for a total of 20 indentations in the cross section of each sample in order to get the average results. The mean values and standard deviations (SDs) of hardness and elastic modulus were calculated by the arithmetic average of the obtained data under different depths, which were measured in the range of 400–900 nm displacements to avoid the effect of surface roughness.

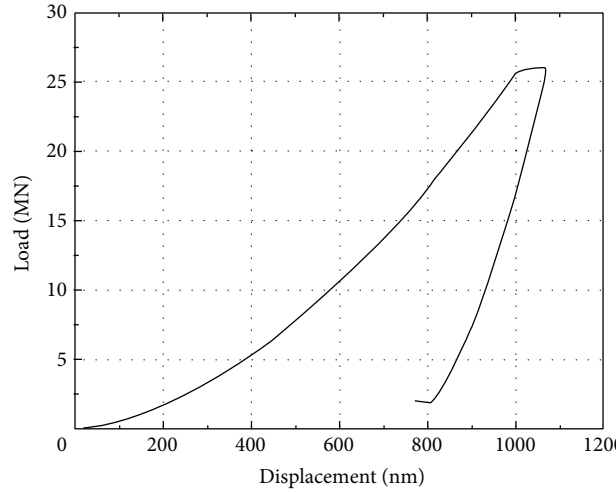
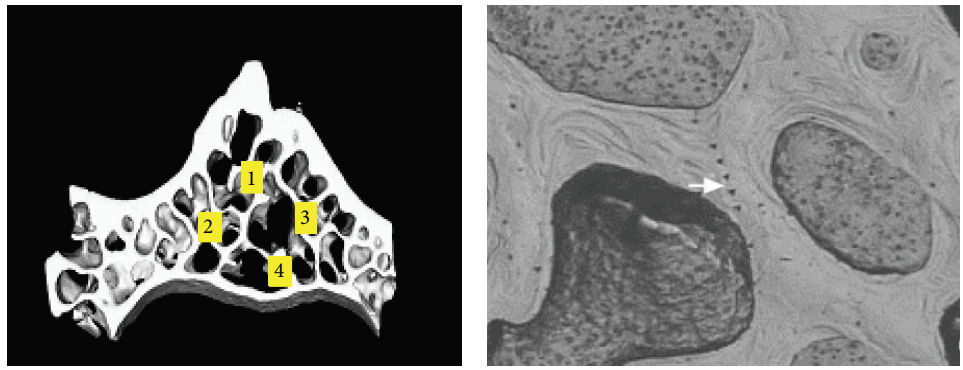


Figure 1: A representative load-displacement curve of nanoindentation.

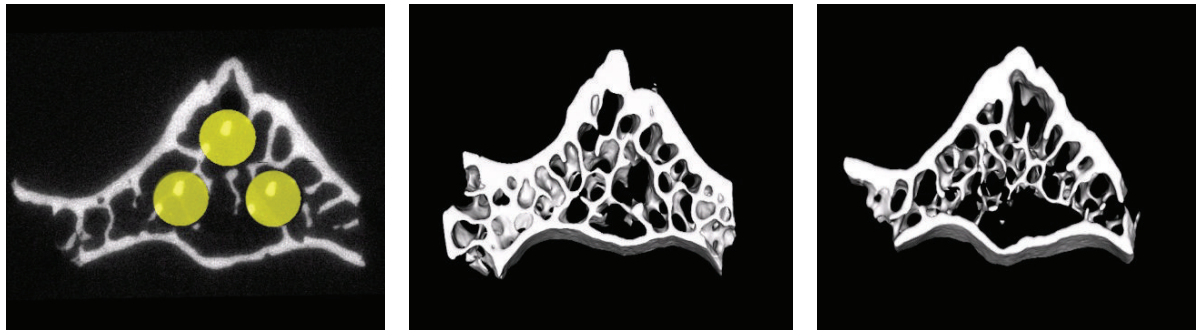
2.4. Atomic Force Microscope (AFM). At 24 h after indentation, the sample surface was cleaned with distilled water for 10 minutes, vacuum-dried at room temperature, and glued to metal disk for AFM (Digital Instruments, NanoScope IV/Dimension 3100; Santa Barbara, CA). The images of the indentations were recorded in air, at room temperature, at a scan rate of 1 Hz, in the tapping mode at the appropriate set point, and with a force constant of 40 N/m and resonant frequency of 300 kHz [36]. A depth profile, local morphological changes due to the indentation process, three-dimensional surface morphology, and roughness of the sample were recorded simultaneously.

2.5. Micro-Computed Tomography (μCT) Morphometry [49]. The vertebral indentation specimens were immerged into deionized water and scanned using a Micro-CT specimen scanner (GE eXplore LocusSP Specimen Scanner; GE Healthcare Company, London, Canada). It is a cone-beam scanning system. The scanning protocol was 80 kV and 80 μA, with an isotopic resolution of 6.5 × 6.5 × 6.5 μm voxel size and an exposure time of 3 seconds per frame. The angle of increment around the sample was set to 0.4° resulting in 900 2D images.



(a) (b)

Figure 2: Schematic representation of the indent areas. On transversal slices of L6 vertebral body, nanoindentation test was performed randomly at four different regions (a). Five indentations were made for each site within 30–50 μm (b).



(a) (b) (c)

Figure 3: Micro-CT images of rat vertebral bodies. (a) Selection of the volume of interest (VOI) in the vertebral body for micro-CT analysis (yellow circles), with 3 VOIs in each vertebral specimen selected to contain the sites of indentations. Compared with sham operation (b), ovariectomy (c) results in pronounced trabecular deterioration of trabecular microstructure of the vertebral body.

To increase the signal-to-noise ratio, each image was averaged over 4 X-ray projections. Both bright fields, that is, an X-ray projection with no object in the field of view, and dark fields, that is, an image acquired without any X-rays, were collected for correction of the acquisition images. A modified Feldkamp cone-beam algorithm was used to reconstruct 2D projections into 3-dimensional (3D) volume [50].

The original 3D image was displayed and analyzed with software (Microview ABA2.1.1; GE Healthcare Company, London, Canada). A fixed threshold value was used to binarize bone from other components.

The volume of interest (VOI) was defined as a cylindrical volume of 110 × 110 × 450 voxel size. Three VOIs in each vertebral specimen were selected to contain the sites of indentations (Figure 3(a)). The method described in the paper is unique, because the purpose of our study was to evaluate the effect of estrogen deficiency on the nano/microscale structural and mechanical properties of vertebral trabeculae in rats. It is critical that the region of each specimen examined by μCT and AFM should contain the sites of indentations that were selected randomly. The method used in this study is different from other studies, because every experiment needs to customize the methodology to test its hypothesis [51–55].

Trabecular bone volume fraction (BV/TV), mean trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), geometric degree of anisotropy (DA), connectivity density, structure model index (SMI), and trabecular volumetric BMD at both organ and tissue levels were determined.

2.6. Statistical Analysis. Statistical analysis was performed using SPSS 11.0 for windows statistical software (SPSS, Chicago, IL, USA). Independent-samples t-test was employed to compare the two groups after determining the normal distribution of the data. A probability (P) value of <0.05 was considered to be significant.

3. Results

3.1. Body Weight and 2D DXA BMD. Body weight and 2D projectional DXA BMD of OVX and SHAM rats were summarized in Table 1. 2D BMD of the L6 lumber vertebrae was significantly less in OVX than in SHAM, by −14% with in vitro measurement and by −12% with in vivo measurement. No statistically significant difference between SHAM and OVX was found in their body weights and total body BMD.

Table 1: Body weight and DXA measurements.

Parameters	SHAM ($n = 10$)	OVX ($n = 10$)	P value
Body weight (g)	332 ± 13	339 ± 9	0.739
Total BMD (mg/cm^2)	170 ± 3	164 ± 2	0.068
L6 2D BMD in vivo (mg/cm^2)	209 ± 6	179 ± 4[a]	0.000
L6 2D BMD2 in vitro (mg/cm^2)	228 ± 6	201 ± 3[a]	0.001

Values are expressed as mean ± SD. [a]$P < 0.01$ versus SHAM.

Table 2: Trabecular volumetric BMD at organ and tissue levels and 3D microstructure evaluated by Micro-CT.

Parameters	SHAM ($n = 9$)	OVX ($n = 9$)	Difference (%)	P value
Organ BMD (mg/mm^3)	478.5 ± 32.4	348.3 ± 41.2	−27.2[a]	0.002
Tissue BMD (mg/mm^3)	820.1 ± 29.7	722.7 ± 36.5	−12.0[a]	0.001
BV/TV (%)	35.2 ± 7.3	25.6 ± 5.3	−28.6[a]	0.007
Tb.Th (μm)	65 ± 7	56 ± 7	−13.8[a]	0.027
Tb.Sp (μm)	177 ± 31	211 ± 47	19.2[a]	0.09
Tb.N (1/mm)	4.65 ± 0.59	3.86 ± 0.48	−17.0[a]	0.006
Structure model index (SMI)	1.02 ± 0.25	1.95 ± 0.31	91.2[a]	0.033
Degree of anisotropy (DA)	1.93 ± 0.29	2.04 ± 0.26	5.7	0.396
Connectivity density (1/mm)	35.6 ± 11.2	28.8 ± 9.8	−19.1	0.699

Values are expressed as mean ± SD. Difference: OVX versus SHAM, [a]$P < 0.05$.

3.2. Micro-CT Analysis of Trabecular Microarchitecture. Table 2 summarizes the data of micro-CT analysis of vertebral trabecular bone. Volumetric trabecular BMD was significantly less in OVX rats than in SHAM rats, both at organ level by −27% and at tissue level by −12%. OVX induced a marked deterioration in microarchitecture of vertebral trabeculae (Figure 3). Trabecular BV/TV (−29%), Tb.Th (−14%), and TB.N (−17%) were significantly lower in OVX than in SHAM. Tb.Sp (19%) and SMI (91%) were significantly greater in OVX rats than in SHAM rats. No statistically significant difference in DA and connectivity density was found.

3.3. Nanoindentation Analysis of Material Properties. The trabecular elastic modulus was 24.609 ± 1.375 GPa for OVX and 25.275 ± 1.457 GPa for SHAM. The trabecular hardness was 1.085 ± 0.135 GPa for OVX and 1.098 ± 0.142 GPa for SHAM. No significant differences in the hardness and elastic modulus between the two groups were found.

3.4. AFM Images Analysis of Nanostructure at the Trabecular Surface. Figure 4 shows typical AFM images of the unindented surfaces and indented impressions of vertebral trabeculae in SHAM and OVX rats. The difference in their surface morphologies can be clearly appreciated. The trabeculae of SHAM rats showed a rough surface with many nodules closely packed to each other, whereas OVX showed relatively smooth surface characterized by some loosely packed larger nodular structures (Figures 4(c) and 4(d)). The OVX bone showed larger composite of collagen and mineral crystals compared with the SHAM, while the interfibrillar space between collagen fibrils of SHAM was more compacted (Figures 4(e) and 4(f)). Little difference between OVX and SHAM was observed in the indentation impressions, indent depths, and residual area caused by plastic deformation. The average impression depth at 24 h after indentation was 172 nm.

4. Discussion

This study identified the structural features and mechanical properties of vertebral trabeculae in OVX rats at the micro- and nanoscales. Estrogen deprivation resulted in a dramatic deterioration in trabecular micro- and nanoarchitectures, while neither the hardness nor the elastic modulus of the vertebral trabeculae measured in longitudinal orientation was different from SHAM. Our observations of the cross sections of bone specimens were consistent with the previous data obtained from the longitudinal section [22, 28, 29]. The data indicate that the effect of estrogen deprivation on these particular intrinsic material properties seems to be insignificant, while effects on bone mineral density measures and on structure and geometry are conspicuous which is compatible with findings in our previous studies in several OVX animal models [3, 49]. Our study also shows that BMD changes after OVX were much greater in micro-CT 3D trabecular BMD at organ level than in 2D DXA BMD. This can be explained by greater loss in trabecular bone than in cortical bone in postmenopausal osteoporosis and by mask effects of relative unchanged cortical bone and end plates included in 2D DXA BMD. The more sensitive detection of bone loss induced by estrogen deprivation using CT than using DXA has been well documented, both in human and in animal models [43].

In this study, we used AFM to image the surface topography of trabeculae in OVX rats. While the composition and size of mineral crystals have been assessed in bone via different spectroscopic or X-ray methods [15, 56], their morphological evaluation through direct visualization in

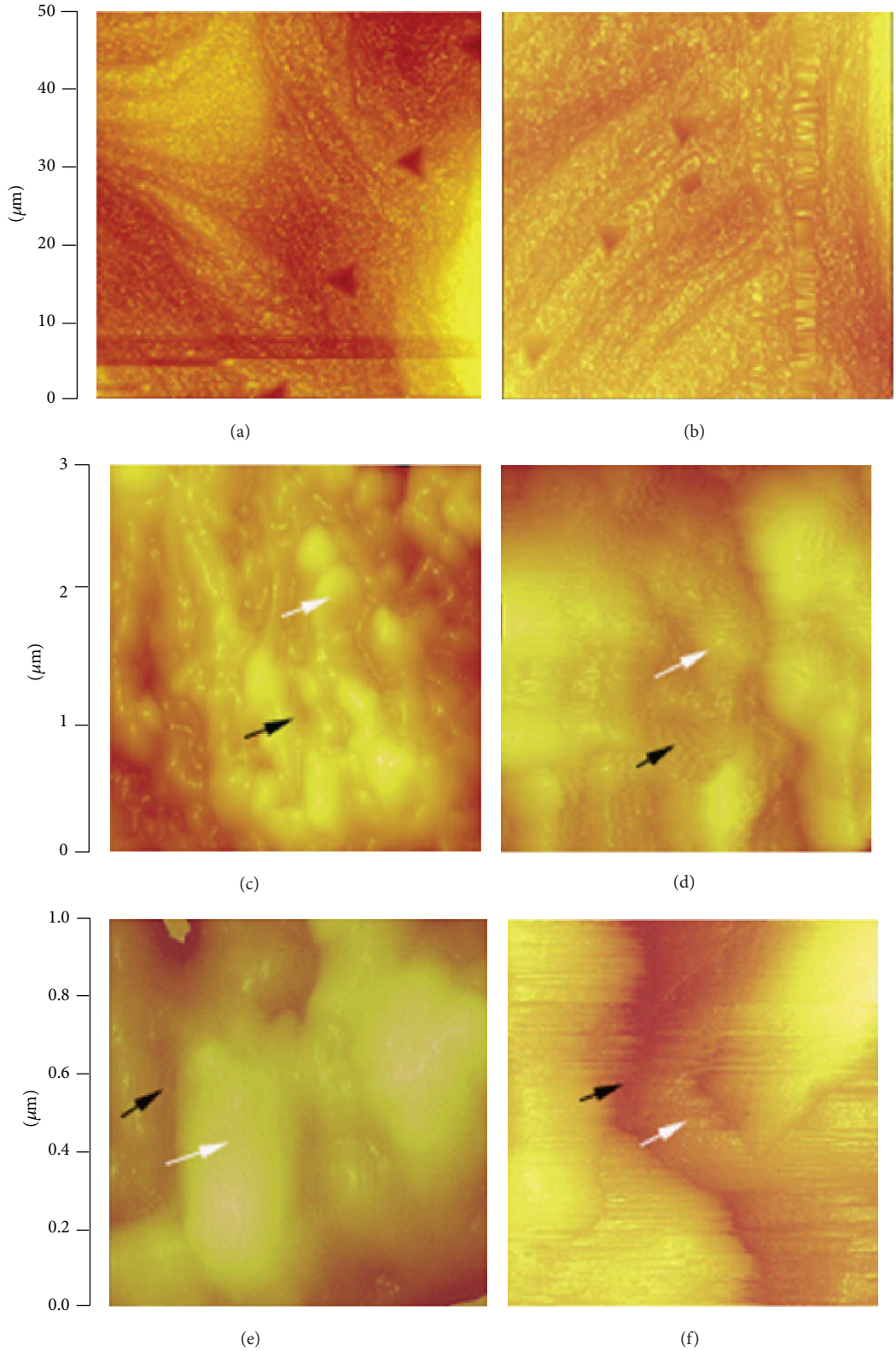


Figure 4: Typical atomic force microscopic topography of trabeculae of rat vertebral bodies of sham operated rats (left panel) and ovariectomized rats (right panel). The top 2 images (a, b) show unindented surface and indented impression. The middle 2 images demonstrate rough surface with many nodules closely packed to each other in sham operation (c), and relatively smooth surface characterized by some loosely packed large nodular structures in ovariectomized rat (d). The woven collagen fibrils of trabeculae from different group of animals have different directions. The interfibrillar space between collagen fibrils of a sham operated rat is more compacted (e), while ovariectomy results in larger size composite of collagen and mineral crystals (f).

bone remains of particular importance [57]. We observed some bundles combining fibrillar collagen and embedded minerals. The collagen fibers and mineral crystals were packed in trabeculae and presented as nodular-like surfaces. OVX induced larger mineral crystals and looser interfibrillar space of collagen, which is consistent with a human study that large mineral grain size was found in the trabeculae of osteoporotic bone or age-related fractures [37]. Generally in various materials including pure hydroxyapatite, there is a size effect that the smaller the grain size, the higher the stiffness, the compressive and tensile strengths, and the fracture toughness [24].

The specimen of SHAM rats in our study showed a rough surface with many nodules closely packed to each other, whereas the surface of the bone sample of OVX rats was relatively smooth characterized by loosely packed large nodular structures. Milovanovic et al. [37] showed that, in contrast to young individuals, lower surface roughness and reduced topographical complexity in the elderly signify a decline in bone toughness. Estrogen-deficiency-induced changes in type-I collagen and collagen cross-linking in bone could be related to lower surface roughness and reduced toughness [58], which make the bone more brittle and susceptible to fracture. Previous studies showed that the physicochemical status of mineral crystals and bone matrix was significantly correlated with the mechanical properties of bone at the organ and material levels [59]. Interestingly, our nanoindentation tests of trabecula bone in OVX rats did not demonstrate a strong relationship between the nanoscale structural features and local tissue mechanical behavior, though our AFM study showed the particular contributions of the mineralized bone matrix from the morphological point of view. Further experimental studies are warranted to evaluate mechanical behavior at the interfibrillar level and to assess the mechanisms of grain enlargement.

The inconsistency between the negligible changes in the nanomechanical properties and the remarkable alterations of trabecular nano- and microarchitectures might be explained from the viewpoint of micromechanics. The negligible differences in the mechanical properties of bone suggest that estrogen deprivation mainly influences the spatial topologies of the constituent organic and mineral phases but not their nanomechanical properties. Though the nanomechanical properties of bone remain the same, changes in geometry and structures compromise macroscopic mechanical properties of bone. Previously reported two studies with changes in bone nanomechanical properties [27, 60] indicated that protein undernutrition associated with estrogen deficiency deteriorated bone tissue properties with improvement upon essential amino acids supplements. Thus, undernutrition rather than OVX was the main causation for the changes of material properties. Guo and Goldstein [22] attributed the unchanged nanomechanics to the unchanged density of trabeculae throughout adult life. Wang et al. [61] reported no difference in elastic moduli or hardness in human cancellous bone between normal and fracture groups. Polly et al. [29] used quasi-static and dynamic nanoindentation techniques to measure elastic and viscoelastic material properties of the trabecular bone and found no difference in bone intrinsic properties between healthy pre- and postmenopausal biopsies. We used AFM to record the residual area and depth profile of indents caused by plastic deformation and found no difference between the two groups, either in the residual area or in the average depth of indents. The material surface of OVX and SHAM bone had similar plastic properties, although different surface morphological characterizations were found. Our study is consistent with all these reports that bone nanomechanical properties identified by nanoindentation remain unchanged following OVX.

Small differences in the obtained elastic modulus values compared to previous published literature could be explained by the differences in the bone sample and the Poisson ratio of 0.3 used in this study. Oliver-Pharr method is based on an assumption that the sample is a perfect isotropic solid. However, bone is a complex multiscale anisotropic medium and is heterogeneous at the organ scale. Its mechanical properties depend on the cross-sectional and axial location [30]. Indentation modulus overestimated the elastic modulus in the directions with lower stiffness, that is, the radial and circumferential axes in long bone, and underestimated it in the direction with highest stiffness, that is, the superior-inferior direction [35]. Our study takes into account the anisotropic character of both the indenter and the sample but not all direction. Guo and Goldstein [22] found elastic modulus of 17.7 ± 4.0 GPa in transversely oriented vertebral trabeculae. Polly et al. [29] found 14.51 ± 3.39 GPa in human trabecular bone in the longitudinal direction. Our study analyzed longitudinally oriented trabeculae with 24.609 ± 1.375 GPa, which compares very favourably with previous nanoindentation studies. Rho and Pharr [48] found that samples of human trabecular bone, in the transverse direction, averaged 19.4 ± 2.3 GPa, while Brennan et al. [62] found an average modulus across the width of the trabeculae of 20.78 ± 2.4 GPa. Interestingly, all these studies agree that in trabeculae the elastic modulus is higher in the longitudinal compared to the transverse direction. Therefore, it is necessary to implement several measurements in different orientations.

Our study has several limitations. The duration of our observation was relatively short, which may also help explain no significant changes in the mechanical material properties of the trabeculae that still existed without enough time for resorbing and forming by remodeling, while newly built trabeculae after OVX would [63]. Quantitative measure of the size of principal topographic elements of the surface by AFM needs to be developed, while our assessment was quantitative. Poisson's ratio of bone might change among different samples and orientations, but in this study Poisson's ratio was assumed to be 0.3. Finally, the bone samples were performed in dry conditions, while a previous study has shown that the hydration testing condition had influence on nanoindentation testing [64]. Nevertheless, interspecimen comparisons in our study were reliable since all the specimens were subject to uniform processing and testing conditions.

5. Conclusion

In conclusion, estrogen deprivation after OVX in aged rat leads to dramatic deterioration in trabecular

micro/nanoarchitectures, 3D volumetric BMD at both organ and tissue levels, and 2D DXA BMD as well, while the nanomechanical properties of trabecular bone remain unchanged. Further studies are warranted to develop quantitative measure of the size of principal topographic elements of the surface, to investigate the effects of mechanical loading or different treatments with various anti-osteoporotic agents on the nanoscale intrinsic material properties, such as long-term administration of bisphosphonates that may result in subtrochanteric insufficiency fracture, to have better understanding of the composition, assembly, organization, and function of the fundamental building blocks of this amazing tissue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Shidi Hu and Jin Li contributed equally to this work.

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Clinical Study

Comparison of the Spine and Hip BMD Assessments Derived from Quantitative Computed Tomography

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Quantification of bone mineral density (BMD) is being used as the main method to diagnose osteoporosis. Dual-energy X-ray absorptiometry (DXA) is the most common tools for measuring BMD. Compared to DXA, quantitative computed tomography (QCT) can determine in three dimensions the true volumetric BMD (vBMD) at any skeletal site. In addition to the spine, the hip is an important site for axial BMD measurement. This study examines lumbar spine and hip BMD of Chinese adults by QCT. Age related changes in bone mass derived by QCT measurements were determined. The osteoporosis QCT detection rates at the spine and hip are assessed in both female and male, and agreement of skeletal status category between the spine and hip in older adults is also assessed.

1. Introduction

Quantification of bone mineral density (BMD) is being used as the main method to diagnose osteoporosis. Several clinical techniques for BMD measurement are available, including dual-energy X-ray absorptiometry (DXA), quantitative computed tomography (QCT), and magnetic resonance (MR) techniques. DXA is the most common tools for measuring BMD. Compared to DXA, QCT can determine in three dimensions the true volumetric BMD (vBMD) at any skeletal site. It is less affected by surrounding tissues, eliminates the posterior vertebrae elements, and facilitates analysis of trabecular and cortical bone separately [1]. QCT has been utilized for measuring spinal BMD [2, 3]. According to the International Society for Clinical Densitometry (ISCD) positions QCT of the spine can be used to predict spinal fractures, to monitor BMD changes, and to initiate treatment [4]. In addition to the spine, the hip is an important site for axial BMD measurement [5], 3D QCT systems for measurement at the hip have been proposed and developed for research. CTXA Hip (a commercial QCT BMD analysis system) uses 3D QCT volume data sets to generate bone projection images that visually look like those generated by DXA. However, CTXA Hip exploits the anatomical detail in the 3D QCT data set to segment bone from surrounding tissues rather than relying on the dual-energy imaging method of DXA. Compared to DXA, CTXA Hip may provide more information. Nevertheless, the higher radiation exposure due to a dedicated QCT exam in comparison to a DXA must be considered.

At present, according to the diagnostic criteria for DXA established by the World Health Organisation (WHO) in 1994 in the clinical diagnosis of osteoporosis, osteoporosis is diagnosed by central DXA if the T-score of the lumbar spine or hip is −2.5 or less. However, the WHO diagnostic classification cannot be applied to T-scores from measurements other than DXA at the femur neck, total femur, lumbar spine, or one-third distal (33%) radius because those T-scores are not equivalent to T-scores derived by DXA. Thus equivalence with a DXA measurement cannot be achieved to QCT of the spine [6]. For the QCT vBMD of spinal trabecular bone, thresholds of 80 mg/cm^3 for osteoporosis QCT (equivalent to a DXA T-score of −2.5) were suggested

by the ISCD in 2007 [4] and by the American College of Radiology [7]. The areal BMD (aBMD) measurements and T-scores derived from CTXA and DXA are probably close [8]. Khoo study concluded QCT aBMD appropriately adjusted can be evaluated against NHANES reference data to diagnose osteoporosis [9].

In this study, CTXA Hip BMD measurements for total hip and femoral neck were compared to QCT Pro volumetric spine BMD measures. The aim of the present study was to determine (1) age related changes in bone mass in eastern Chinese derived by QCT and (2) the osteoporosis QCT detection rates at the spine versus hip and comparison of bone mass diagnostic classification by QCT vBMD and aBMD.

2. Design and Methods

2.1. Participants. The current study included 496 females and 330 males aged 20 to 84 years. All subjects were divided into two groups both females and males: young (age, <50 years), and older (age, ≥50 years). Study exclusion criteria included a history of renal failure, alcoholism, chronic colitis, leukemia, multiple myeloma, rheumatoid arthritis, metabolic and endocrine diseases, or bone tumors. Likewise, none of the participants were taking any medications that were likely to affect bone or soft tissue metabolism, such as glucocorticoids. The study protocol and procedures were approved by the ethics committee of the hospital. All of the participants provided written informed consent before any measurements were obtained.

2.2. QCT Measurements of BMD. All subjects were scanned using CT (*Somatom Sensation 16*, Siemens, Erlangen, Germany). Scan was performed using the following parameters: 120 Kv, 125 mAs, 1 mm slice thickness, and 500 Mm field of view (FOV).

QCT studies were performed using the QCT Pro calibration phantom and software system with the CTXA Hip analysis module (Mindways Software, Inc., Austin, TX). For lumbar spine trabecular BMD measurement, vertebrae from L2 to L4 were scanned in the supine position. Elliptical regions of interest were put in the midplane of three vertebral bodies (L2–L4) in the trabecular bone automatically. In CTXA Hip area BMD measurements, an anterior-posterior computed radiograph was obtained by the scanner from the iliac crest to mid-thigh, and the top of the femoral head to approximately 1 cm below the inferior extent of the lesser trochanter was defined graphically to define the scanning region.

2.3. The Diagnostic Criteria for Osteoporosis QCT. Based on these guidelines [6, 7], volumetric trabecular BMD values from 120 to 80 mg/cm^3 are defined as osteopenic QCT and BMD values below 80 mg/cm^3 as osteoporosis QCT. CTXA Hip BMD estimates provide the same clinical utility as that afforded by DXA. Osteoporosis is diagnosed by central DXA if the T-score of the lumbar spine or hip is −2.5 or less. Low bone mass or osteopenia is classified as a T-score of −1.0 to −2.5. According to WHO definition, these criteria should be restricted to postmenopausal females and aged males. In order to unify expression, all subjects were classified hereby in this study.

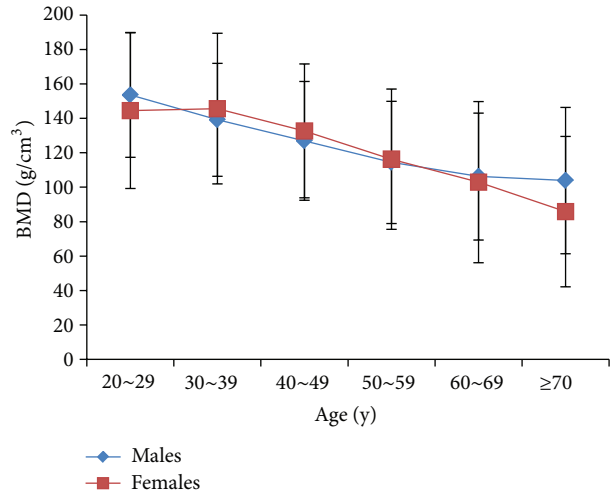

Figure 1: Bone mineral density (in grams per cubic centimeter) changes associated with age for males and females derived by QCT spine.

2.4. Statistical Analysis. All data analysis was performed using SPSS13.0; the incidence rates of osteoporosis QCT detection were calculated by QCT BMD measurements in males and females, the comparison of rates was conducted using chi-square test. The correlations between vBMD and aBMD variables were investigated using the Pearson correlation test for normally distributed variables or Spearman correlation for nonnormally distributed variables. The consistency was checked using Kappa-test. All statistical tests were two-tailed, and $P < 0.05$ was considered significant.

3. Results

3.1. Age Related Changes in Bone Mass of Spine in Males and Females Derived by QCT. The peak vBMD values of the lumbar spine was observed at 30 to 39 years in females (145.73±43.78, mg/cm^3) and 20 to 29 years in males (153.60± 36.18, mg/cm^3). In both sexes, aging was accompanied by a decrease in vBMD after peak bone mass (Figure 1).

3.2. The Osteoporosis QCT Detection Rates with vBMD versus aBMD in Males and Females. In males, of the 330 participants, 46 (13.94%) were found to have osteoporosis QCT by QCT Pro volumetric spine BMD measures; the osteoporosis QCT detection number for CTXA Hip area BMD measurements was 9 (2.73%). There was no significant difference in the osteoporosis QCT detection rates using chi-square test (chi-square value = 2.901; $P = 0.089$). However, of the 196 participants in <50 y males, 11 (5.6%) were found to have osteoporosis QCT by vBMD; 4 (2.0%) were detected to have osteoporosis QCT by aBMD. Roughly 3.6% of them differed in osteoporosis QCT detection rates at the spine and

Table 1: The diagnostic rates classification with vBMD versus aBMD in males.

Location	Osteoporosis QCT (%)	Osteopenia QCT (%)	Normal QCT (%)
Age <50 y (n = 196)			
Lumbar spine (vBMD)	11 (5.6%)	47 (24.0%)	138 (70.4%)
Hip (aBMD)	4 (2.0%)	54 (27.6%)	138 (70.4%)
Age ≥50 y (n = 134)			
Lumbar spine (vBMD)	35 (26.1%)	47 (35.1%)	52 (38.8%)
Hip (aBMD)	5 (3.7%)	43 (32.1%)	86 (64.2%)

Table 2: The diagnostic rates classification with vBMD versus aBMD in females.

Location	Osteoporosis QCT (%)	Osteopenia QCT (%)	Normal QCT (%)
Age <50 y (n = 225)			
Lumbar spine (vBMD)	20 (8.9%)	50 (22.2%)	155 (68.9%)
Hip (aBMD)	2 (0.9%)	57 (25.3%)	166 (73.8%)
Age ≥50 y (n = 271)			
Lumbar spine (vBMD)	89 (32.8%)	80 (29.5%)	102 (37.6%)
Hip (aBMD)	44 (16.2%)	126 (46.5%)	101 (37.3%)

hip by QCT. Moreover, in ≥50 y males, of the 134 participants, 35 (26.1%) were found to have osteoporosis QCT by vBMD; the osteoporosis QCT detection number for CTXA Hip area BMD measurements was 5 (3.7%) (Table 1).

In females, of the 496 participants, 109 (21.98%) were found to have osteoporosis QCT by QCT Pro volumetric spine BMD measures; the osteoporosis QCT detection number for CTXA Hip area BMD measurements was 46 (9.27%). There was a significant difference in the osteoporosis QCT detection rates using chi-square test (chi-square value = 124.86; P = 0.000), with QCT spinal vBMD detecting osteoporosis QCT more frequently than hip aBMD did. Further, of the 225 participants in <50 y females, 20 (8.9%) were found to have osteoporosis QCT by vBMD; 2 (0.9%) were detected to have osteoporosis QCT by aBMD. Roughly 8.0% of them differed in osteoporosis QCT detection rates at the spine and hip by QCT. In ≥50 y females, of the 271 participants, 89 (32.8%) were found to have osteoporosis QCT by vBMD; the osteoporosis QCT detection number for CTXA Hip area BMD measurements was 44 (16.2%) (Table 2).

3.3. Qualitative Skeletal Status Category Agreement between Spine and Hip by QCT Measurement. Osteoporosis QCT, osteopenia QCT, and normal QCT detection rates agreement between QCT spine and CTXA Hip is 3.7%, 32.1%, and 38.8% in older males, respectively, and 16.2%, 29.5%, and 37.3% in older females. Roughly 22.4% and 16.6% discordances was found in osteoporosis QCT detection rates between spine and hip by QCT in older males and females, respectively. 3.0% and 17.0% discordances was found in osteopenia QCT detection rates; 25.4% and 0.3% discordances was found in normal QCT bone mass detection rate (Figure 2).

3.4. Results of the Correlation and Kappa Test between QCT vBMD and aBMD. In both males and females, all BMD variables were nonnormally distributed variables. QCT vBMD

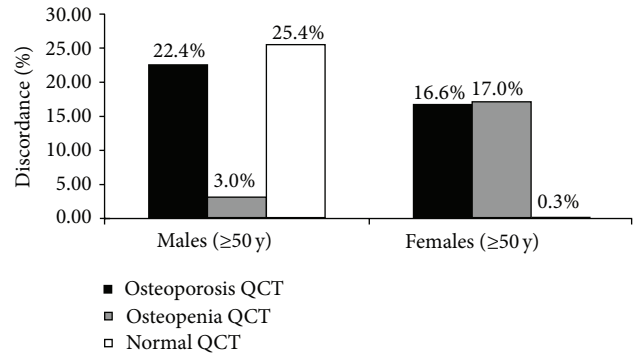

Figure 2: Discordances in skeletal status category between lumbar spine and hip in older males and females.

was positively correlated with aBMD (r = 0.130, P < 0.05) in males; the correlations between QCT vBMD and aBMD were strong positively correlated in females (r = 0.662, P < 0.01). In further consistency analysis, in <50 y males, Kappa coefficient = 0.113 and P = 0.072; in ≥50 y males, Kappa coefficient = 0.110 and P = 0.056; in <50 y females, Kappa coefficient = 0.202 and P = 0.000; in ≥50 y females, Kappa coefficient = 0.360 and P = 0.000.

4. Discussion

QCT could provide the similar results as conventional DXA [10], which may be useful in evaluation of bone mass. It has been generally accepted that peak bone mass at any skeletal site is attained in both sexes during the midthirties. Bone mass decreases significantly with aging both in middle-aged and elderly men and women [11, 12]. In present study, the peak vBMD values of the spine were observed at 30 to 39 years in Chinese women and at 20 to 29 years in Chinese men. Aging

was accompanied by a decrease in vBMD after peak bone mass in both sexes.

BMD measurement for DXA has been used as the gold standard in the clinical diagnosis of osteoporosis. QCT has a number of advantages over DXA in BMD measurement [13]. QCT is able to analyze not only vBMD of trabecular and cortical bone compartment separately, but also geometry and biomechanical parameters in bone such as cross-sectional area, cortical bone thickness, section modulus, and buckling ratio. The analysis of geometry and biomechanical parameters at hip could provide better prediction of hip fracture risk [14]. Despite these, one study showed a significant difference in osteoporosis detection rates between DXA and QCT, providing clinical evidence that QCT has a greater diagnostic sensitivity than DXA [15].

As we know, changes of BMD varied according to skeletal site. The sites of BMD most commonly measured are the lumbar spine and hip. Osteoporotic bone loss occurs mainly in trabecular bone. Many clinical guidelines also recommend lumbar spine measurements to assess skeletal status [16, 17]. In general, QCT is most applied in the lumbar spine to measure trabecular BMD. For the BMD of spinal trabecular bone, thresholds of 80 mg/cm^3 were suggested for osteoporosis. QCT data indicated the detection rate was 46.4% for spinal trabecular BMD in postmenopausal women [15]. In our study, the osteoporosis detection rates for QCT vBMD were 32.8% and 26.1% in ≥50 y women and men, respectively. The lower detection rate of lumbar spine osteoporosis QCT may be due to an unclear distinction between menopausal and postmenopausal status in women.

In addition to the lumbar spine, CTXA for BMD measurement at the hip has been proposed and developed for research [18]. CTXA Hip BMD estimates provides the same clinical utility as that afforded by DXA although the radiation exposure is about 50 times as high [8, 9]. Previous study demonstrated that the precision of CTXA duplicate hip scans was slightly better than DXA [8]. Cheng et al. [19] suggested the CTXA aBMD and *T*-score can be used in the diagnosis and management of osteoporosis as a substitute of DXA aBMD. Based on DXA BMD measurements, in 2005-2006, 49% of older US women had osteopenia and 10% had osteoporosis at the femur neck. In men, 30% had femur neck osteopenia and 2% had femur neck osteoporosis [20]. Based on CTXA aBMD measurements, our study showed 46.5% of older Chinese women had osteopenia and 16.2% had osteoporosis at hip. In older men, 32.1% had hip osteopenia and 3.7% had hip osteoporosis for CTXA BMD. Compared to DXA BMD measurements, the similar osteopenia and the increased osteoporosis detection rate were found by CTXA aBMD measurement in both men and women.

Our study showed QCT vBMD was positively correlated with aBMD both in males and females; nevertheless in further Kappa consistency analysis, Kappa value was less than 0.4 in both men and women; that implied agreement was not found in two diagnostic measures. This study showed the osteoporosis QCT detection rates for CTXA Hip aBMD measures were 9.27% and 2.73% in women and men, respectively. However, those were 21.98% and 13.94% for QCT Pro volumetric spine BMD. Furthermore depending on age, in ≥50 years women and men, the osteoporosis QCT detection rates for CTXA Hip aBMD measures were 16.2% and 3.7%, respectively, and for QCT Pro spine vBMD were 32.8% and 26.1%. There was a significant difference in osteoporosis QCT detection rates between two measurements providing clinical evidence that QCT spine vBMD has a greater diagnostic sensitivity than hip aBMD. This may be due to site-specific differences; trabecular bone can have an advantage of superior sensitivity due to the higher metabolic rate of turnover [9]. Many studies have reported greater discordance in osteoporosis diagnoses between skeletal sites by DXA measurement [21–24]. Recent study indicated that at least half of patients tested by DXA will demonstrate *T*-score discordance between spine and total hip measurement sites; discordance BMD was lower in lumbar spine than total hips [25]. In this study, osteoporosis QCT detection rates discordance between spine and hip measurement sites is 22.4% for males and 16.6% for females. It implied that one site measurement by QCT could be misclassified as not osteoporotic.

Our study has several limitations. There were no BMD measurements of the lumbar spine and hip by DXA in the meantime; there was no clear distinction between menopausal and postmenopausal status in women.

5. Conclusions

In summary, age related changes in bone mass derived by QCT measurements in eastern Chinese were determined. Qualitative skeletal status category was available for reference by QCT BMD of the spine and hip in Chinese adults. QCT vBMD were positively correlated with aBMD. However, poor consistency results were detected in the osteoporosis diagnosis between CTXA Hip aBMD measurements and QCT Pro spine vBMD measurements. Compared to CTXA Hip aBMD, QCT spine vBMD may be more sensitive for detecting osteoporosis QCT.

Conflict of Interests

None of the authors has any conflict of interests to declare.

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Review Article

Mechanism and Treatment Strategy of Osteoporosis after Transplantation

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Osteoporosis (OP) has emerged as a frequent and devastating complication of organ solid transplantation process. Bone loss after organ transplant is related to adverse effects of immunosuppressants on bone remodeling and bone quality. Many factors contribute to the pathogenesis of OP in transplanted patients. Many mechanisms of OP have been deeply approached. Drugs for OP can be generally divided into "bone resorption inhibitors" and "bone formation accelerators," the former hindering bone resorption by osteoclasts and the latter increasing bone formation by osteoblasts. Currently, bisphosphonates, which are bone resorption inhibitors drugs, are more commonly used clinically than others. Using the signaling pathway or implantation bone marrow stem cell provides a novel direction for the treatment of OP, especially OP after transplantation. This review addresses the mechanism of OP and its correlation with organ transplantation, lists prevention and management of bone loss in the transplant recipient, and discusses the recipients of different age and gender.

1. Introduction

Organ transplantation is at present the only effective way to treat the end-stage diseases. But, at the same time, it increases the risk of osteoporosis (OP) and osteoporotic fractures which would have a serious impact on survival and life quality both in children and in adults [1–6]. The preoperative or postoperative factors lead to OP as well as osteomalacia and fracture. Generally, bone damage in transplant patients undergoes four phases: firstly, development of end-stage organ disease before transplantation; secondly, exacerbation immediately after transplantation caused by high-dose immunosuppressive therapy and continuing homeostatic disturbances; thirdly, a phase of stabilization secondary to immunosuppressive dose reduction and reestablishment of microenvironment of bone; fourthly, the return of OP caused by failing graft function. In particular, OP after renal transplantation may thoroughly tend to pass through the process above [7]. Within the different areas of transplantation, the mechanism of OP after transplantation has made considerable progress. Nonetheless, the related drugs for OP after transplantation are limited and lack pertinence in clinical practice. Owing to complex and diverse pathogenesis, strategies in the treatment and management of transplant patients with OP need to be categorized. This review will systematically investigate the prevention and treatment of OP in organ failure patients with different surgical state and population and summarize the progression of OP in scientific research and clinic.

2. Mechanism of Osteoporosis and Its Correlation with Transplantation

OP is characterized by a reduction in bone quality and bone mineral density, which usually gets worse with age. In particular, during the bone remodeling, the imbalance between bone formation and resorption will cause bone loss, which influences architecture of bone and attenuates the whole bone strength. Bone remodeling, which is mediated by osteoclasts (OC) and osteoblasts (OB) activities, is continuous in the whole life [8]. With the further research on the mechanism of OP, the important role of the molecule composed of osteoprotegerin/receptor activator of nuclear

factor-κB ligand (OPG/RANKL) [9, 10] in bone remodeling is striking; up to now, OPG/RANKL acts as a vital coupling factor between OC and OB. OPG and RANKL, produced by osteoblasts or bone marrow stromal cells, inhibit osteoclast differentiation and bone resorption activity. In addition, there are other factors or regulators that can influence the differentiation of OC or OB. An advanced research [11] showed that two important factors, complement component 3a (C3a) and collagen triple helix repeat containing 1 (Cthrc1), establish a bridge between OC and OB. C3a is derived from mature osteoclasts (mOC) and stimulates osteoblastogenesis, while Cthrc1 is secreted from mature active OC (maOC) in the middle of bone resorption and stimulates OB differentiation. The signal transduction pathways between OC and OB have long been shown to exist. Recent studies have demonstrated that several new transcription factors or regulators, such as nuclear factor I-C (NFI-C) [12], omentin-1 [13], and Netrin-4 [14], play different regulating roles in osteoblast proliferation and differentiation; moreover, they could also be used as novel therapeutic approach for treating OP. Some special type of osteoporosis, such as glucocorticoid- (GC-) induced OP (GIO), had already become a hot spot. At the gene regulation level, microRNA-29a (miR-29a) protects against GC-induced disturbance of Wnt and Dkk-1 actions and improves osteoblasts differentiation and mineral acquisition [15]; this study emphatically indicated the detrimental effects of GC treatment in association with reduced miR-29a expression; when the miR-29a function is enhanced, the side effects of GC treatment on mineral acquisition and osteoclast resorption are alleviated, and also RANKL expression is reduced, while knockdown of miR-29a accelerated the process above. Also, microRNA-17/20a inhibits osteoclastogenesis and bone resorption through blocking of RANKL expression in GIO [16]. Accordingly, the gene can regulate the differentiation of OC and OB by signaling pathway; moreover, it can be used as an alternative tactic for alleviating GC-induced bone deterioration. On the cellular level, in a previous study, Aggarwal et al. [17] found that the human umbilical cord blood-derived $CD34^+$ cells induced bone formation in a murine model of OP and also showed that $CD34^+$ transplantation increased trabecular numbers and thickness and increased bone mineral density (BMD), thereby indicating induction of osteoblast in bone. Above these, a safe conclusion to be drawn is that the OPG/RANKL system is the most important regulating mechanism in bone remodeling, while the other signaling pathway has increasingly become the research hot spot.

In the field of organ transplantation, OP is one of the major complications. The OPG/RANKL system is also involved in the pathogenesis of OP after transplantation. Many immunosuppressants directly or indirectly affect the reconstruction and absorption of bone throughout OPG/RANKL system. GC plays a critical role in the mechanisms of bone loss, such as reduced intestinal calcium absorption and renal calcium wasting and both may lead to a secondary hyperparathyroidism. Indeed, GC induces apoptosis of osteoblasts and osteocytes and prolongs lifespan of osteoclast, resulting in low bone mass and microarchitectural deterioration of bone tissue, which leads to severe OP. In this intricate process, van Staa [18] found that GC stimulates osteoclastogenesis by the regulation of OPG/RANKL, and one research has confirmed that an anti-RANKL antibody can protect the bone from loss in mouse model of GIO [19], revealing that OPG/RANKL is crucial for the induction of GIO. At the early period of posttransplantation, an excessive amount of GC must be administered in order to gain the immunosuppressive effect. With regard to GC excess, some researches confirmed that it was directly associated with osteoblast and osteocyte apoptosis in a transgenic mouse model of cell-targeted disruption of GC signaling [20, 21]. Hence, high-dose GC negatively affects osteoblast and osteocyte function. Several studies [22, 23] indicated that these actions include a decrease in the ratio of OPG/RANKL, which increased bone resorption and reduced bone formation, and also demonstrated that the Wnt signaling pathway may be involved in the GC-induced suppression of OPG. Obviously, the OPG/RANKL ratio controls the absorption of osteoclasts on bone; that is to say, the ratio <1 suggests a RANKL predominant activity and bone resorption, while an OPG/RANKL ratio >1 reveals OPG greater activity, and the bone protection process was predominant [24]. This conclusion has been authenticated in the bone marrow microenvironment after allogeneic hemopoietic stem cell transplantation [25]. Only when GCs bind to glucocorticoid receptors (GR) can they exert their functions [26] and then induce the latter conformational change; thus the activated GR can regulate gene expression in a negative way (transrepression), which causes their anti-inflammatory effect (also called immunosuppressive effect). GCs act primarily via the GR in bone cells to induce bone loss [27, 28]. In GIO model, GR in osteoblasts was sufficient to lead to GC-mediated bone loss, while GR in osteoclasts was insufficient. One study [29] confirmed that osteoclastogenesis could be enhanced in the initial phase of GC exposure. The enhanced osteoclastogenesis can decrease the ratio of OPG/RANKL. With regard to excess GC, it can suppress bone formation through inhibition of osteoblastic gene transcription [21]. Additionally, excess GC, through increased protein degradation and decreased protein synthesis, can also adversely affect muscle function; moreover, the riskiness of fragility fractures steadily increases by muscle weakness [30]. However, fortunately, OB can partly regulate the detrimental effects of GC; a frontier research [31] indicated that gene encoding TXNIP may increase the ratio of OPG/RANKL to disfavor OB-mediated osteoclastogenesis. Meanwhile, Epimedium [32], the Chinese patent medicine, can antagonize the abnormal expressions of OPG and RANKL mRNA in the GIO model; thereby it prevents the progression of GIO. Nevertheless, similar studies have not been reported in the GIO after transplantation. Hence, as will be readily seen, on the one hand, GC has direct and indirect pathway to mediate OP and inhibits bone formation (Figure 1); on the other hand, the protection mechanism of OB may play a considerable role in GIO, even treatment for GIO after organ transplantation (Figure 2).

The CI-based immunosuppression regimens, including cyclosporine (CsA) and tacrolimus (FK506), have been linked to OP in adult transplant recipients [33]. The murine experiment [34] suggests that FK506 binding protein 5

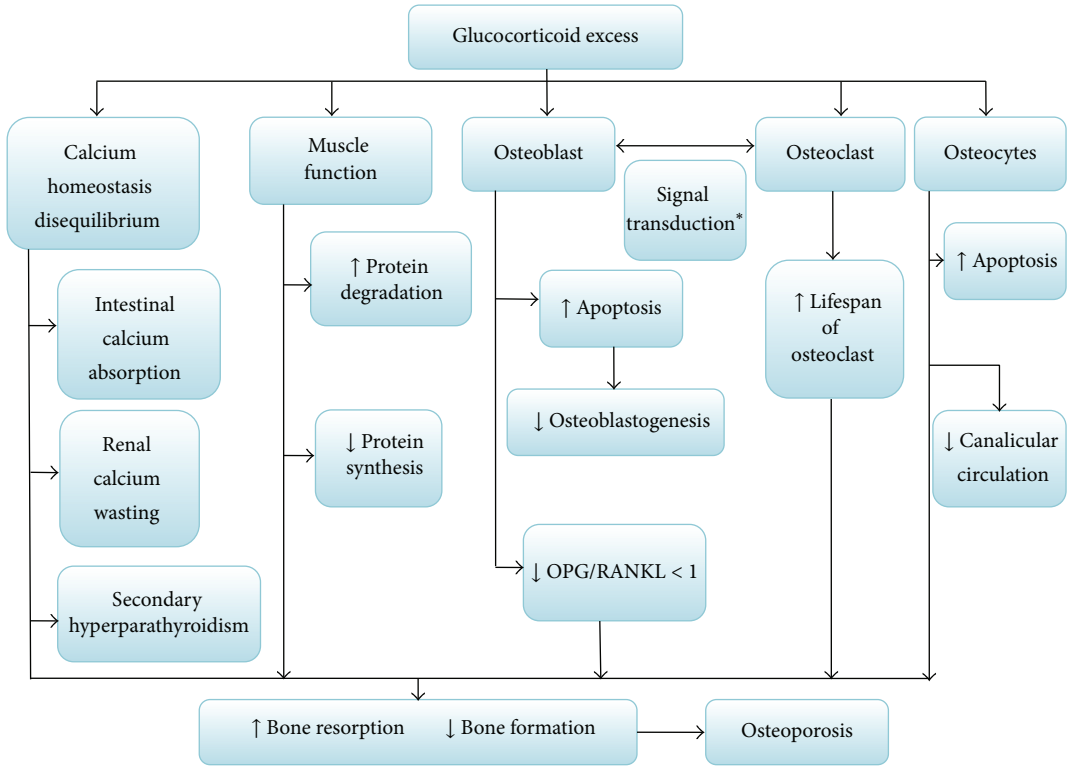

Figure 1: Effect of glucocorticoid excess on bone after transplantation. The atlas has indicated that glucocorticoid has direct and indirect pathway to mediate osteoporosis and inhibits bone formation after transplantation. The signal transduction pathway*: some factors establish a bridge between osteoblast and osteoclast, like complement component 3a (C3a) and collagen triple helix repeat containing 1 (Cthrc1), but there are few literatures after organ transplantation. The upward arrows show promoting effect; the downward arrows show lessening or inhibitory effects.

(FKBP5) messenger RNA (mRNA) in bone marrow can promote osteoclast differentiation by a mechanism distinct from NF-κB activation and might play a role in GIO. Accordingly, FK506 has a negative effect on bone. On the contrary, CsA does not adversely affect bone metabolism or accelerate GIO [35]. The effects of the other immunosuppression regimens like mycophenolate mofetil, sirolimus (SRL), and everolimus on bone have a discrepancy. A recent in vitro study [36] suggests sirolimus might interfere with the proliferation and differentiation of osteoblasts, while a previous research [37] showed SRL was a "bone sparing immunosuppressant," and it can increase the ratio of OPG/RANKL and has a potential to counteract deleterious GC effects on the bone. The function of SRL on bone should be more discussed. Everolimus [38] reduces cancellous bone loss in ovariectomized rats by decreasing osteoclast mediated bone resorption. Mycophenolate mofetil has no influence on bone formation and mass in clinical observations. Other new agents, such as daclizumab, are still being evaluated for their skeletal effects. But, no studies at present have confirmed that the effect of immunosuppressant on bone has a close correlation with OPG/RANKL, except for GC (Figure 3).

The OPG/RANKL system may be involved in the pathophysiological evolution of OP after transplantation. A study confirmed that the inseparable correlation between declined serum OPG levels and the relative bone loss has been observed in the early cardiac posttransplantation period, regardless of effective immunosuppressive therapy [39]. Fábrega et al. [40] revealed the same conclusion that OPG and receptor activator of RANKL may contribute to the development of OP late after orthotopic liver transplantation (OLT), and it is the activation of the immune system produced by the allograft that affects the release of both OPG and RANKL after liver transplantation. It seems that OPG/RANKL system is influenced by immune system in the organ transplantation. In addition to OPG, sclerostin, a circulating inhibitor of the Wnt-signaling pathway, has also received attention. Wnt-signaling pathway [41, 42] has a central role in regulating bone formation, and sclerostin inhibits bone formation. Thus, sclerostin is a protective factor in bone formation and Wnt signaling contributes to the development of OP. A research [43] concluded that the rapid reduction of elevated serum sclerostin levels one year after kidney transplantation parallels the improvement of renal function; the normalization of this hormone could contribute to improved bone health after renal transplantation. Hence, OPG/RANKL or Wnt signaling may be involved in the regulating mechanism of OP after transplantation, which is also influenced by immune system and affects the functions of graft.

All the mechanisms of OP and the relationship with transplantation have been discussed above, but the role of

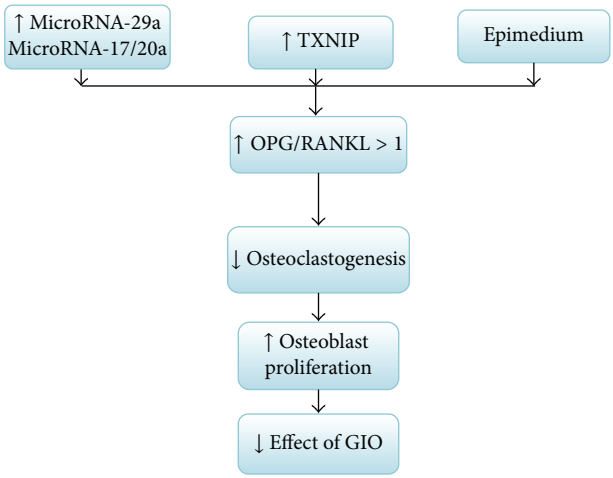

Figure 2: The protection mechanism against glucocorticoid-induced osteoporosis. MicroRNA-29a and microRNA-17/20a can inhibit osteoclastogenesis and promote osteoblast proliferation. Epimedium, which is the Chinese patent medicine, can antagonize the abnormal expressions of OPG and RANKL mRNA. The gene encoding TXNIP may increase the ratio of OPG/RANKL to downregulate osteoblast-mediated osteoclastogenesis. These potential protection mechanisms can prevent the progression of GIO and can provide a feasible and effective guidance to the treatment of osteoporosis after transplantation. The upward arrows show promoting effect; the downward arrows show lessening or inhibitory effects.

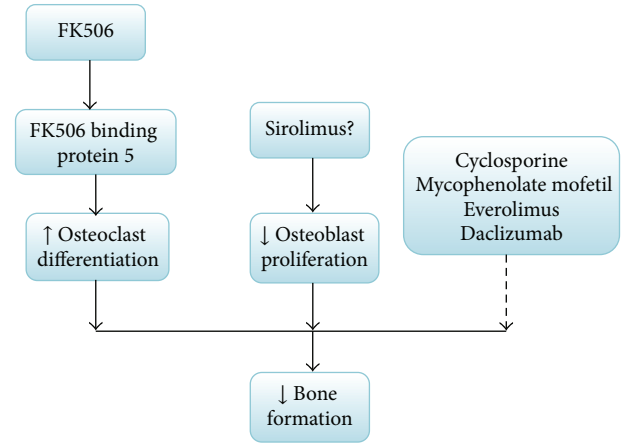

Figure 3: Effect of the nonglucocorticoid immunosuppressant on bone. FK506 binding protein 5 (FKBP5) messenger RNA (mRNA) can promote osteoclast differentiation, involved in glucocorticoid-induced osteoporosis; the function of sirolimus (SRL) on bone should be more discussed. The dashed arrows show that the other nonglucocorticoid immunosuppressants may not influence bone formation and bone mass or are still being evaluated for their skeletal effects.

nonglucocorticoid (non-GC) immunosuppressants in post-transplantation bone disease is less well defined and needs more sophisticated research. Thus, a more comprehensive understanding of bone turnover and remodeling may lead to better therapeutic strategies to control OP in relevant diseases, especially after transplantation.

3. Drugs for the Treatment of Osteoporosis after Transplantation

3.1. Drug Therapy. At global clinical market, more than 20 kinds of drugs for OP have been developed, and they are broadly divided into 4 categories: calcium and vitamin D, antiresorptives, and bone formation stimulating and uncoupling regimens. With the increasing exploitation of new drugs, such as sclerostin inhibitors, bone formation stimulants, $\alpha V\beta 3$ integrin antagonists, cathepsin K inhibitor, calcium sensitive receptor antagonist, chloride channel inhibitors, nitrates, and so forth [44], the safety and effectiveness of these agents have attracted attention.

3.1.1. Bisphosphonates. Within these new therapeutic agents above, some of them have been widely applied in clinical practice, whereas the research relating to the treatment of OP after transplantation is seldom involved. According to the PHARMAPROJECT database, bisphosphonates are the first drugs recommended for the treatment and prevention of postmenopausal OP and are still the hot spot in the OP treatment research. A few novel, longer acting, and more potent bisphosphonates like ibandronate, risedronate, and zoledronic acid may be given as infrequent, intermittent administration, which have been lately approved by US Food and Drug Administration [45]. A randomized case-control study [46] has shown that the use of alendronate sodium (Fosamax) (70 mg per week) for 14 months has a better curative effect without deteriorating renal function. Moreover, Fosamax significantly increased the bone mineral density (BMD) of hip in men more than in women. Abediazar and Nakhjavani [47] have shown that low-dose (30 mg) alendronate combined with vitamin D can increase the BMD immediately after renal transplantation. Also, the recent study [48] put forward the fact that a combination of vitamin D and bisphosphonate is the most effective protocol to improve BMD in renal transplantation recipients, but it also points out that patients who had persistent hyperparathyroidism could not use vitamin D and bisphosphonate only. Consequently, calcitriol (1,25-(OH)2D3) and alfacalcidol are also basic drugs that protect against bone loss. Among bisphosphonates, a research [49] had shown that pamidronate (90 mg, start 3 weeks after transplantation for 3 months) was comparable to alendronate in prevention of bone loss for the first six months after kidney transplantation. However, Torregrosa et al. [50] concluded that the administration of 60 mg pamidronate should be safe and has less adverse effects. Daily administration of bisphosphonates is limited by major gastrointestinal side effects [51]. A newer, orally administered bisphosphonate named risedronate can be well tolerated than others. Torregrosa et al. [52] also found that combination of risedronate (35 mg/week, oral administration) and vitamin D as well as calcium (800 IU cholecalciferol and 2500 mg of $CaCO_3$/d) ameliorates BMD and bone pain in renal transplant recipients with established OP and also improves quality of life. A 1-year randomized, double-blind,

placebo-controlled study [53] demonstrated that ibandronate (i.v. 3 mg, for 3 months) appeared to be safe and well tolerated for 12 months' treatment with early stable renal function (≤28 days following transplantation, GFR ≥ 30 mL/min); use of ibandronate alone did not show any benefit in preventing bone mineral density loss in the lumbar spine. On top of oral calcitriol 0.25 mg/day and calcium 500 mg b.i.d. is virtually maintaining BMD without any loss over 12 months after renal transplantation. In a multicenter, phase II, randomized open-label trial of intravenous zoledronic acid (ZA) (4 mg) to prevent BMD loss in adult recipients of allogeneic hematopoietic cell transplantation (alloHCT) with osteopenia before HCT, it had been confirmed that intermittent ZA is effective in preserving long-term bone health in adult alloHCT recipients at risk for OP [54].

3.1.2. Recombinant Human Parathormone. Teriparatide is a recombinant human parathormone (PTH 1–34), which is an anabolic agent, currently only approved for the treatment of osteoporosis with high risk of fracture. A large sample study [55] has shown that patients who receive long-term GC treatment use teriparatide (20 μg, once daily) to increase more bone mineral density than in those receiving alendronate. Nogueira et al. [56] strongly suggest that refractory hypocalcaemia after renal transplantation in patients with low PTH levels can be successfully treated with teriparatide; PTH analog therapy permits earlier suspension of intravenous calcium supplementation and reduces calcitriol requirements.

3.1.3. Sclerostin Inhibitors. Denosumab, which is called anti-sclerostin antibody, is a RANKL inhibitor for treatment of postmenopausal OP. It can theoretically reduce osteoclastic resorption of trabecular structures, but currently the human data is as yet unproven [57]. Whether anti-sclerostin antibody treatment is efficacious to prevent bone loss after renal transplantation would need to be investigated. Antibodies targeting sclerostin increase bone growth in preclinical studies in osteoporotic monkeys [58]. In a phase I clinical study, a single dose of anti-sclerostin antibody (AMG 785, romosozumab) increased bone density in the hip and spine in postmenopausal women [59]. Hence, the inhibition of sclerostin might be a promising therapeutic strategy for the preservation of bone mass. However, the use of these drugs in the clinical routine of recipients is limited by their poor gastrointestinal tolerance, variable oral bioavailability, and long-term compliance, especially bisphosphonate. Moreover, the efficaciousness of anti-sclerostin antibody treatment to prevent bone loss after transplantation should be more investigated.

3.2. Bone Regenerative Therapy. At present, the regenerative bone therapy acts as source to treat osteoporosis, including embryonic stem (ES) cells and pluripotent stem (iPS) cells. ES cells are created from the inner cell mass of the blastocyst, an early-stage embryo, which has a high proliferative capacity in addition to pluripotency. A recent report confirmed the efficacious use of ES cells for the replacement of lost tissue, like bone [60]. Transplantation of allogenic ES cells also rises the risk of a rejection response in the recipient. However, iPS cells are largely free from ethical issues or the possibility of rejection by the immune system; they can differentiate into any cell type within the body. These induced pluripotent human stem cells were first successfully established from the human in 2007 [61]. A research had shown that transplantation of allogenic adipose-derived stromal cells (ASCs) can restore the BMD and bone histomorphometric properties of rats with glucocorticoid-induced OP (GIOP) and may serve as a potential treatment for GIOP [62]. Another study [63] also supports the use of ASCs as an autologous cell-based approach for the treatment of osteoporosis. Nevertheless, many ethical and technological issues should be more discussed. Not all of the bone regenerative therapies can treat osteoporosis after transplantation and the relative data are still absent. These researches above are anticipated to be extremely useful for the development of new therapeutic strategies, where previous strategies have failed.

4. Management of Immunosuppressive-Induced Osteoporosis

According to its characteristics of immunosuppressive-induced OP, the treatment of OP after transplantation is not only by using therapeutic drugs, but also by adjusting the dosage of immunosuppressive drugs. Numerous studies [64, 65] have also demonstrated that GC is a major contributor to bone loss after transplantation, especially the rapid bone loss that occurs in the first 6–12 months. Therefore, in the first years after transplantation, GC reduction or complete avoidance will be helpful to these patients. But, a randomized controlled trial [66] has shown that GC withdrawal, when carried out weeks to months after renal transplantation, is correlated with an increased risk of acute rejection. Hence, the current Improving Global Outcomes (KDIGO) guidelines [67] do not currently recommend GC withdrawal and avoidance as a routine course of action. So GC therapy should be given at the lowest possible therapeutic window in order to avoid acute rejection and delay the progression of OP. However, the specific data of GC avoidance or withdrawal protocol are conflicting clinically; one retrospective study [68] showed that all liver transplant patients who received GC >3500 mg in the first year have a much higher risk of bone disease than the group of GC <3500 mg and that female patients were worse than the male patients. Another trial [69] comparing early (7 days) GC cessation versus long-term, low-dose (5 mg/d after 6 months of transplant) GC therapy in 386 renal transplant patients showed that there is no discrepancy in the rate of bone loss at 5 years' follow-up. Moreover, for nonrenal transplant patients, there is a lack of evidence supporting GC avoidance or withdrawal protocol and there is no agreement on an ideal protocol. The other immunosuppressants are not proven by experiments that change the dosage of them for treating OP. So, no prescription modifying the immunosuppressive regimens, except for GC, has been significantly influenced in bone, and there is no good clinical evidence for choosing calcineurin inhibitors or other non-GC immunosuppressants to deal with OP.

5. Prevention and Management of Pretransplantation

In order to reduce the OP after transplantation, attention to comprehensive and rigorous preoperative prevention programs should be paid. Most patients undergoing transplantation will have preexisting bone disease, such as renal osteodystrophy, chronic kidney disease-mineral and bone disorder (CKD-MBD), osteitis fibrosa, and chronic obstructive pulmonary disease (COPD). There are many common factors causing the bone disease, which are persistent hyperparathyroidism (PTH), diabetes mellitus, water electrolyte disorder in dialysis, malnutrition, and so on, as well as anxiety, smoking, drinking, obesity, lack of sun exposure, age at menopause (women), and number of falls which became independent risk factors. One study has indicated that low vitamin D levels and bone disease are common among patients with end-stage liver disease awaiting liver transplantation [70]. To control these risk factors above, for example, treating primary bone disease and hyperparathyroidism, controlling blood sugar can effectively reduce the incidence of the osteoporosis and associated bone disease of renal transplant recipients and will extend their lifetime. Tseng et al. [71] found that a significant BMD decreasing was also found in the group of CKD stage ≥III, especially in women, and concluded that osteoporosis screening is necessary in patients with poor renal function. Dorn et al. [72] pointed out that the adolescent smokers are at higher risk for less than optimal bone accrual. Even in the absence of diagnosable depression, depressive symptoms may influence adolescent bone accrual. Physical activity should be encouraged during aging to reduce skeletal structural decay [73]. It can be safely concluded that lifestyle modification including healthy dietary practices and regular exercise, cigarette cessation, and avoiding moderate alcohol intake should be necessary. Vitamin supplementation, particularly vitamin D, should be considered to enhance diet based on patient's need.

6. Osteoporosis Prevention and Management of Posttransplantation

6.1. Paediatric. Transplantation may lead to secondary OP in children. In paediatric renal recipients, preexisting renal osteodystrophy at the time of kidney transplantation, GC treatment, and long-term graft function can be three major contributing factors [74]. A descriptive study [75] on bone histomorphometric findings pointed out that bone quality (i.e., abnormal turnover rate, thin trabeculae) rather than the actual loss of trabecular bone might account for the increased fracture risk in pediatric recipients; in addition, children with a higher present GC dose (≥3 mg/day) had significantly lower osteoclast (OC S/BS) ($P = 0.018$) and osteoid maturation time (Omt, $P = 0.028$) than children with the lower GC dose in this study. Recently, bone biopsy with tetracycline labeling and histomorphometry analysis is still the gold standard in assessing bone quality [76]. However, invasive examinations are not applied to children and noninvasive measures like peripheral quantitative computed tomography (pQCT) are not widely available. Hence, currently, it is recommended that PTH levels should be kept within the range appropriate for the CKD stage. Both native and active vitamin D are used to suppress PTH levels in CKD patients. Native vitamin D should be served as a first-line therapy in patients showing vitamin D insufficiency or deficiency (<30 ng/mL), while active vitamin D should be served as a second-line therapy. Accordingly, paediatric transplant patients should be given optimal nutrition, optimal treatment with vitamin D and calcium, and low dosage of steroids. And then regular physical activity is helpful for improving muscle and bone strength in children. Some studies [77] have indicated that GC withdrawal and recombinant human growth hormone (GH) therapy are helpful for attaining adult height. However, use of GH to treat OP of paediatric renal transplant patients is not yet common. El-Husseini et al. [78] had demonstrated that treatment of established bone loss with alendronate (5 mg/d, oral) is effective in young individuals even after the period of most rapid bone loss has already occurred and also indicated efficacy of intranasal calcitonin (200 IU/day) in the treatment of bone loss in young renal transplant recipients compared to the control group. But, the efficacy and safety of these drugs must be further proven in adequately designed clinical trials.

6.2. Women. Bone loss, especially in women, has been a concern with the long-term use of glucocorticoids and has been one of the driving forces behind steroid minimization and steroid withdrawal protocols. In addition, Brandenburg et al. [79] have confirmed that low estradiol and high luteotropic hormone (LH) levels correlated with the extent of annual BMD loss ($P < 0.05$) in postmenopausal renal transplant women; the lumbar T-scores reduced in the very late period after renal transplantation. Circulating sex hormones influence lumbar BMD. Estrogen supplements have a certain effect, but the side effects should be considered. Toro et al. [80] pointed out that OP was more frequent among female than male patients. The incidence rate of osteoporosis was higher among postmenopausal than premenopausal patients (50% versus 16.1%). In premenopausal women there was a negative correlation between the BMD of the vertebral column and PTH ($P < 0.024$). A cross-sectional study [81] has shown that the decrease of BMD during the menopause is associated with follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels, rather than estradiol (E_2) in Chinese women. A prospective study on the mechanism of postmenopausal women OP indicated that the expression of ER-a36 in bone is positively associated with BMD and negatively associated with serum levels of the bone biochemical marker osteocalcin, and it mediates a bone-sparing effect of the low level of E_2 in postmenopausal women [82]. Another study by Opelz and Döhler [83] corroborated the fact that the posttransplant fracture risk was increased for women, especially for women over 60 years of age who had a 5-fold increased risk of hip fracture. Hence, according to the characteristics of osteoporosis of female transplantation recipients, selecting the appropriate treatment may be important. At present, bisphosphonate therapy is a conventional method, yet there are many side effects. Dietary counseling

to encourage all patients who begin receiving either oral or intravenous injection bisphosphonate therapy should have adequate calcium and vitamin D intake. A daily intake of 1200 mg of calcium is recommended for all women with osteoporosis, but high doses of calcium supplementation may cause increased kidney stone formation [84]. A research suggested that patients, over the age of 50 years, can receive oral administration of vitamin D in 800 IU, including supplements if necessary [85]. Current OP therapies have significant drawbacks; Joshua et al. [86] put forward the fact that cyclic GMP- (cGMP-) elevating agents may have bone-protective effects through nitric oxide/cGMP/protein kinase G (NO/cGMP/PKG) pathway. Accordingly, this provided a concept that soluble guanylate cyclase may act as a novel class of drugs, anabolic treatment strategy for postmenopausal OP. However, there are few clinical studies involving postmenopausal women who had undergone transplantation. Many new drugs cannot be used for clinical purposes, which should be more discussed.

6.3. Elderly People. Currently, although organ transplantation has already extended life expectation in older age groups, elderly renal recipients (defined as patients above 65 years old) need more consideration, in terms of not only selecting and waiting time, but also laying emphasis on their posttransplant and long-term care. Older transplant recipients had worse outcomes than younger recipients [87]. OP is a major concern during the whole life of transplant recipients [88]. So, the management of elderly recipients should be rigorously handled. Consequently, Mallet et al. [89] pointed out the impact of polypharmacy in general and its side effect on mortality and morbidity especially in aged patients. Hence, immunosuppressants have to be adapted to avoid both rejections and adverse effects. On the other hand, older transplant patients seem to have lower incidences of acute rejection episodes than younger patients.

Before transplantation, in elderly patients, there are numerous physiological conditions, such as reduced biomechanical strength, muscle fiber atrophy, calcium intake insufficiency, and vitamin D deficiency. All of which may determine a more complex bone metabolism alteration in elderly patients than in the young. With the research on the mechanism of senile OP, Leucht et al. [90] found that human bone marrow loses its osteogenic potential with age and aged bone grafts show a dramatic reduction in Wnt gene expression and Wnt responsiveness. This provided a new strategy for the treatment of skeletal injuries which is packaging Wnt protein into lipoparticles. Then after transplantation, using glucocorticoids (GCs) will accelerate bone loss, so older people are more likely to experience fracture. That is to say, GCs should be reduced to the appropriately lowest dose. Ahmadpoor et al. [91] showed that a hip or spine Z score of 1 or less had relationship to the total dosage of prednisolone ($P < 0.001$). The other drugs, such as bisphosphonate, had already been used clinically, but the efficacy and side effects have not been systematically and comprehensively evaluated; the side effect of these drugs on the senile people is more prominent than on young people. Although the new mechanism of action of drugs had already made progress, just a few researches had probed into treating the elderly recipients who had undergone transplantation.

7. Conclusion

Osteoporosis following transplantation is an intractable and intricate task; it increases mortality and decreases quality of life. Not only OP after surgery, but also preexisting osteoporosis should be paid more attention, and a healthy lifestyle is necessary. Reasonable and feasible individual treatment program can help improve efficiency.

There have been increasing numbers of studies highlighting the potential benefits of targeting signaling pathway or bone marrow stem cell therapy in OP, especially OPG/RANKL system. But the treatment strategy tailored to clinical use has not been implemented yet. At present, it seems that combination therapy with vitamin D and bisphosphonates and calcitriol (25-OHD) or alfacalcidol and right dose of glucocorticoids was the most effective and efficient regimen to improve BMD of these patients. More studies on animals and further translation to clinical practice should be done to explore more novel mechanisms that could open up new avenues for the treatment of these disorders.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Validity of 12-Month Falls Recall in Community-Dwelling Older Women Participating in a Clinical Trial

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Objectives. To compare 12-month falls recall with falls reported prospectively on daily falls calendars in a clinical trial of women aged ≥70 years. *Methods*. 2,096 community-dwelling women at high risk of falls and/or fracture completed a daily falls calendar and standardised interviews when falls were recorded, for 12 months. Data were compared to a 12-month falls recall question that categorised falls status as "no falls," "a few times," "several," and "regular" falls. *Results*. 898 (43%) participants reported a fall on daily falls calendars of whom 692 (77%) recalled fall(s) at 12 months. Participants who did not recall a fall were older (median 79.3 years versus 77.8 years, $P = 0.028$). Smaller proportions of fallers who sustained an injury or accessed health care failed to recall a fall (all $P < 0.04$). Among participants who recalled "no fall," 85% reported zero falls on daily calendars. Few women selected falls categories of "several times" or "regular" (4.1% and 0.4%, resp.) and the sensitivity of these categories was low (30% to 33%). Simply categorising participants into fallers or nonfallers had 77% sensitivity and 94% specificity. *Conclusion*. For studies where intensive ascertainment of falls is not feasible, 12-month falls recall questions with fewer responses may be an acceptable alternative.

1. Introduction

The major clinical outcome of osteoporosis is an increased risk of fragility fractures [1]. Approximately 90% of hip, forearm, and pelvis fractures result from a fall [2], and so falls monitoring is important in clinical practice and research settings. The incidence of falls among community-dwelling older adults varies widely between studies [3] but it is generally reported that between 30% and 60% fall each year [4]. The wide range is attributable to not only differences in the study populations and definitions of a "fall" but also the methods of falls ascertainment. When ascertained by recall, the interval of recall is obviously important; one 12-month study reported a more than threefold variation in fall rates among older men when falls were ascertained by varying intervals of recall. Men asked to recall falls monthly had a fall rate of 21% compared with 16% for those asked 3-monthly and 6% for those asked 12-monthly. For females in this study the rates were 26%, 18%, and 21%, respectively [5]. Fujimoto and colleagues conclude that the difference in falls rate may be due to differences in the method of recollection since the participants were matched for falls risk factors. Furthermore, accuracy of 12-month falls recall decreases from almost 80% in older adults who do not fall to 20% in older adults who have fallen on three or more occasions during that period [6]. Thus, where accurate data on all falls is crucial to the

study outcomes, it is recommended that falls information be collected at weekly or monthly intervals [7]. Nevertheless, for many studies falls information is not the primary outcome and such labour intensive ascertainment is not practical. In such studies participants are often asked to recall the number of falls in the past 12 months, yet the accuracy of this in older people is often questioned. There is little information available from studies performing a head-to-head comparison of prospective daily falls reporting and 12-month falls recall in older adults.

2. Aim

The aim of this study was to compare 12-month falls recall with falls reported prospectively on daily falls calendars in a clinical trial of women aged ≥70 years.

3. Materials and Methods

This analysis is part of the Vital D study—a randomised, placebo-controlled double blind trial investigating whether a large annual dose of cholecalciferol (vitamin D) reduces falls and fractures in older women. As part of this study, fall events were intensively monitored over the entire intervention period of three to five years (2003–2008) [8]. Using a questionnaire, participants were asked during 2007 to select the category that best described their frequency of falls in the last 12 months. This 12-month recall of falls was compared with results from our database. The database represents a record of falls ascertained each month from a daily calendar for all 2,096 participants, as detailed below. Falls recorded on daily calendars were followed up with a standardised questionnaire administered by telephone regarding the characteristics and consequences of the fall. All participants had completed monthly falls calendar for at least two years before completing the 12-month recall of falls ascertainment. The method of falls ascertainment by prospective calendar returns is referred to as "daily falls calendar" and "12-month recall of falls" refers to results from categorical responses from a question regarding recall of falls in the past 12 months.

3.1. Participants. Between June 2003 and June 2005 we recruited 2,317 older women who were at high risk of falls and fragility fracture. To be eligible, women needed to be at least 70 years of age and not residing in a supported residential aged care facility. All participants scored at least 5 points on a tool based on risk factors for hip fracture identified by Cummings and colleagues [9] including a history of any fracture since the age of 50 years, maternal history of hip fracture, current body weight less than 50 kg, falling in the past year, poor vision (1 point each), and age 80 years or more (2 points).

3.2. Fall Definition and Recording. A fall was defined as "an event reported either by the faller or by a witness, resulting in a person inadvertently coming to rest on the ground or another lower level, with or without loss of consciousness or injury" [10]. This definition was included on our study newsletter sent to all participants twice a year.

3.3. Falls Ascertainment

3.3.1. Daily Falls Calendar. On enrolment into the study participants were given a 15-month falls calendar comprising a set of monthly postcards. The calendars were renewed postannually with a three-month overlap to allow for delays in the mail or other contingencies when the calendar was due for renewal. Participants completed falls calendars daily by writing "*F*" if they had a fall, fracture, or both or "*N*" if not. Calendars were backed with magnetic strips to enable attachment to refrigerators, where they would be seen frequently. Each postcard included the participants' unique study number, the address of the study centre, and prepaid postage for monthly returns. Participants who had not returned their postcard within two weeks of the end of the month were telephoned and asked about falls in the previous month. When a fall or fracture was recorded, a standardised questionnaire was administered by telephone, and fractures were radiographically confirmed.

3.3.2. 12-Month Falls Recall. During 2007 all participants were sent a study questionnaire of eight questions relating to falls, past history of fracture, and sun exposure habits during summertime. The falls recall question was set out as follows.

> *Have you had any falls in the last twelve months?*
>
> *Never* []*(0)*
>
> *A few times* []*(1)*
>
> *Several times* []*(2)*
>
> *Regularly* []*(3)*

Participants who did not return the questionnaire were interviewed over the phone.

3.4. Statistics. To compare the two methods of ascertainment, the continuous falls data from daily falls calendars were categorised into four responses (never; a few times; several times; and regularly) from the recall question using the best agreement between the sum of self-reported daily falls and the 12-month falls recall response. *Sensitivity* was defined as the number of women whose 12-month falls recall response matched the category they were allocated to according to daily falls calendar (a few times; several times; and regularly), divided by the total number of women in that falls category. *Specificity* was defined as the number of women whose 12-month falls recall response was "never" and whose daily falls calendar total was zero, divided by the total number of women who did not report a fall using the daily falls calendar. The *negative predictive value* was defined as the proportion of participants who were "nonfallers" according to both the daily falls calendar and the 12-month recall (true nonfallers), divided by all participants who selected "no fall" on the daily fall calendar regardless of their response on the 12-month falls recall questionnaire (true and false nonfallers). The likelihood ratio was calculated to estimate the probability/likelihood that a "several or regular falls" response from the 12-month

Table 1: Proportion of participants by number of falls.

Daily falls calendar		12-month falls recall question	
Number of falls reported	Proportion of participants (N)	Category selected	Proportion of participants (N)
None	57.2% (1198)	Never	63.4% (1334)
1	25.7% (538)	A few times	31.8% (667)
2	9.8% (205)	Several times	4.1% (86)
3	3.8% (80)	Regularly	0.4% (9)
4	2.1% (43)		
5	0.6% (13)		
6	0.4% (8)		
>6	0.5% (11)		
Total	100% (2096)		100% (2096)

falls recall questionnaire was the same as the categorisation from daily falls calendar (sensitivity/(1 − specificity)). McNemar's test compared the proportion of fallers (determined by daily falls calendars) who did not report a fall in the 12-month falls recall response, according to radiographically confirmed fracture, and self-report of visiting a doctor, hospitalisation, or injury, ascertained from daily falls calendar follow-up interviews.

All statistical analyses were performed using Minitab (version 13) except for McNemar's tests which were performed in SPSS (version 22).

4. Results

The analysis includes 2,096 participants with complete daily falls calendar data and 12-month falls recall data for the same 12-month period. During the twelve months, 43% (n = 898/2096) of participants reported a fall according to the daily falls calendar. Of these, 77% (n = 692/898) recalled having at least one fall according to the 12-month falls recall question (sensitivity 77%). Of the fallers, 40% (n = 360/898) had more than one fall (Table 1). Fallers were slightly older than the nonfallers (median (IQR) fallers versus nonfallers: 78.9 years (75.5 to 82.8 years) versus 77.7 years (75.0 to 81.1 years), $P < 0.001$) and were over three times more likely to have had a fall in the year prior to this 12-month recall interval (odds ratio (95% CI) age-adjusted: 3.21 (2.68; 3.85), $P < 0.001$).

From the daily falls calendar, 26% of participants had one fall, 10% had two falls, and only 7% had more than two falls. The 12-month falls recall data does not allow us to distinguish one and two falls but 32% of all participants recalled falling "a few times" and 4.5% recalled falling "several times" or "regularly" (Table 1). The group of 206 participants who reported a fall in daily falls calendars but did not recall falling in the 12-month falls recall (23%, n = 206/898) was older than others who recalled the same response on both falls ascertainment methods (median 79.3 years versus 77.8 years, $P = 0.028$). Only 6% of participants who did not record a fall on daily falls calendars incorrectly recalled a fall in the past 12 months (94% specificity; n = 1128/1198) and 85% of women who selected "no fall" on the 12-month falls recall were correct (negative predictive value 0.85; n = 1128/1334) (Table 2). Of the 70 women who did not report a fall on daily falls calendars but incorrectly selected that they had fallen on the 12-month falls recall (6%; n = 70/1198), only 36% (n = 25/70) had a fall in the three months prior to the 12-month recall period. Of the 206 participants who did not recall a fall on the 12-month question, 70% reported falls on daily calendars that occurred in the first six-month period of ascertainment. When participants were classified into just two categories of "fallers" or "nonfallers" there was good agreement between the daily falls calendar and 12-month falls recall data (Kappa 0.73, 95% CI: 0.68, 0.79).

Table 2: Table of frequencies: fallers and nonfallers.

12-month falls recall	Daily calendar		
	Falls, N (%)	No falls, N (%)	
Falls	692 (77%)	70 (6%)	762
No falls	206 (23%)	1128 (94%)	1334
	898	1198	2096

The best agreement between the daily falls calendar and the 12-month falls recall questionnaire was achieved by defining "a few times" as one to four falls and "several" as five to seven falls per year (Table 3). The sensitivity of the higher fall categories was low regardless of the number of falls used to define the categories (30% to 36%; Tables 1 and 2). Using our results to calculate sample size [11] we estimate studies need to have at least 2,000 person-years to have the power to detect a difference between the two higher fall categories of "several" and "regular" fallers (80% power and 0.05 significance level). This is based on recruitment of a similar "at risk" cohort. By comparison a total sample size of 400 to 430 person-years would be needed to detect a difference between "fallers" and "nonfallers." This sample size should also have sufficient power to detect a difference between fallers of "a few times" versus "several/regular" fallers since the difference in the proportion of participants classified as "few times" (41%) versus "several/regular" (1.5%) is larger than the difference between fallers (43%) and nonfallers (57%). The likelihood ratio for the two combined categories of several or regular falls is 7.5 (sensitivity/(1 − specitivity): 0.3/(1 − 0.96)). Thus, a woman who recalls falling several times or regularly is 7.5

Table 3: Cross tabulation of falls by best agreement between monthly ascertainment and 12-month recall category question.

12-month falls recall	Daily falls calendar				Sensitivity of 12-month recall
	No falls	Few (1 to 4 falls)	Several (5 to 7 falls)	Regularly (8+ falls)	
No falls	**1128**	203	3	0	
Few	65	**589**	11	2	68%
Several	5	70[1]	**7**	4	30%
Regularly	0	4	2	**3**	33%
	1198	866	23	9	

[1]Only 14% (n = 10/70) had 4 falls, so reclassifying the criteria of the "few" category to be only 1 to 3 falls did not improve the agreement between the daily falls calendar totals and the 12-month falls recall. Sixty-five percent of these women fell only once or twice (n = 46/70).

times more likely to be correct rather than incorrect in her selected falls frequency category.

Among the 898 women who reported one or more falls on daily falls calendars during the study period, 80 (9%) had a radiographically confirmed fracture. There were a total of 1605 falls, 1566 (98%) of which were able to be investigated further by telephone interviews. 414 (26%) and 70 (5%) falls incidents resulted in a doctor visit or hospitalisation, respectively. A total of 705 (79%) participants reported sustaining an injury, 321 (36%) reported visiting a doctor, and 70 (8%) reported being hospitalised, on at least one occasion due to a fall. As reported in Table 4, amongst participants who reported a fall on daily falls calendars, significantly smaller proportions of women who had a radiographically confirmed fracture, or who reported being hospitalised, seeing a doctor, or sustaining any injury, recorded no falls in response to the 12-month falls recall question.

5. Discussion

In our cohort of over 2,000 older women selected on the basis of being at higher risk of falls or fractures, over half (57%) did not prospectively report a fall over a 12-month interval, approximately one-quarter (26%) reported one fall, and fewer than one in five (17%) reported falling twice or more. Fallers were 3.2 times more likely to have fallen in the previous year (prior to the study period) than nonfallers and were slightly older than nonfallers. This proportion of fallers (43%) is consistent with other estimates of 40% for older women [12] and estimates of 10% having two or more falls [13], since the incidence of falls increases with age and our cohort was older and was specifically recruited to be at higher risk of falls and fracture.

The head-to-head comparison of ascertainment methods shows, in response to a 12-month falls recall questionnaire, that 82% (n = 1727/2096) of participants matched prospectively reported daily falls totals. However nonfallers and those that fell only a few times were more likely to match these responses (94% identical for nonfallers and 68% identical for "a few times" fallers). Few participants reported falling more than four times in one year in daily falls calendars (1.5%; n = 32/2096) and, of those, half did not recall having several or regular falls in response to the 12-month falls recall question (n = 16/32), which is consistent with previous Australian data indicating poor 12-month falls recall in older adults who fall three or more times in a year [6]. Although there were 70 (6%) women who reported no falls on daily calendars but reported falling on the 12-month falls recall, the general classification of fallers or nonfallers has a high sensitivity and specificity (77% and 94%, resp.). As participants in the Vital D study had been completing the monthly falls calendar for several years, we were able to ascertain that only 36% of the 70 women who reported a fall on the 12-month recall but not daily falls calendars had sustained a fall in the 3 months prior to the specific 12-month recall interval.

Twenty-three percent of participants who reported a fall on daily falls calendars in our cohort did not recall falling in the past year (n = 206/898). This is consistent with proportions reported by Cummings and colleagues (13% to 32%) but is almost double the 13% reported for their 12-month recall group [14]. There are substantial differences between the two cohorts. Participants in the study by Cummings were 304 men and women aged 60 years and older, whereas our cohort of women only was almost seven times larger and aged at least 70 years (37% of Cummings cohort aged <70 years). Furthermore, our participants who failed to recall fall(s) were slightly older than those who correctly recalled a fall (79.3 versus 78.1 years, P = 0.028).

It has been suggested that participants who fail to recall falling may have difficulty placing the fall in time and that asking whether a fall had occurred since some other dated event that the person remembers may improve the accuracy of recall [14]. This may have contributed to the substantial disparity in the proportion of participants that forgot a fall (current study versus Cumming: 23% versus 13%, resp.) since our recall period did not coincide with the commencement of the study (an "event" often remembered by participants). Nevertheless only 18% (n = 34/206) of those who reported a fall on daily falls calendars but not 12-month falls recall questionnaire reported a fall in the three months prior to the study period although 58% (n = 119/206) reported a fall in the year prior to the recall interval. Similarly only 36% of nonfallers (according to daily falls calendars) who reported falling on the 12-month falls recall questionnaire had sustained a fall in the three months prior to the recall interval. Our sensitivity (77%) was similar to the 6-month recall (74%) but lower than the 12-month recall (87%) reported by Cummings et al. [14], and this may be related to the older age of

Table 4: 12-month falls recall according to injury or health care utilisation.

12-month falls recall	Confirmed fracture		Fall interview					
			Saw a doctor		Hospitalised		Any injury	
	Yes	No	Yes	No	Yes	No	Yes	No
No falls, *N* (%)	8 (10)	198 (24)	45 (14)	161 (28)	9 (13)	197 (24)	133 (19)	73 (38)
P value*	**<0.001**		**<0.001**		**<0.001**		**<0.001**	

*McNemar's test.

our cohort which may be associated with poorer recall. Specificity of both studies was similar (current study and Cummings et al.: 94% and 93%, resp.).

We also observed that fallers (according to daily falls calendars) were around twice as likely to not report a fall in response to the 12-month falls recall question, if they did not sustain a radiographically confirmed fracture or report any injury or health care utilisation at the time of the fall. This is consistent with a previous study of 12-month falls recall in Australian older adults that reported 87% and 62% recall accuracy for injurious and noninjurious falls, respectively [6]. Thus, it is likely that falls rates are underestimated when falls do not result in injury or health care utilisation.

In this study one-quarter of all falls incidents resulted in a GP visit and, for 5% of falls incidents, hospitalisation. This is consistent with previous estimates indicating that approximately 20% of all fall incidents require medical attention [15]. Approximately 15% of all participants reported accessing a GP because of a fall incident in this study but this is significantly higher than recent estimates from the Belgian older adult population indicating that approximately 2.5% of noninstitutionalised general practice patients received GP care for fall-related injuries [16]. The difference is likely attributable to our recruitment of older women identified as having increased risk of falls or fractures who would therefore be more likely to access health care for fall-related injuries than the general older adult population. Conversely, only 8% of our participants reported being hospitalised due to a fall, compared to 31% in the previous study. This may be explained by the similar differences in falls-related fractures; 9% of participants in our study, compared to 32% of participants in the Belgian study [16], sustained a fracture. The higher fracture and hospitalisation rates in the previous study are probably reflective of the fact that the study populations were older adults accessing GP care, indicating that the fall-related injuries captured were in the most severe range (e.g., fracture) and more likely to result in hospitalisation.

A limitation of this study is that half the participants were randomised to high-dose vitamin D supplementation, which was observed to increase risk of falls and fracture in this population [8]. In a post hoc analysis, we observed that, amongst women who were fallers according to daily falls calendars, a smaller proportion of those receiving vitamin D supplementation classified themselves as nonfaller in response to the 12-month falls recall question, compared to those receiving placebo (20 versus 26%; $P = 0.048$). The improved 12-month recall of the vitamin D group may be explained by the higher rate of falls in this group or could be related to an effect of vitamin D, with a recent systematic review indicating that higher vitamin D status is associated with improved cognitive function in older adults [17].

The head-to-head comparison of the falls data also poses an unavoidable limitation of the study since the participants recalling their falls over the past 12 months had also been posting a daily record of their falls each month over the same period. The 23% of participants who reported falls using prospective daily falls calendars but did not report a fall in response to the 12-month falls recall question are likely to be an underestimation of prevalence of forgotten falls, as 12-month recall of falls may be improved by the daily calendar ascertainment in this study. Furthermore, recall of the falls event may have been reinforced by the follow-up telephone interviews. Even with the most rigorous reporting methodology, it is quite likely that falls are underreported [12]. We and others [13] have noted that denial can be a factor in underreporting as some older people take pride in being a "nonfaller" and "do not want to blot their copybook" by reporting a fall. Older people can blame external factors for their fall and not count it as a "true" fall. Overreporting using the daily falls calendar is unlikely since recorded falls were confirmed by telephone and the circumstances of the event were recorded on our database.

Many cohort studies relying on recall of falls over a 12-month period are unlikely to be adequately powered to show differences between groups in the higher falls categories since few participants accurately self-select higher fall frequencies (sensitivities ~30%). Sample size calculations suggest that studies with 2,000 person-years may be powered to detect a difference between fallers of "several times" versus "regular fallers." Studies with 400 person-years may be sufficiently powered to detect a difference between fallers and nonfallers and also between fallers of "a few times" and more frequent fallers. Although we were unable to calculate the reliability of older women selecting between "one fall" and "more than one fall" for the 12-month falls recall question, a choice of three categories is likely to provide a better "spread" of the data since our cohort of over 2,000 older women had 57% nonfallers, 26% with one fall, and 17% with two or more falls. These three categories might offer more insight into risk factor associations than the simple classification of fallers/nonfallers. Nevertheless when the four categories of falls recall were combined, the classification of fallers/nonfallers captured 77% of fallers and correctly identified 94% of nonfallers and may provide a reasonable alternative for smaller studies where falls are not the primary outcome and an intense ascertainment of fallers is not feasible.

6. Conclusions

With the "ageing" of most western populations, the consequences of injurious falls and their impact on both quality of life and the economic burden to the health system continue to grow. We hope, by reporting this head-to-head comparison of prospective daily falls calendars and 12-month falls recall, that researchers can make informed choices in designing studies that incorporate some falls risk data in which a more intensive ascertainment of fall events is not feasible.

Conflict of Interests

None of the authors have any conflict of interests.

Authors' Contribution

Kerrie M. Sanders and Geoff C. Nicholson are chief investigators of the "Vital D" study (ISRCTN83409867). Amanda L. Stuart was the study coordinator and performed some of the statistical analyses. All three contributed to writing the paper. David Scott performed data analyses and revised the paper. Mark A. Kotowicz revised the paper.

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Research Article

Effects of Alendronate Sodium Content on the Interface Strengths of Composite Acrylic Bone Cement

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Objective. Aim to study how the content of alendronate affected shear strengths at bone-bone cement-metal interfaces. *Methods*. All samples were divided into 6 groups, G_0–G_5. On the 1st and 60th day after surgery, bone-bone cement interface shear strengths and bone densities were examined. Interface strengths of metal-bone cement specimens were studied before immersion and 4 weeks after immersion. *Results*. On the 60th day, bone-bone cement interface shear strengths and bone densities showed significant differences ($P < 0.05$), and compared with G_0, G_2–G_5 values increased significantly ($P < 0.05$), and the peak value was met in G_3. Compared with the 1st day, on the 60th postoperative day both factors decreased significantly in G_0 and G_1 ($P < 0.05$). Four weeks after immersion, with the increasing dose of alendronate, the shear strengths decreased gradually and in G_5 decreased significantly ($P < 0.05$). Compared with before immersion, the metal-bone cement interface strengths decreased significantly 4 weeks after immersion ($P < 0.05$). *Conclusions*. 50–500 mg alendronate in 50 g cement powders could prevent the decrease of shear strengths at bone-bone cement interfaces and had no effect on metal-bone cement interface strengths. While the addition dose was 100 mg, bone cement showed the best strengths.

1. Introduction

With the growing aging population, the number of osteoporotic elderly hip fracture patients has been gradually increasing [1]. Bone cement prosthesis replacement has become a very effective method to treat these fractures [2]. Due to the continual friction between the joint prosthesis, aseptic loosening induced by wear particles has become the main reason of the failure in long-term joint replacement [3]. Therefore, how to prevent bone loss and the aseptic loosening after joint prosthesis replacement has become a research focus.

As a class of synthetic analogs of pyrophosphate, bisphosphonate is a potent new drug to inhibit bone resorption [4]. Experiment researches had shown that the drug could inhibit bone loss after joint prosthesis arthroplasty [5], continuously increasing bone densities around the prostheses [6], inhibiting the release of osteolytic factors [7], inhibiting osteolysis induced by wear particles [8], promoting the proliferation and differentiation of osteoblasts [9], enhancing osteoblast activity, inhibiting apoptosis of osteoblasts [10] and bone absorption of osteoclasts, and accelerating apoptosis of osteoclasts [11]. It is supposed that the drug may be an ideal drug to prevent and cure aseptic loosening after prosthesis replacement.

Oral taking is a major administration of bisphosphonates. If these drugs are taken orally for a long time, they have a lot of side effects, such as low bioavailability, high treatment costs, and upper gastrointestinal ulcers [12, 13]. To avoid these side effects, the topical use added in acrylic bone cement may be a better way of administration. Alendronate is a third generation of bisphosphonate and a regular drug used in the treatment of osteoporosis and osteoporotic fractures. In the form of powder, it has some advantages of being mixed easily in bone cement powders, high temperature resistance and remaining drug efficacy in bone cement, and so forth. Literatures reported that acrylic bone cement compounded with alendronate had a favorable biocompatibility [14], and certain contents of alendronate showed no detrimental effects on the fatigue life of composite acrylic bone cement [15].

Bone-bone cement and metal-bone cement interfaces are common sites of aseptic loosening after bone cement joint

(a) (b) (c)

Figure 1: (a) Stainless steel cylinders locating in the center of the axial positioning ring; (b) The metal rod positioning system: stainless steel cylinders locating in the center of hollow polypropylene tubes; (c) Metal-bone cement specimens.

replacement. However, there is no report about whether these interfaces of composite bone cement being affected or not when alendronate is added. To this end, we used composite acrylic bone cement with different dose of alendronate and made the research mentioned above. The aim was to investigate the change of interface strengths, bone densities, and interface microstructure after alendronate was added.

2. Materials and Methods

2.1. Experimental Animals and Materials. Pure alendronate powder (Merck, USA) and Cemex XL bone cement (Tecres SpA, Verona, Italy) were used as received; stainless steel cylinders (diameter 10 mm × length 37 mm), hollow polypropylene tube (inside diameter 16 mm × outside diameter 20 mm × height 20 mm), the axial positioning ring (Figure 1(a)) (inside diameter 10 mm × outside diameter 20 mm × thickness 10 mm), the metal rod positioning system (Figure 1(b)), and the universal tester (type INSTRON 8032) were obtained from Institute of Biological Materials, Central South University. New Zealand rabbits were supplied by the animal Laboratory, the Second Xiang'ya Hospital. The bone density scanner was supplied by the endocrine laboratory of the Second Xiang'ya Hospital. The study design and experimental procedures were approved by our institution's Animal Care and Use Committee.

2.2. Grouping. According to the amount of alendronate added, all drug samples were divided into 6 groups, G_0–G_5 (i.e., 0, 10, 50, 100, 500, and 1000 mg alendronate were added in 50 g bone cement powder, resp.).

2.3. Preparation of Metal-Bone Cement Interface Strength Specimens. Bone cement was mixed with different dose of alendronate according to the dose regimes above. Then the full reacted mixture was injected into hollow polypropylene tubes, respectively. Stainless steel cylinders with positioning rings were slowly inserted into these tubes. The positioning rings were adjusted to their outside diameter overlapping with the outside diameter of the pipes. After bone cement had solidified, the positioning rings were removed, and bone cement-metal interface specimens were prepared (Figure 1(c)).

2.4. Measurement of Metal-Bone Cement Interface Shear Strengths. Specimens were placed on the INSTRON 8032 universal tester, and ten specimens per group, five specimens before immersion, and five specimens 4 weeks after immersion were tested. The metal cylinders were pushed out at the speed of 5.0 mm/min, and the maximum force launched (F) was measured. The metal-bone cement interface shear strengths (E) were calculated by the following formula and its units were MPa. Consider

$$E = \frac{F}{\pi \cdot d \cdot h}. \quad (1)$$

In the equation: F stand for metal cylinder's maximum force launched, in the unit of Newton (N), d for metal cylinder's diameter (10 mm), and h for the height of metal off the bone cement interface (20 mm).

2.5. Microscopic Observation of Metal-Bone Cement Interfaces. Six specimens examined by push-out test were chosen (one specimen before immersion and one specimen 4 weeks after immersion for G_0, G_3, and G_5). These samples were cut longitudinally into four equal parts by electric saws. One part of samples was coated with gold and these interfaces were observed by electron microscopy.

2.6. Preparation of Bone-Bone Cement Interface Shear Strength Specimens. New Zealand rabbits were operated under intraperitoneal anesthesia (1% sodium pentobarbital, 1.5–2.0 mL/kg). After the success of anesthesia, the surgical area was shaved and cleansed well with 5% benzalkonium bromide and draped the operation area. During the surgery, the rabbits were supplemented with 1% lidocaine as local anaesthetics. An incision about 1.5 cm was made to expose the distal femur by the lateral patellar approach. 3.5 mm drill was used to prepare bone holes and it orientated from the femoral attachment point of the lateral collateral ligament to the femoral medial condyle. When the medial skin of knee was lifted, the incision about 1.0 cm was extended to expose the medial condyle. The wound and bone tunnel were repeatedly washed with hydrogen peroxide and saline, and hemostasis was achieved with fine gauze. Bone cement liquid monomer was mixed with its powders containing different amounts of alendronate. When the reaction was full, the mixture was

filled into a volume of 20 mL injector and injected into the bone tunnels in the bilateral distal femurs. Moderate pressure was applied to both ends of the tunnel until the bone cement solidified. The wound was washed twice and closed layer by layer. Penicillin (800,000 U) was injected by intramuscular injection every day after surgery for 7 days. During the observation period, the rabbits were fed under a standard diet and raised in separate cages.

2.7. Preparation of the Bone-Bone Cement Interface Specimens. Twelve rabbits were assigned to each group. Six rabbits were sacrificed on the 1st day, and the other six rabbits on 60th day after surgery. The lower ends of the femurs, which contained the specimens, were removed. The left specimens were used to test the bone-bone cement interface shear strengths, while the right ones were used to scan bone densities surrounding the interfaces.

2.8. Test of Shear Strength at the Bone-Bone Cement Interfaces. Bilateral femur condyles of the rabbits were trimmed to bone-bone cement interface samples with 9.0 mm lengths with scalpel. Then the specimens were loaded on an INSTRON 8032 universal tester, with a loading speed of 5 mm/min. The tester was halted until the load began to decline gradually. The maximum load was recorded, and the interface shear strengths (E), in the unit of MPa, were calculated by the following equation:

$$E = \frac{F}{\pi \cdot d \cdot l}. \quad (2)$$

In the equation: F stand for maximum load force launched, in the unit of Newton (N), d for bone cement cylinder's diameter (3.5 mm), and l for the length of bone-bone cement interface (9.0 mm).

2.9. Bone Densities Surrounding the Bone-Bone Cement Interfaces. Specimens were trimmed to bone-bone cement interface with 3.0 mm thickness with scalpel. Then cut unnecessary bone and make these samples of a standard size (length 6.0 mm × width 6.0 mm × thickness 3.0 mm) and at the same time make sure the bone cement cylinders locate in the center of the specimens. Specimens were scanned using a bone density scanner and the bone densities were calculated by the tester's software.

2.10. Statistical Analysis. SPSS 13.0 for Windows software was used for the statistical analysis. Each set of data were expressed as mean ± standard deviation (SD) and one-way analysis of variance was performed. If there was a significant difference, pairwise comparison was carried out between groups using Scheffe post hoc test. Paired t-tests were used for the shear strengths and the bone densities at bone-bone cement interfaces between the 1st day and 60th day after surgery, and metal-bone cement interface strengths between before immersion and 4 weeks after immersion. Test level bilateral $\alpha = 0.05$ and $P < 0.05$ was considered statistically significant.

Table 1: Shear strengths of bone-bone cement interfaces (MPa) on the 1st day and 60th day after surgery in each group.

Group	1st day	60th day	$P^{\#}$
G_0	5.5372 ± 0.2516	3.6700 ± 0.1341	<0.05
G_1	5.5868 ± 0.1729	3.7600 ± 0.1707	<0.05
G_2	5.5573 ± 0.2041	5.6625 ± 0.2906*	>0.05
G_3	5.5630 ± 0.2708	5.6967 ± 0.2170*	>0.05
G_4	5.6450 ± 0.2843	5.6100 ± 0.2184*	>0.05
G_5	5.5330 ± 0.1787	5.6300 ± 0.1975*	>0.05
$P^{\#\#}$	0.981	<0.05	

Note: ## indicates one-way analysis of variance (ANOVA); # indicates t-test, $P < 0.05$; * indicates Scheffé's post hoc test, $P < 0.05$ compared with G_0.

Table 2: Bone densities surrounding the bone-bone cement interfaces (g/cm^2) on the 1st day and 60th day after surgery in each group.

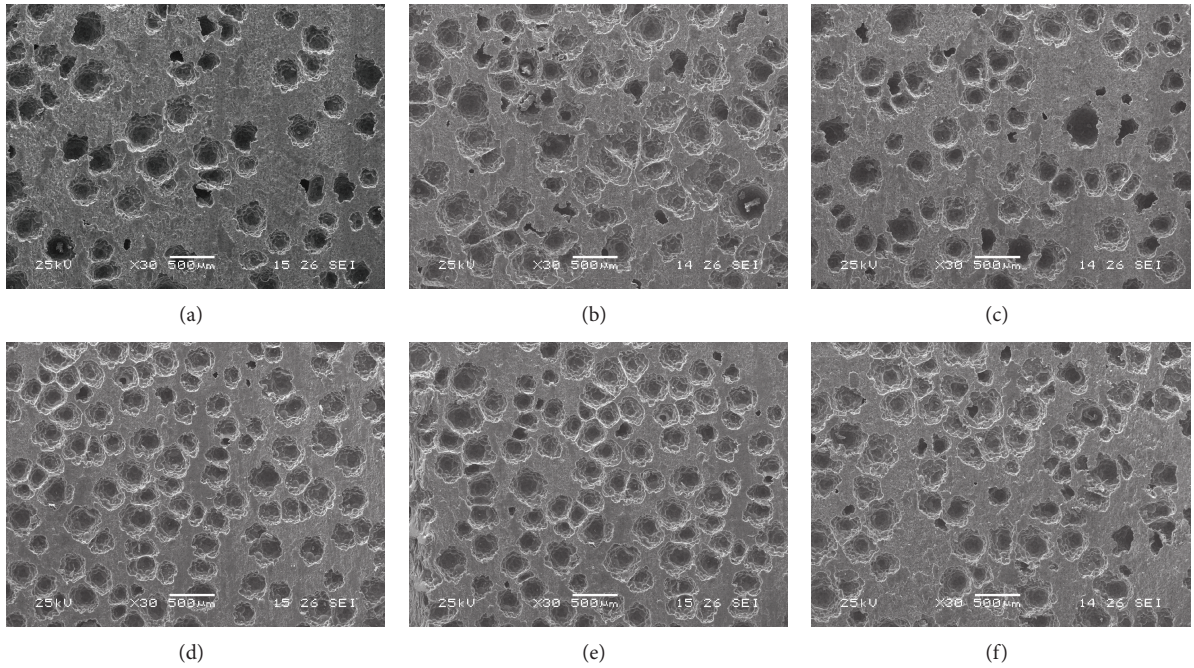
Group	1st day	60th day	$P^{\#}$
G_0	0.2396 ± 0.0527	0.1356 ± 0.0274	<0.05
G_1	0.2455 ± 0.0427	0.1313 ± 0.0095	<0.05
G_2	0.2512 ± 0.0108	0.2509 ± 0.0275*	>0.05
G_3	0.2525 ± 0.0121	0.2584 ± 0.0206*	>0.05
G_4	0.2546 ± 0.0111	0.2554 ± 0.0245*	>0.05
G_5	0.2546 ± 0.0138	0.2512 ± 0.0139*	>0.05
$P^{\#\#}$	0.957	<0.05	

Note: ## indicates one-way analysis of variance (ANOVA); # indicates t-test, $P < 0.05$; * indicates Scheffé's post hoc test, $P < 0.05$ compared with G_0.

3. Results

3.1. Shear Strengths of the Bone-Bone Cement Interfaces. Table 1 showed that on the 1st day after surgery, bone-bone cement interface shear strengths showed no significant differences in all groups ($P > 0.05$). However, on the 60th day after surgery, they showed significant differences ($P < 0.05$), and compared with G_0, bone-bone cement interface shear strengths in G_1 showed no significant differences ($P > 0.05$), but in the other groups their values increased significantly ($P < 0.05$), and the peak value was met in G_3. Compared with the 1st postoperative day, on the 60th postoperative day bone-bone cement interface shear strengths significantly decreased in G_0 and G_1 ($P < 0.05$), not in G_2 to G_5 ($P > 0.05$).

3.2. Bone Densities Surrounding the Bone-Bone Cement Interfaces. Table 2 showed that on the 1st day after surgery, bone densities surrounding bone-bone cement interfaces showed no significant differences in all groups ($P > 0.05$). However, on the 60th day after surgery, they showed significant differences ($P < 0.05$), and compared with G_0, bone-bone cement interface shear strengths in G_1 showed no significant differences ($P > 0.05$), but in the other groups their values increased significantly ($P < 0.05$), and the peak value was met in G_3. Compared with the 1st postoperative day, on the 60th postoperative day bone densities surrounding bone-bone cement interfaces significantly decreased in G_0 and G_1 ($P < 0.05$), not in G_2 to G_5 ($P > 0.05$).



(a) (b) (c)

(d) (e) (f)

Figure 2: Electron microscopy of the metal-bone cement interfaces: (a) and (d) before immersion and 4 weeks after immersion in G_0, respectively; (b) and (e) before immersion and 4 weeks after immersion in G_3, respectively; and (c) and (f) before immersion and 4 weeks after immersion in G_5, respectively.

Table 3: Shear strengths of the metal-bone cement interfaces (MPa) before immersion and 4 weeks after immersion in each group.

Group	Before immersion	4 weeks after immersion	$P^{\#}$
G_0	5.746 ± 0.7701	4.244 ± 0.0709	<0.05
G_1	5.668 ± 0.0864	4.200 ± 0.0632	<0.05
G_2	5.652 ± 0.0834	4.178 ± 0.0581	<0.05
G_3	5.598 ± 0.1188	4.172 ± 0.0286	<0.05
G_4	5.564 ± 0.1250	4.138 ± 0.0835	<0.05
G_5	5.534 ± 0.1043	3.530 ± 0.0418*	<0.05
$P^{\#\#}$	0.053	<0.05	

Note: $^{\#\#}$indicates one-way analysis of variance (ANOVA); $^{\#}$indicates t-test, $P < 0.05$; *indicates Scheffé's post hoc test, $P < 0.05$ compared with G_0.

3.3. Shear Strengths of Metal-Bone Cement Interfaces. Table 3 showed that before immersion, metal-bone cement interface strengths in all groups showed no significant difference ($P > 0.05$), while 4 weeks after immersion, with the increasing dose of alendronate, the shear strengths decreased gradually, and in G_5 the decrease showed significant difference ($P < 0.05$). Compared with that before immersion, the metal-bone cement interface strengths significantly decreased in all groups 4 weeks after immersion ($P < 0.05$).

3.4. Electron Microscopy Observation of the Metal-Bone Cement Interfaces. Figure 2 showed that the porosity of bone cement specimens was similar before immersion, or 4 weeks after immersion in G_0, G_3, or G_5. But compared with before immersion, the porosity in the same group increased obviously 4 weeks after immersion.

4. Discussion

The interfaces between the femoral component and bone cement were known to be a weak area of bone-bone cement prosthesis complex [16]. Previously, Harris and Jasty found that the main mechanism of aseptic loosening on the femoral side was the debonding of the femoral component-bone cement by analyzing the prosthesis removed. Finite element analysis showed that shear stress was a major stress factor for joint prosthesis failure [17]. Interface shear strengths were influenced by a variety of factors, including surface roughness of the femoral stem component, preheating or precoating of the stem component [18], precooling of bone cement monomer, the type of bone cement, the type of prosthesis metal, and the load rate. As a part of this study, we investigated the effects of alendronate on metal-bone cement interface shear strengths. The results showed that before immersion, there is no significant difference in the metal-cement interface strengths in all groups, while 4 weeks after immersion, with the increasing dose of alendronate, the shear strengths decreased gradually, and in group G_5 the decrease showed significant difference. Meanwhile, electron microscopy showed that no significant difference was found with regard to the interface porosity before immersion or 4 weeks after immersion in groups G_0, G_3, and G_5. These results indicated that the decrease of shear strength of metal-bone cement, was more attributed to decrease of the bone cement bonding capacity than the interface porosity. These studies also found that, compared with that before immersion, 4 weeks after immersion the metal-bone cement interface strengths decreased significantly in the same group. Therefore, it was proposed that the main factor decreasing

bone cement bonding capacity might be related to immersion in saline.

Bone-bone cement interfaces were another common site of aseptic loosening after joint prosthesis replacement. According to published reports, aseptic loosening about approximately 50–79% was found 15 years after total hip arthroplasty for young active patients, and 16% of these patients needed revision arthroplasty [19]. Because of the impossible bonding between hydrophobic bone cement and hydrophilic bone tissues, bone cement was used as fillers instead of binders [19]. Instead, interfaces between bone cement and bone tissue become stable fixation by a mechanical intercourse locking. Some studies showed that the shear strengths of bone-bone cement interfaces could be increased by enhancing microlocking between bone cement and bone, and precoating with a layer of an amphiphilic substance on the bone surface. There are many factors that can influence the interface shear strengths, including bone porosity [20], trabecular orientation [20], continuous pressures on the cement [20], preparation of bone surface, and viscosity of bone cement. Moran et al. found that shear strengths of bone-bone cement interfaces were not influenced by gentamicin (0.5 g, 1.0 g, 2.0 g, or 4.0 g) added in 40 g bone cement powders [21]. Moreover, the shear strengths in bone cement were higher than that at bone-bone cement interfaces. Therefore, it is obvious that shear strengths of bone-bone cement interface are a key factor for joint prosthesis service life.

This study found that on the 1st day after surgery shear strengths of bone-bone cement interface in all groups showed no significant difference. However, significant differences were observed on the 60th day after operation. Compared with the 1st day, the interface strengths decreased significantly after 60 days after surgery in G_0 and G_1, but no obvious changes were shown in G_2–G_5. To investigate the reason of shear strengths' changes, we scanned the bone densities surrounding the bone-bone cement interfaces on the 1st and 60th day after surgery. The results showed that there were similar changes between bone densities and shear strengths at bone-bone cement interfaces. According to the phenomenon above, we inferred that the bone densities might be an important factor to decide the shear strengths at bone-bone cement interfaces. The reason was that bone cement had better mechanical strengths than trabecular bone tissue. Meanwhile, in G_0 and G_1, bone densities had significant reduction after 60 days after operation. It might be related to surgical trauma, thermal damage from bone cement, and activities reduction of rabbits. However, in G_2–G_5, bone densities at bone-bone cement interfaces showed no significant change. This might result from mineralization capacity enhancement of osteoblast and function inhibition of osteoclast, which was caused by the topical release of alendronate and offset of the negative effects on bone densities.

The advantages and disadvantages of this study also deserved discussion: (1) In this study, we used distal femurs of New Zealand rabbits to prepare the bone-bone cement interfaces. Compared with diseased femoral heads used by Moran [21], the advantages included convenience of obtaining specimens, and an increase in sample volume, and avoiding negative impact from structure differences in diseased bone. However, using healthy tissue also had some drawbacks. These specimens obtained were smaller and more difficult to prepare bone-bone cement interfaces. When alive specimens subjects were used, the bleeding at the interfaces could affect the study results. (2) As artificial femoral stem substitute, stainless steel cylinder had different morphology and surface friction coefficient, which resulted in different shear strength values. However, we had already homogenized these factors that might affect the interface strengths in our experiments, therefore the study results were reliable.

In conclusion, these results showed that a certain amount of alendronate in bone cement had a remarkable effect on interface strengths of composite acrylic bone cement and interfacial bone densities. A dose of 50–500 mg alendronate in 50 g bone cement powder could prevent the decrease of interface strengths at bone-bone cement interfaces, and it had a similar effect on bone densities around these interfaces. However, the same doses of alendronate showed no effect on the interface strengths of metal-bone cement interfaces before immersion and 4 weeks after immersion. While the addition dose was 100 mg, bone cement showed the best strengths. The results of this study indicated that alendronate-loaded bone cement could be made, but alendronate amount must be controlled to below a certain level which had no effect on the shear strengths at metal-bone cement-bone interfaces.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Low Magnesium Exacerbates Osteoporosis in Chronic Kidney Disease Patients with Diabetes

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The aim of this study is to investigate the impact of serum Mg on bone mineral metabolism in chronic kidney disease (CKD) patients with or without diabetes. A total of 56 CKD patients not receiving dialysis were recruited and divided into two groups, one group of 27 CKD patients with diabetes and another group of 29 CKD patients without diabetes. Biochemical determinations were made, and the estimated glomerular filtration rate (eGFR) was measured. Bone mineral density was measured by dual-energy X-ray absorptiometry. Serum Mg was inversely correlated with serum Ca ($P = 0.023$) and positively correlated with serum parathyroid hormone (PTH) ($P = 0.020$), alkaline phosphatase ($P = 0.044$), and phosphate ($P = 0.040$) in the CKD patients with diabetes. The CKD patients with diabetes had lower serum albumin and a higher proportion of hypomagnesemia and osteoporosis than the nondiabetic patients did ($P < 0.05$). Serum Mg was inversely correlated with eGFR in the CKD patients with or without diabetes ($P < 0.05$). Serum Mg showed an inverse correlation with 25-hydroxyvitamin D in CKD patients without diabetes ($P = 0.006$). Furthermore, the diabetic CKD patients with low serum Mg had a lower iPTH ($P = 0.007$) and a higher serum Ca/Mg ratio ($P < 0.001$) than the other CKD patients. The lower serum Mg subgroup showed a higher incidence of osteoporosis than the moderate and higher serum Mg subgroups did (66.7%, 39.4%, and 29.4%, resp.). In conclusion, low serum Mg may impact iPTH and exacerbates osteoporosis in CKD patients, particularly with diabetes.

1. Introduction

Osteoporosis is a skeletal disorder characterized by a low bone mass and disruption of bone architecture that leads to decreased bone strength and increased fracture risk [1]. Many factors are associated with osteoporosis, including nutritional, hormonal, and clinical factors [1, 2]. Low calcium (Ca) status is associated with a reduced bone mass and osteoporosis [3, 4]. Vitamin D deficiency impairs the absorption of Ca and leads to osteomalacia [5]. Magnesium (Mg) is also a major regulator of bone homeostasis [6]. Low Mg levels may impair the activity of parathyroid hormones (PTH) [2] and reduce serum vitamin D levels [7, 8]. In addition, chronic kidney disease (CKD) will cause abnormality of bone mineral metabolism and therefore result in complications of bone disease [9–11]. Besides, serum Mg levels may be reduced or raised with poor diabetic control or renal functional decline [12, 13], which may exacerbate bone disease [6]. However, the effect of Mg status, dietary Mg, serum Mg, and urine Mg in patients with diabetic nephropathy or CKD on Ca and bone metabolism remains unclear.

Low or high serum Mg levels may result in unwanted neuromuscular, cardiac, nervous, metabolic, or bone disorders [14, 15]. A deficit in Mg increases the risks for several diseases, including diabetes, hypertension, and cardiovascular diseases [16, 17]. Moreover, Mg deficiency is associated with low bone mass and osteoporosis [6]. Indeed, Mg deficiency affects the secretion and sensitivity of PTH. Therefore, low magnesium status reduces the activity of the 25-hydroxycholecalciferol-1-hydroxylase resulting in low serum concentrations of

1,25-dihydroxyvitamin D ($1,25(OH)_2D$) and Ca [2, 7, 8]. In contrast, hypermagnesemia causes vasodilation and neuromuscular blockade [14]. Furthermore, high serum Mg levels may inhibit parathyroid hormone (PTH) secretion and also have adverse biologic effects on bone mineral metabolism [6]. However, the relationship between low or high serum Mg levels with PTH levels, vitamin D, and bone mineral metabolism remains unclear.

Chronic kidney disease causes a progressive decline in renal function over time. In the early stages, there may be no specific symptoms. Moderate-severe renal decline causes abnormality in bone and mineral metabolism, which is one of the common complications in patients with CKD [9–11]. Abnormal levels of PTH, serum vitamin D, serum phosphate (P), and serum Ca contribute to renal bone disease [9–11]. Serum Mg levels may raise with renal functional decline [12], and this eventually may be harmful to bone health [6]. In addition, diabetes is related to an increased risk of hypomagnesemia and osteoporosis [18, 19]. The patients with diabetes presented lower levels of serum Ca, vitamin D, PTH, and serum Mg [20, 21]. Furthermore, lower PTH concentration resulted in low bone formation, which may increase the risk of vertebral fractures in diabetes patients of both sexes [22]. However, there is little information about the effects of Mg on PTH, Ca, vitamin D, and bone metabolism in patients with CKD, particularly in diabetic CKD patients.

Now growing evidence shows that low Mg is associated with diabetes and nephropathy [13, 23]. Low Mg and impaired secretion and function of PTH decrease the levels of $1,25(OH)_2D$, cause low serum Ca, and are linked to bone and mineral metabolism disorders [2, 24]. Thus, low Mg may exacerbate bone disease in patients with CKD. However, it remains unclear whether moderate-severe CKD patients with diabetes still have a higher prevalence of hypomagnesemia than those of nondiabetic CKD patients because serum Mg levels may rise with renal function decline. Moreover, differences in the correlations between serum Mg with Ca, PTH, and bone mineral metabolism between CKD patients with and without diabetes have not been fully explored.

In the present study, serum Mg levels, bone mineral metabolism parameters, bone mineral density, and renal function indicators were measured. The objective of this study was to evaluate the impacts of serum Mg levels on PTH and bone mineral metabolic parameters among CKD patients with or without diabetes.

2. Materials and Methods

2.1. Study Design and Subjects. This study involved 56 stage 3–5 CKD patients not receiving dialysis who were divided into two groups as 27 CKD with diabetes and 29 CKD without diabetes. Patients were residents in a rural area and were diagnosed with chronic kidney disease at the hospital clinic of Central Taiwan. All patients were without a history of symptomatic ischaemic heart disease, heart failure, liver disease, current malignancy, and hypoparathyroidism. The study protocol was approved by the Changhua Christian Hospital Institutional Review Board (CCHIRB 090605), and informed consent was obtained from each participant.

2.2. Biochemical Determination. Blood samples were collected after an overnight fasting for the determinations of serum Mg, Ca, P, intact PTH (iPTH), alkaline phosphatase (ALP), 25-hydroxyvitamin D (25(OH)D), and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) levels. Serum Mg levels between 1.82 and 2.31 mg/dL were defined as the normal range [25]. For patients with stages 3, 4, and 5 CKD, PTH should be maintained in the range of 35–70 pg/mL, 70–110 pg/mL, and 150–300 pg/mL, respectively [26]. For patients with stages 3 to 4 CKD, serum Ca should be maintained within normal range, 8.9–10.1 mg/dL, and serum P should be within 2.7–4.6 mg/dL [26]. For patients with stage 5 CKD, serum Ca should be 8.4–9.5 mg/dL, and serum P target should be 3.3–5.5 mg/dL [26]. For patients with stages 3 to 5 CKD, Ca-P product should be $<55\ mg^2/dL^2$ [26]. The reference range of ALP is 50–136 (U/L) for laboratory used. Furthermore, vitamin D deficiency is defined as a serum 25(OH)D level of less than 20 ng/mL and vitamin D insufficiency is defined as a serum 25(OH)D level of 20 to 30 ng/mL [27]. Serum $1,25(OH)_2D$ deficiency is defined as a serum $1,25(OH)_2D$ level of less than 25.1 ng/mL for laboratory used.

In addition, based on the formula recommended by the Taiwan Society of Nephrology, estimated glomerular filtration rate (eGFR) was calculated as eGFR (mL/min/1.73 m2) (Simplified Modification of Diet in Renal Disease (MDRD)) = $186 \times \text{serum creatinine}^{-1.154} \times \text{Age}^{-0.203}$ in men, and $186 \times \text{serum creatinine}^{-1.154} \times \text{Age}^{-0.203} \times 0.742$ in women. The definition of chronic kidney disease (CKD) stages was based upon guidelines for the management of CKD [28]. Blood urea nitrogen (BUN) was also measured.

2.3. Bone Mineral Density. Bone mineral density at the left femoral neck, right femoral neck, and lumbar spine (L1–L4) was measured by dual-energy X-ray absorptiometry. Results were expressed as g/cm^2 or as a T-score, which represents the number of standard deviations (SD) of the difference between a patient's BMD and that of a gender-matched young adult reference population. By definition from the World Health Organization, we have the following: (1) normal: T-Score at or above −1.0 SD; (2) osteopenia: T-Score between −1.0 and −2.5 SD; and (3) osteoporosis: T-Score at or below −2.5 SD [29].

2.4. Statistical Analysis. The Kolmogorov-Smirnov test was used to assess the normality of the distribution of investigated parameters. Continuous data were expressed (mean ± SD) and differences were tested by a 2-tailed t-test. Categorical data were analyzed by the Chi-square test. Continuous data of skewed distribution were presented in median, and range (25th pctl–75th pctl) and differences were examined by the Wilcoxon rank-sum test or the Kruskal-Wallis test. Multivariate analysis of the general linear model was used to analyze the association between variables. The serum Mg, bone metabolism parameters, serum Ca/Mg ratio, serum Ca × P values, and renal function indicators were log transformed before analysis because of the data's skewed distribution. Multiple regression was used to analyze all variables and presented in unstandardized coefficients (B),

Table 1: Characteristics of the 56 nondialysis CKD patients with or without diabetes.

Variables	Total (n = 56)	Diabetes (n = 27)	Nondiabetes (n = 29)	P
Age (y)	69.0 (58.8–75.8)	68.0 (62.0–77.0)	70 (57.0–74.5)	0.928
Gender				
Female	24 (42.9)	15 (55.6)	9 (31.0)	0.064
Male	32 (57.1)	12 (44.4)	20 (69.0)	
Estimated GFR (mL/min)	16.5 (10.5–28.8)	13.7 (9.4–29.8)	18.4 (12.0–24.8)	0.302
Chronic kidney disease				
Stage 3	13 (23.2)	7 (25.9)	6 (20.7)	0.102
Stage 4	18 (55.4)	5 (18.5)	13 (44.8)	
Stage 5	25 (44.6)	15 (55.6)	10 (34.5)	
Albumin (g/dL)	3.8 ± 0.4	3.7 ± 0.4	3.9 ± 0.4	0.046
Serum Mg (mmoL/L)				
<1.82 mg/dL low	6 (10.7)	6 (22.2)	—	0.023
1.82–2.31 mg/dL normal	33 (58.9)	13 (48.1)	20 (69.0)	
>2.31 mg/dL high	17 (30.4)	8 (29.6)	9 (31.0)	
Osteoporosis				
With	22 (39.3)	15 (55.6)	7 (24.1)	0.016
Without	34 (60.7)	12 (44.4)	22 (75.9)	

(1) eGFR, estimated glomerular filtration rate.
(2) Comparisons of continuous data between two groups were analyzed by Wilcoxon rank-sumtest. Data are median and range (25th pctl–75th pctl). The t-test was used for the difference in the means of two groups. Data are means ± SD.
(3) Categorical data were analyzed by the Chi-square test. When cells have expected count less than 5, data were analyzed by Fisher's Exact test. Data are number (n), percent (%).
(4) A P value less than 0.05 was considered statistically significant.

in standardized coefficients (β), and at a 95% confidence interval (CI) for B. The value $P < 0.05$ was considered statistically significant. Statistical analysis was done using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. The Characteristics of the Subjects. The characteristics of the 56 nondialysis CKD patients with or without diabetes are shown in Table 1. The CKD patients with diabetes had significantly lower serum albumin ($P = 0.046$) and lower serum magnesium ($P = 0.023$) and osteoporosis ($P = 0.016$) when compared to those CKD patients without diabetes. In addition, age, gender, eGFR, and CKD stages were not significantly different between CKD patients with diabetes and without diabetes.

3.2. Relationships of Serum Mg with Renal Function and Bone Metabolism Parameters in CKD Patients with or without Diabetes. As shown in Table 2, after adjusting for confounding factors, serum Mg was inversely correlated with serum Ca ($P = 0.015$) and positively correlated with serum iPTH ($P = 0.041$) and ALP ($P = 0.027$) in the CKD patients with diabetes. Moreover, serum Mg was inversely correlated with eGFR ($P = 0.014$) and positively correlated with creatinine ($P = 0.007$) and BUN ($P = 0.044$) in the CKD patients with diabetes. However, serum Mg was not significantly associated with serum P, 25(OH)D, and $1,25(OH)_2D$ in the CKD patients with diabetes. For CKD patients without diabetes, serum Mg showed an inverse correlation with 25(OH)D ($P = 0.006$). Moreover, serum Mg showed a positive correlation with serum creatinine ($P = 0.040$) and an inverse correlation with eGFR ($P = 0.034$) in the CKD patients without diabetes. There was a marginal inverse correlation between serum Mg and serum Ca in the CKD patients without diabetes ($P = 0.065$). However, serum Mg had no significant correlation with serum urea nitrogen, P, iPTH, ALP, and $1,25(OH)_2D$ in the CKD patients without diabetes. On the other hand, iPTH was inversely correlated with serum Ca ($P = 0.003$) and 25(OH)D ($P = 0.023$) and positively correlated with serum ALP ($P = 0.005$) and in the CKD patients with diabetes. However, iPTH was not significantly correlated with bone metabolism parameters in CKD patients without diabetes. In addition, iPTH also was not significantly correlated with renal function indicators in the CKD patients with or without diabetes.

3.3. Correlation of Different Serum Mg Levels with Renal Function and Bone Metabolism Parameters. The correlation of different serum Mg levels and bone metabolism parameters is shown in Table 3 and Figure 1. Although serum Mg levels were not statistically significantly correlated with osteoporosis, the low serum Mg subgroup presented a higher proportion of osteoporosis than that of moderate and high serum Mg subgroups (66.7%, 39.4%, and 29.4%, resp.). Furthermore, the low serum Mg subgroup had a lower serum iPTH when compared with the moderate or high serum Mg subgroup. In contrast, the high serum Mg subgroup had a higher iPTH ($P = 0.007$), lower serum Ca ($P = 0.018$), elevated serum P ($P = 0.026$), and lower serum Ca/Mg

Table 2: Relationships between serum Mg with renal function and bone metabolism parameters in CKD patients with or without diabetes.

Dependent variables	log serum Mg (mg/dL)				log iPTH (pg/mL)			
	B	*β*	*P*	95% CI for *B*	*B*	*β*	*P*	95% CI for *B*
With diabetes								
Renal function indicators[†]								
log eGFR (mL/min)	−1.616	−0.473	0.014	(−2.869, −0.363)	−0.191	−0.282	0.220	(−0.504, 0.122)
log creatinine (mg/dL)	1.539	0.528	0.007	(0.461, 2.616)	0.175	0.175	0.202	(−0.101, 0.451)
log BUN (mg/dL)	0.952	0.391	0.044	(0.026, 1.878)	0.027	0.056	0.810	(−0.202, 0.255)
Bone metabolism parameters[‡]								
log iPTH (pg/mL)	2.296	0.454	0.041	(0.109, 4.483)				
log Ca (mg/dL)	−0.134	−0.546	0.015	(−0.239, −0.028)	−0.030	−0.626	0.003	(−0.049, −0.012)
log P (mg/dL)	0.272	0.312	0.106	(−0.063, 0.608)	0.040	0.231	0.222	(−0.026, 0.106)
log ALP (U/L)	0.719	0.449	0.027	(0.093, 1.345)	0.168	0.529	0.005	(0.056, 0.279)
log 25(OH)D (ng/mL)	−0.369	−0.221	0.420	(−1.305, 0.567)	−0.188	−0.570	0.023	(−0.348, −0.029)
log $1,25(OH)_2D$ (pg/mL)	−0.286	−0.081	0.745	(−2.094, 1.523)	−0.077	−0.110	0.649	(−0.422, 0.269)
Without diabetes								
Renal function indicators[†]								
log eGFR (mL/min)	−1.091	−0.381	0.034	(−2.092, −0.090)	0.040	0.084	0.665	(−0.150, 0.231)
log creatinine (mg/dL)	0.922	0.404	0.040	(0.046, 1.798)	−0.045	−0.119	0.577	(−0.210, 0.120)
log BUN (mg/dL)	0.426	0.172	0.368	(−0.531, 1.382)	−0.020	−0.047	0.814	(0.814, 0.149)
Bone metabolism parameters[‡]								
log iPTH (pg/mL)	1.057	0.176	0.413	(−1.566, 3.679)				
log Ca (mg/dL)	−0.153	−0.407	0.068	(−0.318, 0.012)	0.007	0.118	0.597	(−0.021, 0.026)
log P (mg/dL)	0.148	−0.135	0.508	(−0.605, 0.308)	−0.065	−0.355	0.065	(−0.134, −0.004)
log ALP (U/L)	0.221	0.155	0.469	(−0.400, 0.841)	0.085	0.357	0.078	(−0.010, 0.179)
log 25(OH)D (ng/mL)	−1.250	−0.580	0.002	(−1.993, −0.507)	−0.079	−0.219	0.273	(−0.223, 0.066)
log $1,25(OH)_2D$ (pg/mL)	0.895	0.255	0.245	(−0.656, 2.446)	0.107	0.183	0.392	(−0.147, 0.362)

(1) eGFR, estimated glomerular filtration rate; BUN, blood urine nitrogen; iPTH, intact parathyroid hormone; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D.
(2) All outcomes of the multiple regression analysis are presented in unstandardized coefficients (*B*) and standardized coefficients (*β*) and at a 95% confidence interval (CI) for *B*. A *P* value less than 0.05 was considered statistically significant. [†]Adjusted gender and age. [‡]Adjusted gender, age, albumin, and eGFR.

Table 3: Correlation of different serum Mg levels with osteoporosis and bone metabolism parameters.

Variables	Serum Mg (mg/dL)			*P*
	<1.82 (*n* = 6)	1.82–2.31 (*n* = 33)	>2.31 (*n* = 17)	
Osteoporosis				
With	4 (66.7)	13 (39.4)	5 (29.4)	0.314
Without	2 (33.3)	20 (60.6)	12 (70.6)	
Bone metabolism parameters				
iPTH (pg/mL)	32.2 (20.0–62.4)	86.4 (45.9–133.0)	126.0 (80.8–221.5)	0.007
Serum Ca (mg/dL)	9.0 (8.7–9.5)	8.9 (8.6–9.3)	8.7 (8.2–8.9)	0.018
Serum P (mg/dL)	3.5 (3.1–4.1)	3.9 (3.4–4.1)	4.4 (3.7–5.0)	0.026
ALK-P (U/L)	88.5 (74.3–102.4)	79.0 (66.5–89.8)	87.0 (79.0–116.5)	0.061
25(OH)D (ng/mL)	27.2 (17.7–37.3)	22.7 (18.5–28.0)	23.6 (14.6–28.0)	0.631
$1,25(OH)_2D$ (pg/mL)	24.0 (15.5–29.4)	16.6 (11.9–31.2)	15.4 (11.8–23.9)	0.516
Serum Ca/Mg ratio	5.1 (5.0–5.6)	4.1 (3.8–4.4)	3.4 (2.8–3.6)	<0.001
Serum Ca × P value	31.2 (27.8–38.1)	33.8 (29.5–38.3)	37.4 (34.6–41.9)	0.128

(1) iPTH, intact parathyroid hormone; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D.
(2) Comparisons of continuous data between three groups were analyzed by Kruskal Wallis test. Data are median and range (25th pctl–75th pctl).
(3) Comparisons of categorical data between two groups were analyzed by Chi-square test. When cells have expected count less than 5, data were analyzed by Fisher's Exact test. Data are number (*n*), percent (%).
(4) A *P* value less than 0.05 was considered statistically significant.

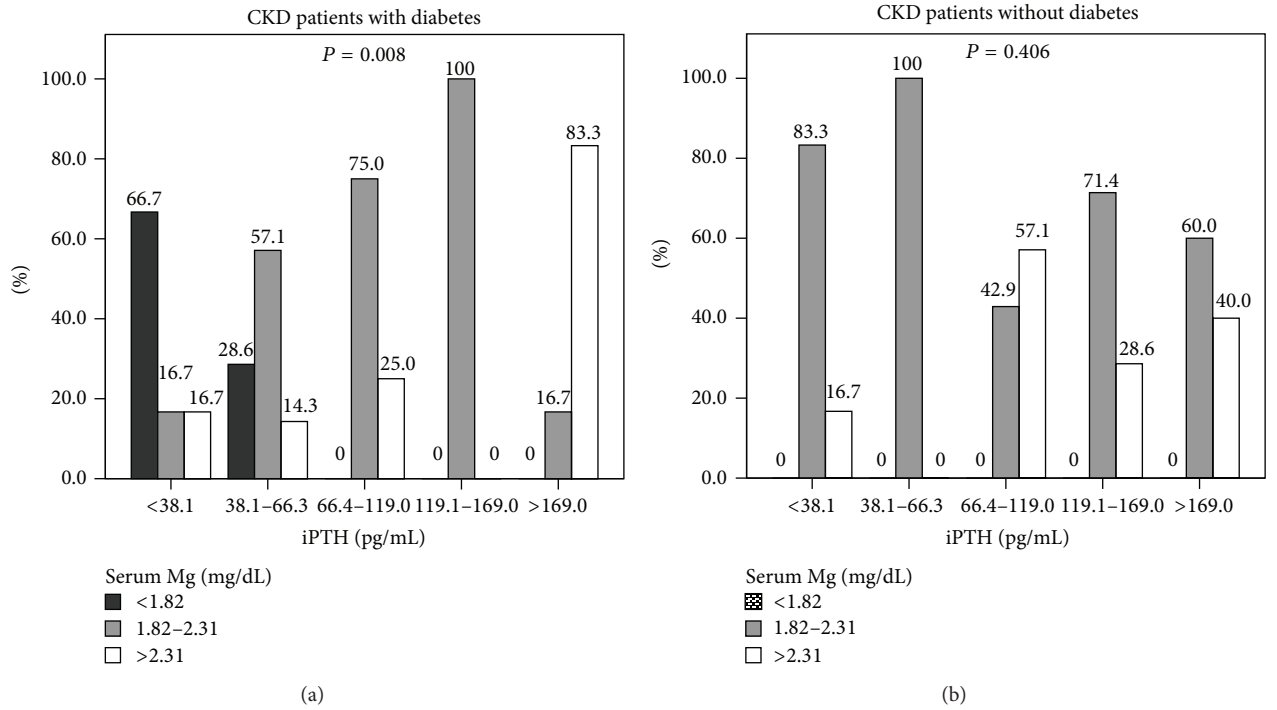

Figure 1: Correlation between serum Mg levels and iPTH levels. (a) CKD patients with diabetes. (b) CKD patients without diabetes. Comparisons of categorical data between two groups were analyzed by Chi-square test. When cells have expected count less than 5, data were analyzed by Fisher's Exact test. Data are presented as percentage (%). A P value less than 0.05 was considered statistically significant.

ratio ($P < 0.001$) when compared with the moderate or low serum Mg subgroup. However, serum Mg levels were not significantly associated with serum Ca × P value, 25(OH)D, and $1,25(OH)_2D$. After stratifying the CKD patients based on presence or lack of diabetes, serum Mg levels were significantly correlated with iPTH levels in the CKD patients with diabetes ($P = 0.008$) but not in those without diabetes (Figure 1). Moreover, among six diabetic CKD patients with hypomagnesemia, four patients had low iPTH levels and four patients had osteoporosis.

3.4. Bone Metabolic Parameter Levels for the CKD Patients with Osteoporosis by Low and High Serum Mg Levels. As shown in Table 4, of six diabetic CKD patients with hypomagnesemia, four patients had low iPTH levels (range of 7.8–38.1 pg/mL), four patients had osteoporosis, and one patient had osteopenia. Three patients had 25(OH)D insufficiency-deficiency (range of 11.3–21.6 ng/mL), and three patients had $1,25(OH)_2D$ deficiency (range of 5.1–22.1 pg/mL). Of the five CKD patients with high serum Mg and osteoporosis, four patients had diabetes and one patient did not have diabetes. The nondiabetic CKD patient with high serum Mg and osteoporosis had high iPTH levels and low levels of serum Ca, 25(OH)D, and $1,25(OH)_2D$. Furthermore, of the four diabetic CKD patients with high serum Mg and osteoporosis, three patients had also high iPTH (range of 83.8–293.0 pg/mL), 25(OH)D insufficiency-deficiency (range of 13.2–23.8 pg/mL), $1,25(OH)_2D$ deficiency (range of 5.2–19.7 pg/mL), and tendency to low serum Ca (range of 8.4–9.0 mg/dL).

3.5. Relationships of PTH Levels with Serum Ca : Mg Ratio and Serum Ca × P Value. The relationships of PTH levels with the serum Ca/Mg ratio and serum Ca × P were analyzed via multivariate analysis using the General Linear Model, with sex, age, diabetes, and eGFR as the adjusted variables. Data are adjusted for mean and SE. The serum Ca/Mg ratio of Q1 (<38.1), Q2 (38.1–66.3), Q3 (66.4–119.0), Q4 (119.1–169.0), and Q5 (>169.0), based on quintiles of PTH levels, was 4.5 ± 0.2, 4.0 ± 0.2, 3.7 ± 0.2, 4.2 ± 0.2, and 3.5 ± 0.2, respectively ($P = 0.019$). Following Bonferroni's *post hoc* comparisons, the Q5 group had a lower serum Ca/Mg ratio than the Q1 group ($P < 0.05$). However, PTH levels were not associated with the serum Ca × P values ($P = 0.483$). In addition, linear regression analysis was performed to examine the impact of the serum Ca/Mg ratio on the PTH, also using sex, age, diabetes, and eGFR as adjusted variables. Our data indicated that the serum Ca/Mg ratio was inversely correlated with PTH levels ($P = 0.027$) and B was −1.455, whereas β was −0.305, and the 95.0% confidence interval for B was −2.740 to −0.170. However, PTH levels were not correlated with the serum Ca × P values ($P = 0.182$).

4. Discussion

Appropriate management of abnormal bone mineral metabolism may reduce CKD patients' risk of developing some complications [26]. The aim of the present study is to investigate the impacts of serum Mg levels on bone mineral metabolism in the CKD patients with and without diabetes. Our findings show that the hypomagnesemia may

Table 4: Bone metabolic parameters levels for the CKD patients with osteoporosis by low and high serum Mg levels.

Case	CKD stage	iPTH (pg/mL)	Ca (mg/dL)	P (mg/dL)	25(OH)D (ng/mL)	$1,25(OH)_2D$ (pg/mL)	Bone health status
Low serum Mg							
Diabetes							
1	3	61.3	9.0	3.1	21.6	22.1	Osteoporosis
2	3	26.2	9.4	3.3	37.8	25.9	Osteoporosis
3	4	7.8	9.0	3.1	37.1	35.2	Osteoporosis
4	5	38.1	8.6	4.4	19.9	27.4	Osteoporosis
5	4	24.1	9.7	4.1	32.8	19	Osteopenia
6	3	65.8	8.7	3.6	11.3	5.1	Normal
High serum Mg							
Diabetes							
1	3	51.5	8.7	3.6	23.8	26.8	Osteoporosis
2	4	83.8	9.0	3.9	13.2	15.9	Osteoporosis
3	5	184.0	8.4	5.9	25.1	19.7	Osteoporosis
4	5	293.0	8.7	4.7	15.9	5.2	Osteoporosis
Nondiabetes							
1	5	169.0	8.7	4.2	17.8	3.4	Osteoporosis

(1) For patients with stages 3, 4, and 5 CKD, PTH is in the range of 35–70 pg/mL, 70–110 pg/mL, and 150–300 pg/mL, respectively.
(2) For patients with stages 3 to 4 CKD, serum Ca should be maintained within normal range, 8.9–10.1 mg/dL, and serum P should be within 2.7–4.6 mg/dL. For patients with stage 5 CKD, serum Ca should be 8.4–9.5 mg/dL and serum P target should be 3.3–5.5 mg/dL.
(3) Vitamin D deficiency is defined as a serum 25(OH)D level of less than 20 ng/mL and vitamin D insufficiency is defined as a serum 25(OH)D level of 20 to 30 ng/mL. Serum $1,25(OH)_2D$ deficiency is defined as a serum $1,25(OH)_2D$ level of less than 25.1 ng/mL.

cause low iPTH levels and may aggravate bone mineral disorders in CKD patients with diabetes. Serum Mg levels were inversely correlated with serum Ca levels and positively correlated with iPTH, ALP, and P levels in the CKD patients with diabetes. Moreover, the CKD patients with diabetes had lower serum albumin and a higher proportion of hypomagnesemia and osteoporosis. In addition, serum Mg levels were inversely correlated with eGFR and positively correlated with serum creatinine levels in the CKD patients with or without diabetes. Serum Mg showed an inverse correlation with 25(OH)D in CKD patients without diabetes. Furthermore, serum Ca/Mg ratios were inversely correlated with the PTH levels.

4.1. Serum Mg and Osteoporosis in the CKD Patients with or without Diabetes. In the present study, CKD patients with diabetes had lower serum albumin and a higher proportion of hypomagnesemia and osteoporosis than those of CKD patients without diabetes. The possible explanation for this is that diabetic nephropathy is characterized by decreased renal function and significant albuminuria [30]. Serum albumin acts as a transport protein for numerous substances, including Mg, Ca, and zinc [31]. Therefore, the amount of total Mg and Ca may lower with the albumin decreasing in CKD patients with diabetes. Indeed, our data was consistent with findings from other studies [13, 32, 33]. A retrospective cohort study reported that the subjects with low serum Mg had higher proteinuria and lower serum albumin levels among CKD patients with or without diabetes. In particular, CKD patients with diabetes had more serious proteinuria and low levels of serum albumin and Mg than those of nondiabetic CKD patients [13]. Dewitte et al. also reported that renal failure patients with diabetes had lower blood Mg levels than nondiabetic patients [32]. Furthermore, hypomagnesemia is common in patients with diabetes [33], and it is associated with osteoporosis [6]. Our findings suggest that CKD patients with diabetes had lower serum albumin and a higher proportion of hypomagnesemia, and this may be related to the development of osteoporosis.

4.2. Serum Mg and Bone Mineral Metabolism. The differences in the relationship between serum Mg levels with bone mineral metabolism among CKD patients with and without diabetes are still under controversy. Our findings showed that serum Mg levels were positively correlated with iPTH and inversely correlated with serum Ca and 25(OH)D before stratifying the CKD patients depending on whether or not they had diabetes. After stratifying the CKD patients based on presence or absence of diabetes, there remains an inverse correlation between serum Mg and 25(OH)D in CKD patients without diabetes. Nevertheless, serum Mg levels were inversely correlated with serum Ca levels and positively correlated with iPTH and ALP in the CKD patients with diabetes but not in those without diabetes. Recently, Kanbay et al. reviewed the literature and concluded that serum Mg is inversely correlated with PTH in the general population [34]. Sakaguchi et al. reported that serum Mg levels had no correlation with serum Ca levels in CKD patients. Conversely, serum Mg levels were positively correlated with P levels [13]. Navarro et al. discovered that hypermagnesemia is common in peritoneal dialysis patients, and there was an inverse correlation between serum Mg and PTH [35]. Furthermore, the relationships of serum Mg with PTH and mineral metabolism have yielded conflicting

data in hemodialysis (HD) patients. An inverse correlation between Mg levels and PTH in HD patients was claimed in some studies [36, 37]. However, another study showed that serum Mg levels positively correlated with plasma P, but no correlations between serum Mg and serum Ca or PTH in HD patients were found [38]. Our findings and data from other studies indicated that serum Mg levels may play an important role in regulating the PTH levels and bone mineral metabolism in CKD patients [13, 35]. However, relationship between serum Mg with PTH and bone mineral metabolism may vary with the presence of diabetes and different renal replacement therapies. Therefore, the effect of serum Mg levels on PTH and bone mineral metabolism in CKD patients needs further exploration.

4.3. Low Serum Mg Levels, Low iPTH Levels, and Osteoporosis. The most commonly encountered types of bone disease in CKD are lower turnover bone disease (adynamic bone disease), high-turnover bone disease (bone resorption), and mixed bone disease [26]. We further focus on the impacts of low or high serum Mg levels on PTH and bone mineral metabolism in CKD patients. Our data showed that serum Mg levels were correlated with iPTH levels in the CKD patients with diabetes. The subgroup with low serum Mg levels had lower iPTH levels and higher serum Ca/Mg ratios when compared with the moderate or high serum Mg levels subgroup. Moreover, our data showed six patients found hypomagnesemia in the CKD with diabetes patients but not in non-diabetes patients. Of six diabetic CKD patients with hypomagnesemia, four patients had low iPTH levels, four patients had osteoporosis, and one patient had osteopenia. Three patients had 25(OH)D insufficiency-deficiency, and three patients had $1,25(OH)_2D$ deficiency. Indeed, hypomagnesemia affects the secretion and activity of PTH as well as tissue sensitivity to PTH [39, 40] and reduces the activity of the 25-hydroxycholecalciferol-1-hydroxylase, hence resulting in low serum concentrations of $1,25(OH)_2D$ [2, 24]. Therefore, these diabetic CKD patients with low iPTH levels and osteoporosis may belong to lower turnover bone disease (adynamic bone disease) [26], and the low serum Mg may be a major cause. A cross-sectional study also has shown that type 2 diabetes patients have lower levels of bone resorption markers and PTH compared with subjects without diabetes [41]. Yamamoto et al. indicated that decreased PTH levels accompanied by low bone formation are related to vertebral fractures in postmenopausal women with type 2 diabetes. Therefore, lower levels of PTH may induce a lower turnover state, and this status may be correlated with the higher risk of fracture in patients with diabetes [22]. Our findings suggest that low serum Mg levels may cause insufficient PTH action, and this may eventually cause lower turnover bone disease in CKD patients with diabetes.

4.4. High Serum Mg, High PTH, and Osteoporosis. In contrast, our findings showed that the subgroup with high serum Mg had a higher iPTH, lower serum Ca, elevated serum P, and lower serum Ca : Mg ratio when compared with the moderate or low serum Mg subgroup. Of the five CKD patients with high serum Mg and osteoporosis, four patients have diabetes and one patient has no diabetes. The nondiabetic CKD patient with high serum Mg and osteoporosis had high iPTH levels and low levels of serum Ca, 25(OH)D, and $1,25(OH)_2D$. In addition, of the four diabetic CKD patients with high serum Mg and osteoporosis, three patients also had high iPTH, 25(OH)D insufficiency-deficiency, $1,25(OH)_2D$ deficiency, and a low serum Ca trend. These CKD patients with high serum Mg levels, raised PTH levels, vitamin D deficiency, low Ca levels, and osteoporosis may be considered as having a high-turnover bone disease (bone resorption) [26]. Although high serum Mg levels may inhibit PTH secretion [6], Mg is able to reduce PTH secretion mainly when moderate to low Ca levels are present [40]. Moreover, the stimulus to produce PTH by low serum Ca levels may be more strongly influenced, rather than inhibited, by the effect of high serum Mg levels on PTH secretion [42]. Furthermore, elevated PTH levels can lead to increased bone resorption [26]. Hypermagnesemia may also cause complications in CKD patients [6]. Our data suggests that high serum Mg levels may no longer be enough to suppress PTH secretion when moderate-severe CKD patients with low serum Ca levels do not receive dialysis. Patients who maintain Ca levels within the normal range and avoid excess P and Mg levels may be key points for clinical care.

4.5. Serum Ca/Mg Ratio and PTH Levels. Recently, more and more studies are paying attention to the importance of Ca and Mg balance on preventing disease [43–45]. An inadequate Ca/Mg ratio may cause inflammation, cardiovascular disease, and cancer [44, 45]. However, impacts of Ca/Mg ratio on bone mineral metabolism have not been fully investigated. Our data and several studies have shown that serum Mg level was associated with serum PTH and Ca levels. Moreover, varied serum Mg and Ca levels may affect the suppression or production of PTH levels [6, 40, 42]. Therefore, interactions between serum Mg and Ca or the serum Ca/Mg ratio may be an important factor in the modulation of PTH levels and reducing the development of bone mineral disease. In the present study, we further analyze the relationship between PTH levels with serum Ca/Mg ratio and serum Ca × P values in CKD patients. Our findings showed that the serum Ca/Mg ratio was inversely correlated with the PTH levels. However, PTH levels were not associated with the serum Ca × P values. The CKD patients with low PTH levels had higher serum Ca/Mg ratios of 4.5 than those of other subgroups. In contrast, the CKD patients with high PTH levels had lower serum Ca/Mg ratios of 3.5 than those of other subgroups. In addition, CKD patients with low serum Mg and low PTH indeed had higher serum Ca/Mg ratio of 5.1. The CKD patients with high serum Mg, elevated PTH, and low serum Ca had lower serum Ca/Mg ratios of 3.4 (Table 3). Thus, we are speculating that serum Ca/Mg ratios greater than 4.5 may cause insufficient parathyroid hormone action and deteriorate lower turnover bone diseases in CKD patients. In contrast, serum Ca/Mg ratios less than 3.5 may cause stimulation to produce PTH and lead to high-turnover bone diseases in CKD patients. Our data suggest that the serum Ca/Mg ratio may be a novel determinant of PTH level, and this may be correlated with lower or high-turnover status

in CKD patients. Future research shall pay much attention to the effects of serum Ca/Mg ratios on PTH levels and bone mineral metabolism as well as what the adequate serum Ca/Mg ratios in moderate-severe CKD patients are.

4.6. Serum Mg and Renal Functional Declines. In a recent study, serum Mg levels were inversely correlated with eGFR and positively correlated with serum creatinine levels in the CKD patients with and without diabetes. Our finding in CKD patients without diabetes was consistent with that of Sakaguchi et al., who reported that serum Mg levels had a negative correlation with creatinine clearance in patients without type 2 diabetes [13]. Dewitte et al. also found that serum Mg levels increase when creatinine clearance from 115 falls to 30 mL/min in renal failure patients without diabetes [32]. Conversely, serum Mg levels were not correlated with renal functional parameters including creatinine and GFR in the diabetic patients [13, 32]. Nevertheless, even if CKD patients with diabetes had higher proteinuria levels and lower serum Mg levels than those of nondiabetic CKD patients [13, 32], the serum Mg levels may rise when renal functional declines to moderate-severity CKD patients [12]. As the GFR falls below 30 mL/min, urinary Mg excretion may be insufficient to balance the intestinal Mg absorption [12]. Thus, the CKD patient with diabetes may be similar to the CKD patient without diabetes when declining renal function was accompanied by increases in serum Mg levels. Our data suggested that there is an inverse correlation between serum Mg levels with eGFR in the CKD patients with and without diabetes. Therefore, the dietary Mg intake should be a major determinant of serum Mg levels when the serum Mg level is raised with renal functional declines.

4.7. Policy Implications for Medical Care. In general, CKD disrupts Ca and P homeostasis and causes alterations of PTH and vitamin D levels [9–11]. These alterations may lead to complications, including bone and mineral metabolic diseases [9–11]. Nevertheless, the importance of Mg imbalance in disorders of bone mineral metabolism has been neglected in the clinical management of patients with CKD. The major findings of our study may have guideline implications for medical care in CKD patients, with and without diabetes. Our findings suggest that low serum Mg levels may cause insufficient parathyroid hormone action and may further lead to bone diseases in CKD patients with diabetes. For these patients, adequate Mg intake by diet or supplement may reduce the development of lower turnover bone disease. In contrast, the inhibitory effect of a high serum Mg level on PTH secretion may be offset by the stimulation produced through low serum Ca in moderate-severe CKD patients, who are not receiving dialysis. We suggest that these patients maintain a serum Ca level within the optimal range and avoid consuming excess amounts of Mg and P to reduce the risk of high-turnover bone diseases. Moreover, there should be routine monitoring of serum Mg levels, and paying attention to the balance of serum Ca and Mg is important in the assessment and management of bone mineral disorders in CKD patients with or without diabetes.

4.8. Limitations. Our study has several limitations. First of all, the most major one is the relatively small sample size, which may decrease the power of statistical analysis among subgroups. Inadequate statistical power may provide only pilot results for data analysis. Secondly, the generalizability of the results may be limited because the patients were residents in a rural area. Thirdly, the cross-sectional design may not conclude Mg deficiency as a cause of insufficient parathyroid hormone action in CKD patients with diabetes. Despite these limitations, our findings provide important implications for moderate-severe CKD patients. Our findings showed that low serum Mg levels may impact PTH levels and deteriorate osteoporosis. These data are inconsistent with those reported by several previous studies [2, 39]. The present data may also be applicable to nonrural CKD patients with low Mg and PTH levels. Thus, clinical staff may educate these patients with nutrition knowledge of increasing dietary Mg intakes from food or Mg supplements. They may improve the low Mg status, modulate secretion and activity of the PTH, and reduce the risk of developing osteoporosis. Further prospectively designed and supplementary studies with a large sample size may help us to reveal the effects of the Mg status on PTH and bone mineral metabolism in CKD patients with or without diabetes.

5. Conclusions

The CKD patients with diabetes have a higher prevalence of hypomagnesemia and osteoporosis. Low serum Mg may cause insufficient PTH action and deteriorate osteoporosis in CKD patients, particularly those with diabetes. Clinical care should focus on monitoring and managing the serum Mg levels to reduce the development of bone mineral disease in moderate-severe CKD patients who are not receiving dialysis, particularly, CKD patients with diabetes.

Abbreviations

ALP: Alkaline phosphatase
BUN: Blood urea nitrogen
Ca: Calcium
CKD: Chronic kidney disease
eGFR: Estimated glomerular filtration rate
HD: Hemodialysis
iPTH: Intact parathyroid hormone
MDRD: Modification of Diet in Renal Disease
Mg: Magnesium
$1,25(OH)_2D$: 1,25-Dihydroxyvitamin D
25(OH)D: 25-Hydroxyvitamin D.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Jui-Hua Huang participated in the design of study, implementation of study, and analysis and interpretation of data and initiated the first draft of the paper. Fu-Chou Cheng

participated in the interpretation of the original data and in revision and editing of the final paper and initiated the first draft of paper,. Hsu-Chen Wu has substantial contributions to the conception and the original design of study, conducting the clinical study, and furnishing the final paper. All the authors have read and approved the final paper.

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Research Article

The Inhibitory Effect of Alisol A 24-Acetate from *Alisma canaliculatum* on Osteoclastogenesis

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Osteoporosis is a disease that decreases bone mass. The number of patients with osteoporosis has been increasing, including an increase in patients with bone fractures, which lead to higher medical costs. Osteoporosis treatment is all-important in preventing bone loss. One strategy for osteoporosis treatment is to inhibit osteoclastogenesis. Osteoclasts are bone-resorbing multinucleated cells, and overactive osteoclasts and/or their increased number are observed in bone disorders including osteoporosis and rheumatoid arthritis. Bioactivity-guided fractionations led to the isolation of alisol A 24-acetate from the dried tuber of *Alisma canaliculatum*. Alisol A 24-acetate inhibited RANKL-mediated osteoclast differentiation by downregulating NFATc1, which plays an essential role in osteoclast differentiation. Furthermore, it inhibited the expression of DC-STAMP and cathepsin K, which are related to cell-cell fusion of osteoclasts and bone resorption, respectively. Therefore, alisol A 24-acetate could be developed as a new structural scaffold for inhibitors of osteoclast differentiation in order to develop new drugs against osteoporosis.

1. Introduction

Bone is a living, dynamic tissue that is constantly remodeled in the process of bone turnover. Bone health in adults depends on the synchronized performance of bone-resorbing osteoclasts and bone-forming osteoblasts that function together on the bone surface [1]. Bone remodeling is important in vertebrates to maintain bone volume and calcium homeostasis [2]. An imbalance in the activities of bone-resorbing osteoclast cells and bone-depositing osteoblast cells upon aging or reaching menopause leads to osteoporosis [3]. Osteoporosis, Paget's disease, and rheumatoid arthritis are the result of overactive osteoclasts, which resorb bone [4]. This disorder has been increasing in frequency along with the increase in life expectancy [5].

Osteoclasts are tissue-specific macrophage polykaryons created by the differentiation of monocyte/macrophage precursor cells at or near the bone surface [6]. These cells have essential roles in the balance of skeletal homeostasis. Osteoclast differentiation from bone marrow-derived macrophages (BMMs) is needed for receptor activator of nuclear factor-κB ligand (RANKL), which is known to play an important role in osteoclast development [7]. The nuclear factor of activated T cells c1 (NFATc1), noted master transcription factor for osteoclast differentiation, is induced by RANKL [8]. NFATc1 promotes the expression of osteoclast differentiation-related factors including tartrate-resistant acid phosphatase (TRAP), cathepsin K, and dendritic cell-specific transmembrane protein (DC-STAMP) [9–11].

Plants are valuable sources of medicinal compounds with a broad range of biological activity. Approximately 25 to 50% of current pharmaceuticals are derived from plants [12]. Traditional oriental herbal medicines have been reevaluated by clinicians [13] because these medicines have fewer side effects and are more suitable for long-term use compared to chemically synthesized medicines [14].

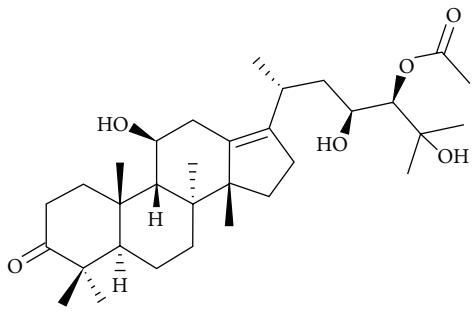

Figure 1: Molecular structure of alisol A 24-acetate.

Alisma canaliculatum, a member of the plant family Alismataceae, is a herb commonly used in traditional Korean medicine. *Rhizoma Alismatis*, a dried tuber of *A. canaliculatum*, is the main medicinal part of the plant. *A. canaliculatum* has diuretic hepatoprotective, antitumor, and antibacterial effects [15]. Previous phytochemical and pharmacological investigations of this plant reported the isolation of protostane- and seco-protostane-type triterpenes [16] such as alisols A, B, and C, alisol A 24-acetate, alisol B 23-acetate, alisol C 23-acetate, and alismalactone 23-acetate, and guaiane-type sesquiterpenes [17] such as alismols A and B, sulfoorientalol A, and orientatols AB, C, E, and F.

In our ongoing investigation of biologically active compounds from natural products, the dried rhizomes of *A. canaliculatum* were examined, and bioactivity-guided fractionations and HPLC yielded a triterpenoid, alisol A 24-acetate (Figure 1).

Herein, we report the isolation and the biological activities of alisol A 24-acetate.

2. Materials and Methods

2.1. Reagents. Recombinant mouse receptor activator of nuclear factor-κB ligand (RANKL) and recombinant mouse macrophage-colony stimulating factor (M-CSF) were purchased from R&D Systems (MN). Cell culture medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen Life Technologies (NY). The CCK-8 assay kit was obtained from Dojindo Molecular Technologies (ML). All reagents used in the reverse transcription (RT) and real-time PCR master mix were from Enzynomics (KR). NFATc1 monoclonal and actin polyclonal antibody were from Santa Cruz Biotechnology (CA, USA).

2.2. Plant Material. Alisma canaliculatum was purchased from Dongbu plant market in Suncheon in the South Sea in Korea.

2.3. Extraction and Isolation. The dried rhizomes of *Alisma canaliculatum* (wet weight, 1.2 kg) were minced and extracted with ethanol at room temperature for five days; the ethanol was concentrated under vacuum and then partitioned between EtOAc and H_2O (1 : 1). The EtOAc-soluble layer was concentrated under vacuum to give 18.0 g, which was subjected to silica gel (0.040–0.063 mm) column chromatography using a stepwise gradient with solvents of increasing polarity, from 100% CH_2Cl_2 to 100% MeOH. The fraction containing triterpenoid mixtures eluting with 2% CH_2Cl_2 in MeOH was further purified by RP-HPLC [Phenomenex Luna RP-C18(2), 5 μm, 250 × 10 mm, 2.5 mL/min] using an isocratic solvent system with 85% acetonitrile in H_2O to afford alisol A 24-acetate (**1**, 7.0 mg, t_R 14 min).

2.4. Alisol A 24-Acetate (1). ^{1}H NMR ($CDCl_3$, 700 MHz): δH 4.65 (1H, s, H-24), 3.89 (2H, overlapped, H-11 and H-23), 2.81 (2H, dd, J = 13.8, 5.9 Hz H-12), 2.68 (1H, m H-20), 2.35 (2H, ddd, J = 15.5, 9.6, 3.3 Hz, H-2), 2.25 (1H, m, Ha-1), 2.20 (3H, s, -$COCH_3$), 2.15 (1H, m, Hb-1), 2.16 (2H, m, H-16), 2.10 (1H, m, H-5), 2.02 (2H, m, H-7), 1.89 (1H, m, H-15a), 1.74 (1H, d, J = 10.8 Hz, H-9), 1.45 (1H, m, H-6a), 1.39 (1H, m, H-6b), 1.38 (2H, m, H-22), 1.36 (1H, m, H-15b), 1.30 (3H, s, H-27), 1.16 (3H, s, H-26), 1.15 (3H, s, H-30), 1.07 (3H, d, J = 11.0 Hz, H-21), 1.06 (3H, s, H-28), 1.00 (3H, s, H-18), 0.99 (3H, s, H-19), 0.98 (3H, s, H-29); ^{13}C NMR (175 MHz, $CDCl_3$): δC 220.5 (qC, C-3), 171.5 (-$\underline{CO}CH_3$), 138.3 (qC, C-13), 135.5 (qC, C-17), 78.6 (CH, C-24), 73.9 (qC, C-25), 70.0 (CH, C-11), 69.0 (CH, C-23), 57.0 (qC, C-14), 49.6 (CH, C-9), 48.5 (CH, C-5), 47.0 (qC, C-4), 40.5 (qC, C-8), 39.7 (CH_2, C-22), 36.9 (qC, C-10), 34.5 (CH_2, C-12), 34.3 (CH_2, C-7), 33.8 (CH_2, C-2), 30.9 (CH_2, C-1), 30.5 (CH_2, C-15), 29.6 (CH_3, C-28), 29.1 (CH_2, C-16), 27.9 (CH, C-20), 27.5 (CH_3, C-26), 26.6 (CH_3, C-27), 25.7 (CH_3, C-19), 24.1 (CH_3, C-30), 23.2 (CH_3, C-18), 20.1 (-$COCH_3$), 20.1 (CH_3, C-29), 20.1 (CH_3, C-21), 20.0 (CH_2, C-6); LCMS m/z: 515 [M-H_2O+H]$^+$, 497 [M-2H_2O+H]$^+$.

2.5. Osteoclast Differentiation. This study was carried out in strict accordance with the recommendations outlined in the Standard Protocol for Animal Study from the Korea Research Institute of Chemical Technology (KRICT; Permit number 2012-7D-02-01). The protocol (ID number 7D-M1) was approved by the Institutional Animal Care and Use Committee of KRICT (IACUC-KRICT). All efforts were made to minimize the suffering of animals. Bone marrow cells (BMCs) were collected from femur and tibia of 5-6-week-old male ICR mice by flushing femurs and tibias with α-MEM supplemented with antibiotics. BMCs were cultured with M-CSF (10 ng/mL) in α-MEM containing 10% fetal bovine serum (FBS) and antibiotics in a culture dish for 1 day. Nonadherent BMCs were cultured for 3 days in a Petri dish in M-CSF (30 ng/mL), and the adherent cells were used as bone marrow-derived macrophages (BMMs). For the formation of osteoclasts, BMMs were cultured with RANKL (10 ng/mL) and M-CSF (30 ng/mL) in the presence or absence of alisol A 24-acetate for 4 days. The culture medium was changed once per three days. Osteoclast formation was assessed by TRAP (tartrate-resistant acid phosphatase) staining.

2.6. TRAP Staining Assay. Cells were fixed with 3.7% formalin for 5 min, permeabilized with 0.1% Triton X-100 for 10 min, and stained with TRAP solution (Sigma-Aldrich, MO, USA) for 10 min. TRAP$^+$-MNCs (3 ≤ nuclei) were counted as multinucleated osteoclasts.

2.7. Cytotoxicity Assay. The BMMs were cultured with M-CSF (30 ng/mL) at a density of 1×10^4 cells/well on 96-well

Table 1: Primer sequences used in this study.

Target gene	Forward (5′-3′)	Reverse (5′-3′)
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
TRAP	GATGACTTTGCCAGTCAGCA	ACATAGCCCACACCGTTCTC
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

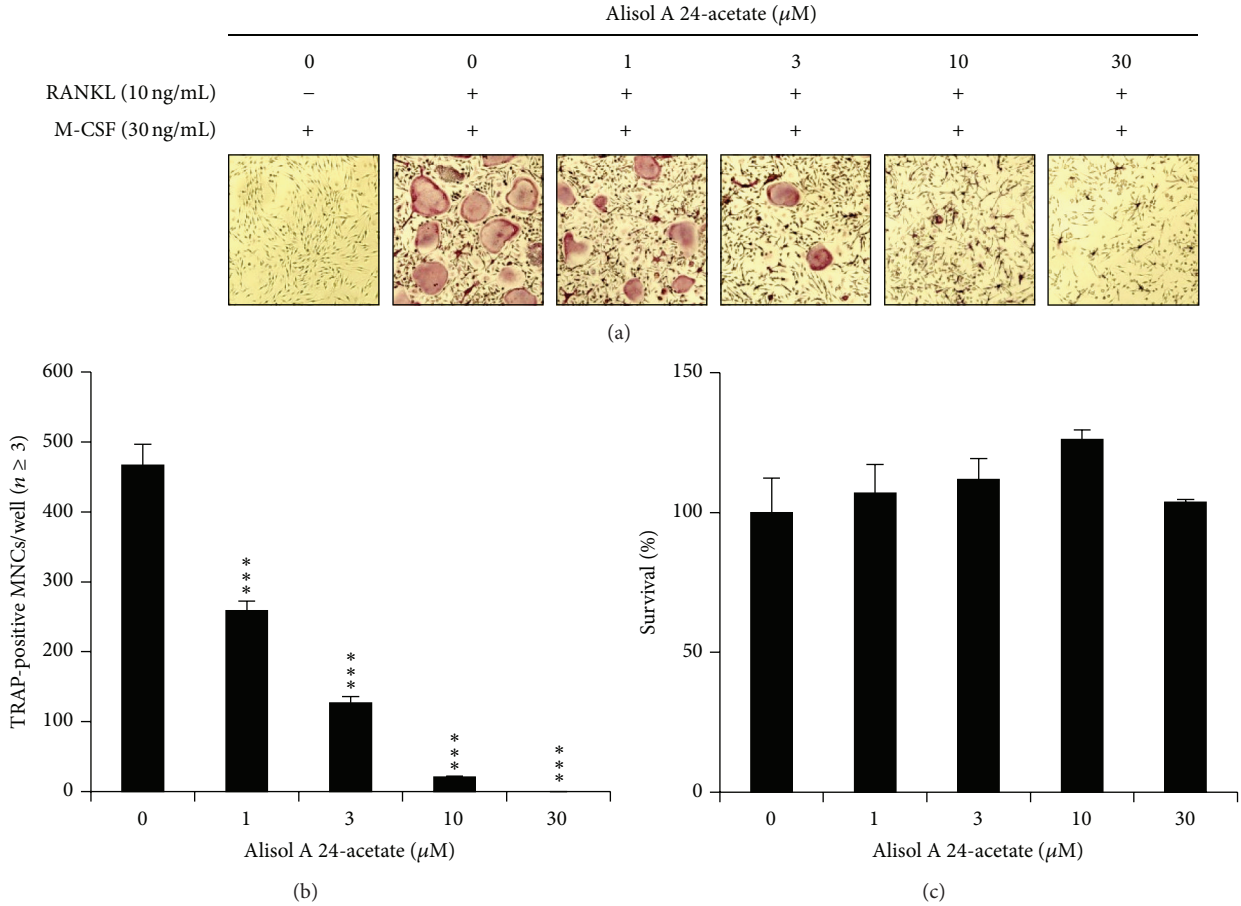

Figure 2: Effects of alisol A 24-acetate on osteoclastogenesis. (a) BMMs prepared from bone marrow cells were cultured for 4 days with RANKL (10 ng/mL) and M-CSF (30 ng/mL) in the presence of the indicated concentrations of alisol A 24-acetate. Cells were fixed in 3.7% formalin, permeabilized in 0.1% Triton X-100, and stained for TRAP, a marker enzyme of osteoclasts. (b) TRAP-positive multinuclear cells with three or more nuclei were counted as osteoclasts. $^{**}P < 0.01$; $^{***}P < 0.001$ ($n = 3$). (c) Effect of alisol A 24-acetate on the viability on BMMs was evaluated by CCK-8 assay.

plates in the presence of alisol A 24-acetate (indicated concentration) for 3 days. The cells were incubated for 3 hours in α-MEM containing 10% CCK-8 reagent. The optical density (OD) values were measured at 450 nm.

2.8. Real-Time PCR Analysis. Primers were chosen with Primer3 design program [18]. The primer sets used in this study are shown in Table 1. Total RNA was isolated from cells with TRIzol reagent according to the manufacturer's protocol. First-strand cDNA was synthesized with 0.5 μg of total RNA, 1 μM oligo-dT18 primer, and the M-MLV cDNA synthesis kit (Enzynomics, KR) according to the manufacturer's protocol. SYBR green-based QPCR was performed with the Stratagene Mx3000P real-time PCR system and TOPrealTM qPCR 2X PreMIX (Enzynomics, KR), with the first-strand cDNA diluted 1 : 10 and 20 pmol of primers according to the manufacturer's protocol. The polymerase was activated at 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 s (extension). This was followed by the generation of PCR-product temperature-dissociation curves (also called melting curves) at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$ method [19]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

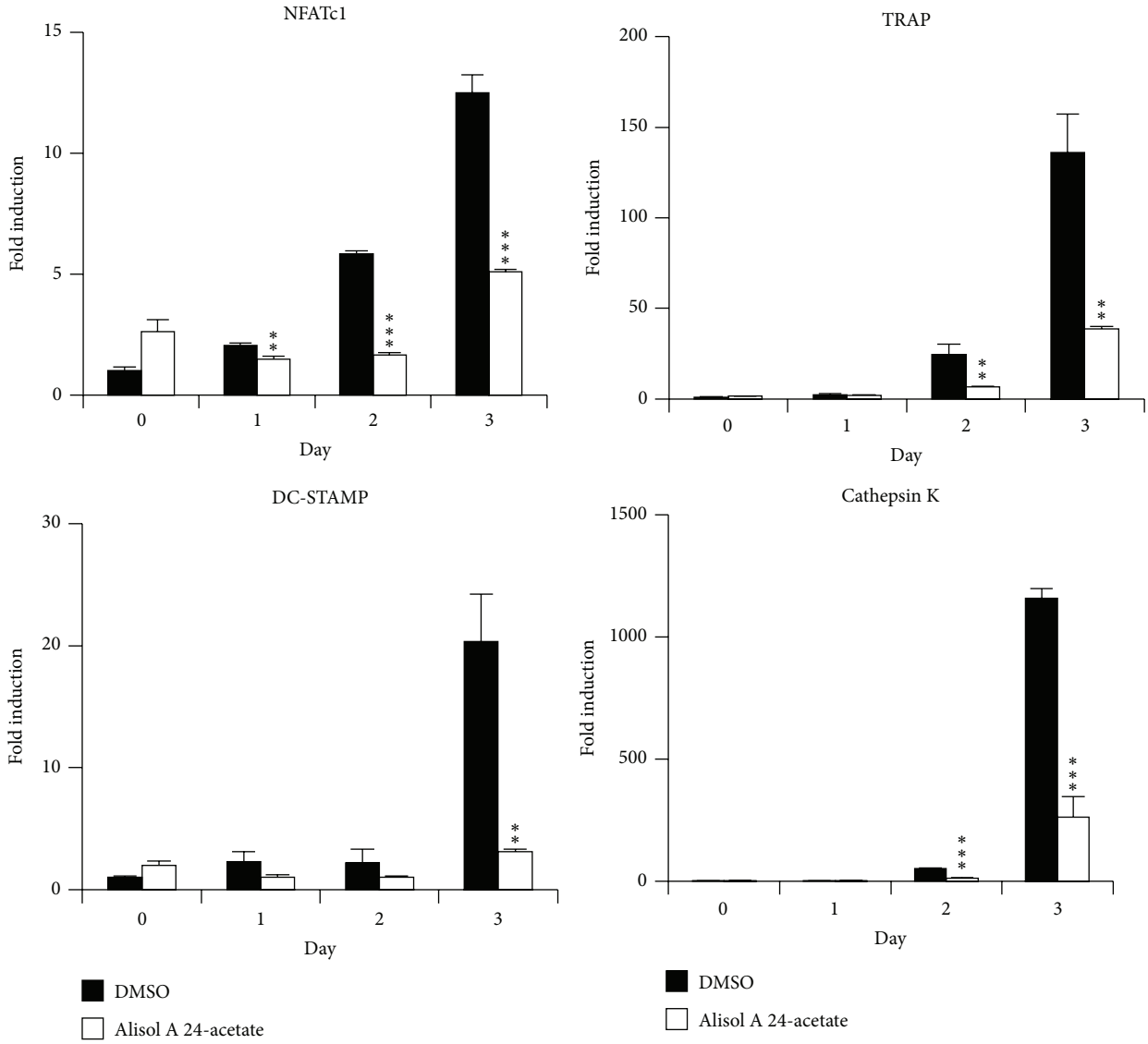

Figure 3: Alisol A 24-acetate decreased NFATc1 transcriptional expression by RANKL stimulation. BMMs were pretreated with vehicle (DMSO) or alisol A 24-acetate (10 μM) for 30 minutes and then stimulated with RANKL (10 ng/mL) for the indicated number of days. Expressed mRNA levels were analyzed by real-time PCR compared with the vehicle control. $^{**}P < 0.01$; $^{***}P < 0.001$ ($n = 3$).

2.9. Western Blotting Analysis. Cells were incubated in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, and 1% deoxycholate, 1 : 1000 proteinase inhibitor) for 30 minutes on ice. Cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were washed with TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and incubated in blocking buffer (5% nonfat milk in TBST) for 1 hour at room temperature. The membranes were incubated with anti-NFATc1 (1 : 500) and anti-actin (1 : 1000) overnight. After three 30 min wash, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase for 2 hours at room temperature and then washed three times for 30 min. Specific bands were visualized by chemiluminescence using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan).

2.10. All Quantitative Values Are Presented as Mean ± SD. Each experiment was performed three to five times, and the results from one representative experiment are shown. Statistical differences were analyzed using Student's t-test of Microsoft Excel. The P values were described by the comparison between the control and one of the test groups ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). A value of $P < 0.05$ was considered significant.

3. Results

3.1. Alisol A 24-Acetate Inhibited the Differentiation of BMMs by RANKL. To determine the effect of alisol A 24-acetate

on osteoclast differentiation, alisol A 24-acetate was added during osteoclast differentiation with RANKL (10 ng/mL) and M-CSF (30 ng/mL). The addition of alisol A 24-acetate inhibited the differentiation of BMMs into osteoclasts (Figure 2(a)). In addition, the number of TRAP-positive multinucleated cells (3 ≤ nuclei) was significantly decreased in a dose-dependent manner by alisol A 24-acetate (Figure 2(b)). Osteoclasts were completely inhibited at a concentration of 10 μM alisol A 24-acetate. These results implied that alisol A 24-acetate could inhibit RANKL-induced osteoclastogenesis.

3.2. The Cytotoxic Effect of Alisol A 24-Acetate. The cytotoxicity of alisol A 24-acetate during osteoclast differentiation was measured by CCK-8 assay. BMMs were incubated in the presence of M-CSF (30 ng/mL) and DMSO (vehicle) or alisol A 24-acetate for 3 days. Alisol A 24-acetate had no cytotoxic effects at the indicated concentration (Figure 2(c)). These results suggested that osteoclastogenesis suppression by alisol A 24-acetate was not due to toxic effects on BMMs.

3.3. Alisol A 24-Acetate Inhibited RANKL-Induced mRNA Expression of Osteoclast-Specific Genes. We investigated mRNA expression of osteoclast-specific genes in osteoclast differentiation by real-time PCR. Expressed mRNA levels of NFATc1, TRAP, DC-STAMP, and cathepsin K were analyzed compared with the control (DMSO) for 3 days. Alisol A 24-acetate significantly suppressed mRNA expression of transcription factors such as NFATc1. Furthermore, it decreased osteoclast-related molecules including TRAP, DC-STAMP, and cathepsin K (Figure 3).

3.4. Alisol A 24-Acetate Inhibited RANKL-Induced Protein Expression of NFATc1. The inhibitory effect of alisol A 24-acetate on the translational expression of NFATc1, a master regulator of osteoclast differentiation, was evaluated by western blot analysis. Protein expression of NFATc1 was significantly increased by RANKL without alisol A 24-acetate but was dramatically inhibited by alisol A 24-acetate (Figure 4). This result indicated that alisol A 24-acetate could inhibit the translational expression of NFATc1 and suppress osteoclastogenesis.

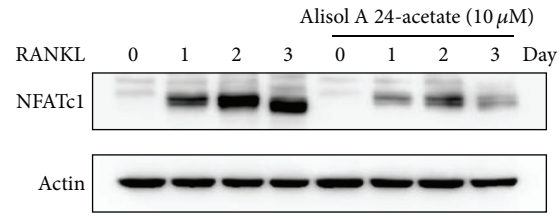

Figure 4: Alisol A 24-acetate inhibits RANKL-induced NFATc1 expression. BMMs were pretreated with alisol A 24-acetate (10 μM) for 1 h and then stimulated with RANKL (10 ng/mL) for the indicated time. Cell lysates were resolved by SDS-PAGE, and western blotting was performed with anti-NFATc1 and anti-actin antibodies as indicated.

4. Discussion

Osteoporosis is a bone disease characterized by low bone mass and structural deterioration of bone tissue. Osteoporosis causes nearly nine million new osteoporotic fractures annually worldwide [20]. Low bone mineral density (BMD) is a major cause of bone fracture. BMD is affected by bone-resorbing osteoclasts and bone-forming osteoblasts.

Bone remodeling is an important process in sustaining healthy bones. It is carried out by osteoblasts and osteoclasts. The balance between osteoblastic bone formation and osteoclastic bone resorption is crucial for bone homeostasis. Generally, bone disorders such as osteoporosis and rheumatoid arthritis involve overactive osteoclasts and/or their increased number. Osteoclasts, derived from pluripotent hematopoietic stem cells, are bone-resorbing multinucleated cells [6, 21, 22]. RANKL, an osteoclasts differentiation factor, is related to the TNF superfamily and expressed by stromal cells in bone marrow and osteoblasts [23, 24]. It contains a C-terminal receptor-binding domain and a transmembrane domain and binds to its receptor, RANK, which is expressed on osteoclasts [25]. RANKL/RANK binding activates NFATc1, which regulates many osteoclast-specific genes, such as cathepsin K, TRAP, and DC-STAMP [24, 25]. TRAP is the principal cytochemical marker for osteoclasts [26], DC-STAMP plays an essential role in cell-cell fusion of osteoclasts [11, 27], and cathepsin K is a major protease in bone resorption [28].

Here, we tested the effect of alisol A 24-acetate on osteoporosis, specifically RANKL-mediated osteoclast differentiation. The alisol A 24-acetate, isolated from the dried tuber of *Alisma canaliculatum*, completely inhibited osteoclast differentiation and had no cytotoxic effects at concentrations over 10 μM. These results suggested that the alisol A 24-acetate has antiosteoclastogenic activity without cytotoxicity to BMMs. As mentioned earlier, the expression of NFATc1 is the key factor in osteoclastogenesis. So we investigated the transcriptional expression level of NFATc1 and some osteoclast-specific genes for osteoclastogenesis. The mRNA expression of NFATc1 was inhibited by alisol A 24-acetate. Furthermore, the mRNA expression levels of osteoclast-specific genes for osteoclast differentiation such as TRAP, DC-STAMP, and cathepsin K were significantly reduced by alisol A 24-acetate. Thus, alisol A 24-acetate inhibited the signal cascade from RANKL/RANK binding to NFATc1, and osteoclast differentiation was inhibited because of the inhibitive mechanism of alisol A 24-acetate. Moreover, alisol A 24-acetate blocked cell-cell fusion of osteoclasts by inhibiting the expression of DC-STAMP. The decreased expression of DC-STAMP and cathepsin K was related to the decreased expression of NFATc1 [29, 30]. We confirmed the inhibition of alisol A 24-acetate on the translational expression of NFATc1 by western blotting. Like the transcriptional inhibition of NFATc1, the translational expression of NFATc1 was strongly inhibited by alisol A 24-acetate. Our results suggest that alisol A 24-acetate may be a potential therapeutic molecule for bone disorders and could be utilized as a new structural scaffold for inhibitors of osteoclast differentiation.

5. Conclusions

This is the first report of alisol A 24-acetate, isolated from *Alisma canaliculatum*, and its antiosteoclastogenic activity. Alisol A 24-acetate inhibited RANKL-induced osteoclast differentiation by downregulating NFATc1, a master factor for osteoclast differentiation, without cytotoxicity and also inhibited the expression of DC-STAMP and cathepsin K. Therefore, alisol A 24-acetate could be used as a scaffold for the development of a new osteoporosis drug.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Kwang-Jin Kim and Alain Simplice Leutou contributed equally to the work.

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Review Article

Vitamin D and Osteoporosis in HIV/HCV Coinfected Patients: A Literature Review

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Vitamin D deficiency further increases the risk of osteoporosis in HIV-positive patients coinfected with hepatitis C virus (HCV); however, it is still unclear whether HCV-related increased fracture risk is a function of the severity of liver disease. The aim of this review was to identify studies on associative vitamin D deficiency patterns in high-risk populations such as HIV/HCV coinfected patients. We did this by searching MEDLINE and EMBASE databases, from inception to August 2014, and included bibliographies. The final 12 articles selected are homogeneous in terms of age but heterogeneous in terms of sample size, participant recruitment, and data source. Most of the HIV/HCV coinfected patients have less than adequate levels of vitamin D. After reviewing the selected articles, we concluded that vitamin D deficiency should be regarded as a continuum and that the lower limit of the ideal range is debatable. We found that vitamin D deficiency might influence liver disease progression in HIV/HCV coinfected patients. Methodological issues in evaluating vitamin D supplementation as a relatively inexpensive therapeutic option are discussed, as well as the need for future research, above all on its role in reducing the risk of HCV-related fracture by modifying liver fibrosis progression.

1. Introduction

Clinicians and researchers are currently using available data sets to assess the balance of beneficial and harmful effects of vitamin D not only on skeletal health but also on its potential role in nonskeletal outcomes such as cardiovascular disease, death, and quality of life [1–3].

The effects of vitamin D on immune function [4] and its immunomodulatory and anti-inflammatory properties have been recognized, and a nontraditional role of vitamin D has been reported in cancer patients and autoimmune disease [4–6].

These effects have also been reported in chronic liver disease and among chronic hepatitis C patients in whom vitamin D is involved in regulating the immune system, inflammatory response, and fibrogenesis [7–10].

Recently, low 25-Hydroxyvitamin D serum levels have been associated with the severity of liver fibrosis in genotype 1 chronic hepatitis C patients (G1CHC) [11, 12].

In vitro studies have shown that vitamin D is an antiviral agent that inhibits HCV production in a human hepatoma cell line [13]; a synergistic effect of vitamin D and interferon-alfa on HCV production has also been reported. In HCV monoinfected patients with recurrent hepatitis C after liver transplant, higher rates of virologic response were observed in those receiving vitamin D supplementation [14].

Finally, in a randomized prospective trial including only CHC treatment-naive HCV-genotype (HCV-GT) 1 patients, virologic response rates were again higher in the group receiving vitamin D supplementation [11].

Hepatitis C virus (HCV) infection has become a major health problem among the HIV-infected population [15, 16]. Approximately 30% of all human immunodeficiency virus

(HIV-) positive patients are also infected with hepatitis C virus (HCV) [16–18].

Among the multifactorial mechanisms underlying skeletal disorder in the HIV and HCV setting, vitamin D deficiency is considered a risk factor for osteoporotic fracture [7, 18–20]; moreover, the finding that HCV-related increased fracture risk is a function of the severity of liver disease has generated a lot of attention so far [7, 21–23].

The fact that HIV and HIV/HCV coinfected patients are at risk of vitamin D deficiency because a wide variety of medications used to treat AIDS/HIV enhance the catabolism of 25(OH)D and 1,25(OH) 2D [23, 24] has been acknowledged. Vitamin D deficiency and bone disease in HAART patients [25] have been associated with NNRTIs [26], as well as tenofovir [27] and PIs [27, 28].

While there are recommendations for the evaluation, treatment, and prevention of vitamin D deficiency in healthy patients at risk of deficiency [3, 24] and in specific HIV populations such as young HIV-positive adults with 25-hydroxyvitamin D (25-OHD) < 20 ng/mL [29], there is still some debate about the evaluation, treatment, and prevention of vitamin D deficiency in the HIV population [30] and in particular in HIV/HCV coinfected subjects [7, 8, 31, 32].

Finally, recent literature offers recommendations on screening and treating vitamin D deficiency and osteoporosis in HIV-positive patients [30] but there is not a great deal of literature and/or consensus on cost-effective management of this patient population, especially HIV/HCV coinfected patients [22, 31–33].

This paper aims to summarize the prevalence of vitamin D deficiency in HIV/HCV coinfected patients, review available data on the association between vitamin D levels and severity of liver disease, and discuss the impact of this relatively inexpensive therapy on reducing liver fibrosis and improving sustained virologic response rate (SVR) in HCV patients.

2. Methods

We searched the Medline (PubMed) database for articles that matched any combination of the following keywords: vitamin D, vitamin D deficiency, 25-hydroxyvitamin D, HIV/HCV coinfections and diagnosis and treatment, and hypovitaminosis. Studies were identified through searching MEDLINE and EMBASE databases, from their inception to August 2014.

Articles were screened and those that reported on the relationship between vitamin D insufficiency/deficiency and HIV/HCV coinfections were included. We limited the search to language (i.e., Spanish, French, or English) and abstract availability. Because the terms HIV infection and HIV/HCV infection are frequently associated in the scientific literature, for this study the term HIV/HCV coinfections was used as a medical subject heading (MeSH) and the other terms (together with their linguistic variations) were used as keywords. Some articles that appeared with keyword searching were excluded because they were not relevant to the purpose of this review and tackled other topics such as anti-HCV therapy in HIV/HCV coinfected patients.

3. Results

Forty-four studies fit the criteria; 15 of these were duplicates and were removed. After screening titles and abstracts, we excluded 9 articles on studies involving HCV monoinfected or HIV monoinfected participants. Applying the eligibility criteria, the full texts of 12 articles were reviewed.

We selected 12 studies (see Table 1): 10 original [21, 31–39], 1 systematic review and meta-analysis article [22], and 1 review manuscript [20].

We found 5 cross-sectional [31, 32, 35, 37, 39], 3 retrospective [21, 34, 36], and 2 prospective cohort studies [33, 38]; most control groups included patients with HIV-mono- or HCV-monoinfection.

Overall, the articles were highly heterogeneous in terms of sample size, participant recruitment, and data source (Table 1). The patients' age in all the studies is relatively homogenous (median age 45 years old), reflecting the worldwide aging of the HIV population after the widespread availability of combination antiretroviral therapy (cART).

In general, the articles analyzed the prevalence of vitamin D levels in HIV/HCV coinfected patients and the association between vitamin D deficiency and liver disease variables such as severity of liver disease [21, 32, 34, 39] and the influence of vitamin D levels on virological response [32, 35, 36].

In fact, two recent studies showed a significant association between hypovitaminosis D, severity of liver disease, and response to interferon- (IFN-) based treatment in HIV-HCV patients [11, 35]. However, the association between 25(OH)D levels and SVR rates is thought to be limited to difficult-to-treat patients in whom treatment failure may depend on other factors (IL28 B, HCV genotype, hepatic expression of vitamin D receptor) [11, 12, 33, 35].

In their HIV/HCV coinfected setting, Branch et al. [36] found that baseline levels of 25(OH)D in patients treated with ritonavir are not predictors of EVR and SVR because ritonavir may influence conversion of 25(OH)D to the active metabolite. Other articles showed a significant negative association between longer duration of ART, especially PI exposure and bone mineral density (BMD) and osteoporosis [22, 28, 38]. However, in one large cohort study, HCV coinfection remained an independent predictor of osteoporotic fractures after checking for the presence of cirrhosis [21, 22].

Two articles [33, 39] showed no association between hypovitaminosis D, low BMD, and liver fibrosis (histological fibrosis staging according to METAVIR scores 0 [no fibrosis] to 4 [cirrhosis]) in HIV/HCV coinfected patients. The analysis of patient setting showed that most of the study populations included HIV/HCV coinfected African Americans. How race affects the impact of vitamin D on bone health has recently been investigated in African American men and women, revealing differences due to socioeconomic and genetic factors, such as resistance to the bone resorbing effects of PTH in the black population [33, 40–42].

Table 1: Characteristics of the selected studies.

Study, year	Country	Total number	HIV+/HCV+ number	Control Group(s)	Age (yrs) HIV+/HCV+	Study design	Population study/setting	Topics
Mandorfer et al., 2015 [34]	USA	86	86		38.7 median	Cohort retrospective	HIV/HCV coinfected	Vitamin D levels. Other variables and severity of liver disease
Dong et al., 2014 [22]	USA					Systematic review and meta-analysis	HIV/HCV coinfected	Osteoporosis and fractures
Guzmán-Fulgencio et al., 2014 [31]	Spain	174	174	HIV/HCV coinfection	40.8 median	Cross-sectional	HIV/HCV coinfected	Prevalence of vitamin D levels and association with other parameters
Avihingsanon et al., 2014 [32]	Australia	331	130	Monoinfected HCV–HIV/HCV coinfection	42 median	Cross-sectional	HIV/HCV coinfected	Vitamin D levels and virological response in coinfection treatment
Luetkemeyer et al., 2013 [20]	USA					Review	monoinfected HIV and HIV/HCV coinfected	Bone metabolisms and vitamin D deficiency
Mandorfer et al., 2013 [35]	Austria	65	65	HIV/HCV coinfection	38.6 median	Cross-sectional	HIV/HCV coinfected	Vitamin D levels and virological response in coinfection treatment
Branch et al., 2013 [36]	USA	144	144	non-EVR* HIV/HCV coinfected	48 median	Cohort retrospective	HIV/HCV coinfected genotype 1 treated in ACTG study	Vitamin D levels and virological response in coinfection treatment
El-Maouche et al., 2013 [33]	USA	116	116	HIV/HCV coinfection	49.9 median	Cohort prospective	HIV/HCV coinfected	Vitamin D levels and bone mineral density (BMD)
Linari et al., 2013 [37]	Italy	78	26	Monoinfected (HIV+)/Uninfected	45.8 mean	Cross-sectional	Haemophilia	Prevalence of hypovitaminosis D and BMD markers
Maalouf et al., 2013 [21]	USA	56.660	17.734	Monoinfected (HIV+)	44 median	Cohort retrospective	HIV-infected population	HCV-associated risk of osteoporotic fractures and severity of liver disease
Vecchi et al., 2012 [38]	Italy	120	41	Monoinfected (HIV+)/Uninfected	47 mean	Cohort prospective	HIV-infected population	Vitamin D levels and BMD; dairy calcium intake
Milazzo et al., 2011 [39]	Italy	237	93	Monoinfected HIV/Uninfected	45 median	Cross-sectional	HIV-infected population	Vitamin D levels and severity of liver disease

*Early virologic response.

Most of the patients enrolled in Milazzo et al.'s study [39] had HCV GT1 genotype and low levels of vitamin D that varied seasonally, as reported in another Italian study [9].

On the contrary, Avihingsanon et al. [32] found significant liver fibrosis in patients with HIV/HCV coinfection and low levels of 25(OH)D. These results may be influenced by race (Asian), HCV genotype (GT) [the most prevalent circulating genotype was HCV GT3 (47%)], and IL28B polymorphism (the major allele [CC genotype] of rs12979860 position was found in 88% of HIV/HCV patients).

Regarding the prevalence of fracture in this group of patients with liver disease, Dong et al.'s systematic review and meta-analysis [22] highlights that fracture incidence rate ratios (IRRs) are higher for coinfected than HIV monoinfected and uninfected patients. In multivariate analyses of HIV/HCV coinfected individuals, older age, lower BMI, postmenopausal status, and time on protease inhibitor were significantly associated with osteoporosis [18–20, 22, 27, 28, 38].

Among the HIV/HCV coinfected patients, haemophiliacs are considered to be at the highest risk for fracture. Table 1 includes Linari et al.'s study [37] on the prevalence of osteoporosis in this group of patients. The authors divided 78 haemophiliac patients into three groups (uninfected, HIV monoinfected, and HIV/HCV coinfected); hypovitaminosis D and low BMD were present in all patients, with lower L-DXA scores in coinfected patients and a more evident increase of bone resorption markers in HIV and HIV/HCV coinfected patients.

4. Discussion

Our literature review indicated that vitamin D deficiency is common in HIV/HCV coinfected patients and that hypovitaminosis D occurrence among patients with an average age of 45 years should raise concerns about the risk of developing bone fractures.

Low levels of vitamin D were also found in HIV monoinfected [21, 38, 39] and HCV monoinfected control groups [32, 37].

However, the selected articles reveal some aspects that prompt us to reconsider the definition of hypovitaminosis D. In fact, the significance of vitamin D deficiency has several limitations because 25-hydroxyvitamin D concentrations varied by age, season, study sample size, and methodological assay approach [25(OH)D assay used] [37, 39, 40]. Moreover, black and Hispanic individuals synthesize less vitamin D per unit of sun exposure than white individuals [33, 41, 42]. Therefore, levels of vitamin D in HIV/HCV coinfected patients should be monitored according to the reference range for the sample setting. In fact, further validation of the reported results is needed, since studies conducted on larger cohorts, as well as in Italian coinfected patients [39], have revealed low vitamin D levels consistent with those recently reported for healthy populations from Western countries [38, 39, 43].

Milazzo et al. found that season and severity of fibrosis were predictors of low 25(OH)D in an Italian HIV/HCV coinfected population and that median 25(OH)D serum levels below 25 ng/mL were similar in the HIV monoinfected, HIV/HCV coinfected, and healthy controls [39]. In a recent, large study on a general healthy population in Central Europe, Pludowski et al. [43] reported an average concentration of less than 30 ng/mL of 25(OH)D.

Guidelines specify that 25(OH)D concentrations are the best indicator of overall vitamin D status in the general population and define vitamin D insufficiency as a 25(OH)D level of below 75 nmol/L (30 ng/mL) and deficiency as below 50 nmol/L (20 ng/mL) [40]. However, the Endocrine Society Clinical Practice Guidelines (ESCPG) suggest that the vitamin D requirements of sick patients may be greater than those of healthy individuals, and blood levels above 30 ng/mL may carry additional health benefits by reducing the risk of various disease conditions [24, 40].

The Hormone Foundation's Patient Guide to Vitamin D Deficiency suggests that patients with chronic (long-term) liver disease are at high risk of deficiency; therefore vitamin D testing is recommended and patients should be given advice about adequate dietary intake and medical supplementation to prevent and treat deficiency [44].

The influence of diet on vitamin D status is minimal (accounting for 3.7–5.9 μg or 148–236 IU daily) as only a few foods, such as sardines, tuna, and mushrooms, naturally contain vitamin D [24, 40]. Today, the main sources are foods which have been fortified with vitamin D2 and/or vitamin D3, such as milk, orange juice, yoghurt, cheese, and breakfast cereals, which people of all ages can include in their daily diet. Although it has been suggested that a Mediterranean-style diet or a diet rich in fish and other foods containing vitamin D has health benefits, further evaluation is necessary [24, 38, 44, 45].

Our selected articles investigated the possibility of an association between vitamin D deficiency and hepatic fibrosis in HIV/HCV coinfected patients. One particular study that involved mostly African American HIV–HCV coinfected patients showed that increasing vitamin D levels does not improve bone or liver outcome [33], whereas other studies illustrate how vitamin D influences virological response to antiviral treatment in HIV-HCV coinfected patients and has a role in liver fibrosis progression [31, 34, 35].

Older and more recent research has investigated the link between vitamin D homeostasis and bone loss in patients with liver disease [7, 8, 22, 46]. Vitamin D deficiency in chronic liver disease is only partly the result of a synthesis dysfunction of the liver or/and decreased vitamin D absorption caused by intestinal edema due to portal hypertension or to cholestasis.

Recently, significantly lower levels of 25-hydroxyvitamin D were observed in patients with liver cirrhosis, admitted for acute decompensation, suggesting that systemic inflammation or liver dysfunction has an impact on 25(OH)D level [46].

Regarding vitamin D synthesis, parathyroid hormone (PTH) is involved in its activity and expression; disturbance of the parathyroid hormone-vitamin D axis with bone mass loss in chronic liver disease has recently been reported in

cirrhotic postmenopausal women and in geriatric patients with vitamin D deficiency [47, 48].

A systematic review and meta-analysis included in our literature review found that HIV/HCV-coinfected patients are at higher risk for osteoporosis and fractures than HIV-monoinfected controls and are at a substantially higher risk than uninfected controls. HCV and viral hepatitis coinfection remained an independent predictor of osteoporosis [22].

HCV and HIV infections are both associated with increased levels of proinflammatory cytokines that can promote osteoclastogenesis or inhibit osteoblast differentiation and collagen synthesis [7, 8, 22].

The mechanisms responsible for osteopenia and osteoporosis are uncertain and multifactorial, but exposure to certain antiretroviral drugs (in particular a NRTI: tenofovir-TDF-and the PI class), aging, HIV itself, parathormone (PTH) increase, and vitamin D deficiency may be implicated.

Moreover, in a cohort of GlCHC patients, the hepatic expression of VDR protein is associated with severity of both liver fibrosis and inflammation [12, 34].

Guidelines for the management of osteoporosis in HIV-negative [3, 24, 40, 44] and HIV-positive patients identified adequate vitamin D status, in addition to calcium from diet or supplements, as essential for the prevention of osteoporosis [24, 29, 30, 49]. Adjunct therapy with high-dose, daily vitD3 for HIV-infected subjects and for those on/off highly active antiretroviral therapy was recently investigated in a high-risk, adult HIV-infected group and in HIV-infected children [29, 49]. However, these levels have not been supported by adequate dose-finding RTC studies.

Our review highlights important areas to explore for future prevention strategies. Future interferon-free direct-acting agents may have a better effect on bone metabolism and decrease fracture incidence after successful treatment. Although the risk of fracture is clearly higher in HIV/HCV coinfected individuals, it is not clear if DXA screening of these individuals before the age of 50 is a cost-effective prevention method and requires further study.

Lifestyle-related factors appear to have a substantial impact on the risk of fractures in HIV/HCV coinfected individuals but, based upon available studies, this cannot solely be attributed to alcohol and substance use [22, 38].

Recent data indicate that vitamin D supplementation is a relatively inexpensive therapeutic option to reduce liver fibrosis and improve SVR [22, 34]. Interestingly, two potentially modifiable factors, CD4+ nadir and serum 25(OH)D levels, were both independent modulators of liver fibrosis progression and determinants of portal pressure [34].

Currently, practitioners are often concerned about the lack of well-characterized data on the therapeutic value of vitamin D supplementation to reduce liver fibrosis progression in HIV/HCV coinfected patients. Increasing vitamin D intake may positively modulate response to antiviral treatment in HCV-infected or HIV/HCV coinfected patients, and, in association with standardized treatment for chronic liver disease, it could be of benefit in reducing liver fibrosis progression in HIV/HCV coinfected patients.

In general, there is no evidence to suggest that increasing the recommended vitamin D intake for the general population to 20–50 μg (800–2000 IU) would cause any medical problems. However, the authors recommend careful clinical observation and laboratory monitoring when higher doses of vitamin D supplements are administered because of the long half-life of vitamin D accumulation in tissues; excessive intake of vitamin D can cause chronic toxic effects, which present ashypercalcemia and renal damage.

Further controlled randomized trials on the effects of vitamin D supplementation are warranted to assess the relevance of vitamin D for liver fibrosis progression in HIV/HCV coinfected patients.

5. Conclusions

Our review indicates that vitamin D supplementation can be considered a relatively inexpensive therapeutic option to lower HCV-related fracture risk, owing to its benefic effect in reducing liver fibrosis progression in HIV/HCV coinfected patients. Other determinants of HCV-related increased fracture risk have still to be defined.

A good compromise between different opinions could be to start with relatively higher doses of vitamin D in HIV-HCV infected patients, skipping some steps of dose supplementation until more information is available to settle the question.

What Is New?

What Is Known?

(i) Experimental evidence suggested a hepatoprotective role of vitamin D.

(ii) Practitioners are often concerned about the lack of well-characterized data on the therapeutic value of Vitamin D supplementation to reduce liver fibrosis progression in HIV/HCV coinfected patients.

What Is New?

(i) Only particularly low levels of vitamin D should be considered in HIV/HCV coinfected patients.

(ii) This systematic review reveals a recent interest in vitamin D supplementation as a relatively inexpensive therapeutic option to reduce HCV-related increased fracture risk by modifying liver fibrosis progression.

What Does This Mean?

(i) Among HIV-infected patients, the association between 25(OH)D levels and severity of liver disease partly explains the HCV-associated increased risk of osteoporotic fractures, while other determinants have still to be defined.

(ii) Further RCTs are warranted to determine what level of vitamin D insufficiency and deficiency places an individual at risk of cirrhosis evolutions and what is the optimal dosage of vitamin D3 to exert sufficient antifibrosis effects in HIV/HCV coinfected populations.

Conflict of Interests

The authors state that there are no conflict of interests and that they have not received any payment for the preparation of this paper.

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Research Article

Sarco-Osteoporosis: Prevalence and Association with Frailty in Chinese Community-Dwelling Older Adults

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The aim was to apply AWGS criteria to estimate the prevalence of sarco-osteoporosis and investigate its relationship with frailty, in a sample of 316 community-dwelling Chinese older people. Regression analysis was performed using frailty as the dependent variable. The results showed that the prevalence rate of sarco-osteoporosis was 10.4% in older men and 15.1% in older women. ≧80 years old (OR 4.8; 95% CI, 3.05–10.76; $P = 0.027$), women (OR 2.6; 95% CI, 1.18–2.76; $P = 0.036$), and higher level of comorbidity (OR 3.71; 95% CI, 1.61–10.43; $P = 0.021$) were independently associated with the likelihood of being sarco-osteoporosis. In the frail group, sarco-osteoporosis occurred in 26.3% of men, in 38.5% of women, and in lower proportion in the prefrail (13.6% of men; 16.2% of women) and nonfrail group (1.6% of men; 1.9% of women) ($P < 0.05$, resp.). Furthermore, the likelihood of being frail/prefrail was substantially higher in the presence of sarco-osteoporosis (OR 4.16; 95% CI, 2.17–17.65; $P = 0.019$ in men; and OR 4.67; 95% CI, 2.42–18.86; $P = 0.007$ in women). The results indicate that patients with sarco-osteoporosis are more likely to be ≧80 yrs with higher burden of comorbidities and to have frailty/prefrailty, especially for women.

1. Introduction

Population ageing is accelerating rapidly worldwide. Frailty in the elderly is a major public health problem. It is a state of increased vulnerability to poor resolution of homoeostasis after a minor stressor event, which increases the risk of adverse outcomes, including falls, delirium, disability, long-term care, and death [1, 2]. Between a quarter and half of people older than 85 years are estimated to be frail [3]. For older community residents, effective frailty prevention may potentially reduce serious frailty-related injuries. Reducing frailty risk in older individuals is, therefore, an important public health objective. So a clinical need exists to optimally identify those who will develop frailty.

Sarcopenia and osteoporosis are two distinct characteristics seen in older patients and are highly prevalent among elderly patients with frailty [4, 5]. Sarcopenia, the age-related loss of skeletal muscle mass, is characterized by the deterioration of muscle quantity and quality leading to a gradual slowing of movement, a decline in strength and power, increased risk of fall-related injury, and often, frailty [4]. Osteoporosis is a progressive bone disease that is characterized by a decrease in bone mass and density which can lead to an increased risk of fracture. In osteoporosis, the bone mineral density (BMD) is reduced, bone microarchitecture deteriorates, and the amount and variety of proteins in bone are altered. Osteoporosis is a common condition in elders and a powerful risk factor for adverse health outcomes such as fracture [5]. Intuitively, having a low muscle mass and strength with low bone mineral density (BMD) seems likely to lead to more physical functional limitations and frailty. However, we do not know the clinical characteristics of the individuals with both osteoporosis and sarcopenia, so-called "sarco-osteoporosis." Despite sharing common risk factors and biological pathways, the relationship between frailty and sarco-osteoporosis is not clear. More research is needed to better understand sarco-osteoporosis. The objective of our present study was to investigate the prevalence

of sarco-osteoporosis among community-dwelling Chinese elders and the relationship between sarco-osteoporosis and frailty.

2. Material and Methods

2.1. Participants. From August 2012 to August 2014, the patients who conducted comprehensive geriatric assessment (CGA) from community-dwelling Chinese elders (≧65 years) were recruited in Changsha city and its surrounding area of China. The enrollment work was done by a full-time nurse responsible for CGA. Individuals were originally excluded if unable to walk without the assistance of another person, or their renal function and liver function was abnormal, or their heart function classification was of grades III and IV according to New York Heart Association (NYHA) standard. Patients with severe parkinsonism were also excluded who had signs of postural instability. A total of 360 subjects were screened and 316 of them had sufficient data for analysis, and their characteristics are presented in Table 1. The study protocol was approved by the Second Xiangya Hospital of Central South University Ethics Committees in accordance with the Declaration of Helsinki and Good Clinical Practices Guidelines.

2.2. Assessment Methods

2.2.1. Questionnaire about Health Status. Participants completed a questionnaire and were interviewed by a CGA nurse at the examination center and asked about health status, educational achievement, and smoking status. A selected medical history including a history of a physician diagnosis of diabetes mellitus, hypertension, coronary heart disease, dementia, parkinsonism, stroke, cancer, and chronic obstructive lung disease was obtained. Body weight and height measurements were used to calculate a standard body mass index (BMI).

2.2.2. Frailty Status. Participants were classified as frail, prefrail, and nonfrail according to a validated screening tool based on the presence or absence of five measurable characteristics by Fried and colleagues [6]: weakness, low physical activity, slowed walking speed, exhaustion, and weight loss. (1) Weakness was defined as grip strength in the lowest quintile within groups defined by sex and BMI. Participants reported their level of daily leisure physical activity in the past year; (2) low physical activity was defined as either complete inactivity or performing low-intensity activities less than 1 h/wk; (3) slowed walking speed was defined as usual walking speed in the slowest quintile within groups defined by sex and height. Walking speed was measured on a 4 m course using photocell recordings at the course start and finish. The final measure averaged two walks; (4) exhaustion was indicated by a response of "occasionally" or "often/always" to the statement, "I felt that everything was an effort"; (5) weight loss was measured as self-reported unintentional weight loss more than 4.5 kg within the past year. Individuals with three or more of the five components were defined as frail, those with one or two components were defined as prefrail, and those with none were defined as nonfrail.

2.2.3. Sarcopenia. Sarcopenia was defined as proposed by the Asian Working Group for Sarcopenia (AWGS) [7] with cutoff values for muscle mass measurements (7.0 kg/m^2 for men and 5.7 kg/m^2 for women by using bioimpedance analysis), handgrip strength (<26 kg for men and <18 kg for women), and usual gait speed (<0.8 m/s).

2.2.4. Osteoporosis. Bone mineral density (BMD) of lumbar spine and femoral neck of all elderly adults were measured by DXA using QDR 4500A fan beam bone densitometer (Hologic Inc., Bedford, MA, USA), according to the manufacturer's recommended standard analysis procedures for the PA lumbar spine (vertebrae L2–L4) and hip femoral neck. A long-term (exceeding 15 years) coefficient of variation (CV) for the BMD was not greater than 0.40%. With reference to the World Health Organization (WHO) definition [5], the diagnosis of osteoporosis was established when a BMD of 2.5 SD was lower than the peak mean of the same gender ($T \leq -2.5$), and secondary osteoporosis was excluded.

2.2.5. Covariates. Covariates were selected if they were considered to be related to frailty status, low bone mineral density, or sarcopenia. The covariates used were age, education (less than 9 years and more than 9 years), drinking and smoking (current drinking or current smoking or if stopped less than 4 years prior to the interview), supplemental Vitamin D use or supplemental calcium use (less than twice every week and more than twice every week), physical activity (less than 30 minutes per day and more than 30 minutes per day), and the number of chronic diseases including diabetes mellitus, hypertension, coronary heart disease, dementia, parkinsonism, stroke, cancer, and chronic obstructive lung disease (less than 3 and more 3).

2.3. Statistical Analysis. Descriptive statistics were reported as mean ± standard deviation. Data were analyzed including distribution of means and proportions of variables of interest across sex, musculoskeletal diseases, or frailty categories were compared using the student t-test and Chi-square and trend tests. Logistic regression using sarco-osteoporosis as the dependent variable for characteristics of the subjects, or using frailty/prefrailty (or nonfrailty) as the dependent variable for groups of musculoskeletal diseases classification, and adjusted for covariates was performed. "No sarcopenia and no osteoporosis" was set as the reference group. Results were presented as odds ratio (OR) with 95% confidence intervals (CIs). All analyses were performed using the SPSS 19.0 package (SPSS, Chicago, IL). $P < 0.05$ was considered to be statistically significant.

3. Results

This study analyzed 316 elders who had sufficient data for analysis. The percentage of women was 48% ($n = 152$). Characteristics of the subjects by sex are shown in

Table 1: Characteristics of the subjects by sex.

	Men $n = 164$	Women $n = 152$	P value
Age (years)	75.6 ± 4.8	76.9 ± 5.2	Matched
BMI (kg/m^2)	23.1 ± 2.5	23.6 ± 3.4	0.357
AMI (kg/m^2)	7.7 ± 0.6	6.2 ± 0.7	0.031
Body fat (%)	23.3 ± 4.9	29.9 ± 5.7	0.035
Grip strength (kg)	34.9 ± 5.2	23.6 ± 3.8	0.017
Usual walking speed (m/s)	1.56 ± 0.3	1.41 ± 0.3	0.012
BMD (g/cm^2)			
Lumbar spine	0.81 ± 0.07	0.76 ± 0.07	0.037
Femoral neck	0.63 ± 0.06	0.58 ± 0.07	0.013
Education (>9 years)	36 (22.0)	32 (21.1)	0.583
Supplemental Vitamin D use	38 (23.1)	41 (27.0)	0.089
Supplemental calcium use	43 (26.2)	44 (28.9)	0.237
Lifestyle related habits			
Current drinker	25 (15.2)	7 (4.6)	0.027
Current smoker	32 (19.5)	5 (3.3)	0.019
Physically active	37 (22.6)	41 (27.0)	0.174
Chronic medical history			
Diabetes	38 (23.1)	32 (21.1)	0.318
Hypertension	53 (32.3)	49 (32.2)	0.671
Coronary heart disease	31 (18.9)	27 (17.8)	0.484
Dementia	3 (1.8)	3 (2.0)	0.375
Parkinsonism	6 (3.7)	5 (3.3)	0.278
Stroke	4 (2.4)	3 (3.3)	0.069
Cancer	2 (1.2)	2 (1.3)	0.427
Chronic obstructive lung disease	19 (11.6)	12 (7.9)	0.038
Musculoskeletal diseases classification			
Sarcopenia and no osteoporosis	26 (15.9)	28 (18.4)	0.387
Osteoporosis and no sarcopenia	31 (18.9)	47 (30.9)	0.016
Sarco-osteoporosis	17 (10.4)	23 (15.1)	0.024
No sarcopenia and no osteoporosis	90 (54.8)	54 (35.6)	0.018

Notes. Values are mean ± standard deviation (SD) and number (percentage). BMI: body mass index; AMI: appendicular muscle mass index; BMD: bone mineral density.

Table 1. The mean age was 75.6 ± 4.8 years for men and 74.9 ± 5.2 years for women, and the average BMI value was 23.1 kg/m^2 and 23.6 kg/m^2 in men and women, respectively, with no significant difference between the two groups. In the total body bioimpedance analysis, women presented a higher percentage of total fat mass as compared to men. Men had significantly higher values than did women for all BMD measurements, grip strength, and walking speed. The proportions of current drinker or current smoker and chronic obstructive lung disease were higher in men than in women.

However, the percentage of physically active individuals was lower in men than in women. There was no significant difference in education, vitamin D and calcium supplementation, proportions of diabetes, hypertension coronary heart disease, dementia, parkinsonism, stroke, and cancer among the two groups.

Subject characteristics for sarco-osteoporosis are shown in Tables 2 and 3. 26.2% of men ($n = 43$) and 33.6% of women ($n = 51$) were classified as having sarcopenia (having osteoporosis or no osteoporosis); 29.3% ($n = 48$) of men and 46.1% ($n = 70$) of women were classified as having osteoporosis (having sarcopenia or no sarcopenia). Sarco-osteoporosis was prevalent in 10.4% of men ($n = 17$) and 15.1% of women ($n = 23$) among the study population. The mean age of men or women in the sarco-osteoporosis group was significantly higher than the mean age of those in the other groups. The percentage of drinkers, smokers, or parkinsonism was significantly higher in the sarco-osteoporosis group than in the other groups among men.

In a logistic regression model, ≧80 years old or women had an increased likelihood of sarco-osteoporosis compared

Table 2: Subject characteristics of men by musculoskeletal diseases classification.

Men $n = 164$	No sarcopenia and no osteoporosis $n = 90$	Sarcopenia and no osteoporosis $n = 26$	Osteoporosis and no sarcopenia $n = 31$	Sarco-osteoporosis $n = 17$	P value
Age (years)	71.1 ± 3.4	78.6 ± 3.8	74.8 ± 4.3	83.1 ± 2.9	0.012
BMI (kg/m^2)	24.7 ± 2.4	22.6 ± 2.1	24.5 ± 3.3	21.7 ± 2.8	0.273
AMI (kg/m^2)	8.6 ± 1.4	6.7 ± 0.3	7.1 ± 1.1	6.1 ± 0.4	0.013
Body fat (%)	21.6 ± 2.7	28.9 ± 2.2	23.7 ± 2.6	30.7 ± 2.4	0.011
Grip strength (kg)	35.9 ± 5.2	23.9 ± 2.3	30.6 ± 3.1	21.1 ± 2.0	0.001
Walking speed (m/s)	1.61 ± 0.22	0.72 ± 0.13	1.56 ± 0.17	0.63 ± 0.19	0.016
BMD (g/cm^2)					
Lumbar spine	0.84 ± 0.06	0.82 ± 0.06	0.73 ± 0.09	0.72 ± 0.11	0.041
Femoral neck	0.71 ± 0.07	0.69 ± 0.05	0.62 ± 0.08	0.60 ± 0.13	0.028
Education (>9 years)	16 (17.8%)	7 (27.0%)	8 (25.9%)	5 (29.4%)	0.156
Supplemental Vitamin D use	15 (16.7%)	10 (38.5%)	9 (29.0%)	4 (23.6%)	0.231
Supplemental calcium use	17 (18.9%)	12 (46.2%)	10 (32.3%)	4 (23.6%)	0.369
Lifestyle related habits					
Drinker	7 (7.8%)	4 (15.4%)	6 (19.4%)	8 (47.1%)	0.023
Smoker	10 (11.1%)	6 (23.1%)	9 (29.0%)	7 (41.2%)	0.027
Physically active	18 (20.0%)	5 (19.2%)	8 (25.8%)	6 (15.3%)	0.159
Chronic medical history					
Diabetes	13 (14.4%)	9 (34.6%)	10 (32.3%)	6 (35.3%)	0.057
Hypertension	16 (17.8%)	14 (53.9%)	13 (42.0%)	10 (58.8%)	0.063
CHD	14 (15.6%)	6 (23.1%)	8 (25.8%)	3 (17.6%)	0.326
Dementia	0	1 (3.8%)	1 (3.2%)	1 (5.9%)	0.417
Parkinsonism	1 (1.1%)	2 (7.7%)	1 (3.2%)	2 (11.8%)	0.046
Stroke	1 (1.1%)	1 (3.8%)	2 (6.5%)	0	0.132
Cancer	1 (1.1%)	0	1 (3.2%)	0	0.413
COPD	4 (4.4%)	7 (27.0%)	5 (16.1%)	3 (17.6%)	0.247

Notes. Values are mean ± standard deviation (SD) and number (percentage). BMI: body mass index; AMI: appendicular muscle mass index; BMD: bone mineral density; CHD: coronary heart disease; COPD: chronic obstructive lung disease.

to <80 years old or men (OR 4.8; 95% CI, 3.05–10.76; $P = 0.027$; OR: 2.6; 95% CI, 1.18–2.76; $P = 0.036$, resp.). Moreover, higher level of comorbidity (the number of chronic diseases was more than 3) was associated with sarco-osteoporosis (OR 3.71; 95% CI, 1.61–10.43; $P = 0.021$).

Subject characteristics for frailty/prefrailty stratified by sex are shown in Tables 4 and 5. Frailty status was detected in 11.6% ($n = 19$) of the elderly men, prefrailty in 49.4% ($n = 81$), and nonfrailty in 39.0% ($n = 64$) of the elderly men and frailty in 17.1% ($n = 26$) of the elderly women, prefrailty in 48.7% ($n = 74$), and nonfrailty in 34.2% ($n = 52$) of the elderly women. In the frail group, sarco-osteoporosis occurred in 26.3% of men ($n = 5$), 38.5% of women ($n = 10$), and in lower proportion in the prefrail (13.6% of men, $n = 11$; 16.2% of women, $n = 12$) and the nonfrailty groups (1.6% of men, $n = 1$; 1.9% of women, $n = 1$) (Tables 4 and 5). The mean age of men or women in the frailty/prefrailty group was significantly higher than that of those in the nonfrailty group.

The associations between sarco-osteoporosis and frailty/prefrailty in men and women, as assessed by logistic regression analysis, are shown in Tables 6 and 7, respectively. After adjusting for subjects aged 80 years or more, drinking and smoking, education, body mass index and percentage of fat in the whole body scan, chronic medical history, sarcopenia (OR 3.11; 95% CI, 1.65–6.63; $P = 0.018$ in men; OR 3.38; 95% CI, 1.41–7.62; $P = 0.025$ in women), and osteoporosis (OR 2.07; 95% CI, 1.09–13.12; $P = 0.037$ in men; OR 2.41; 95% CI, 1.26–14.15; $P = 0.024$ in women) were independently associated with frailty. Furthermore, the likelihood of being frail was substantially higher in the presence of sarco-osteoporosis (OR 4.16; 95% CI, 2.17–17.65; $P = 0.019$ in men; and OR 4.67; 95% CI, 2.42–18.86; $P = 0.007$ in women.) (Tables 6 and 7).

4. Discussion

This cross-sectional study examined the association between sarco-osteoporosis (the individuals with both sarcopenia and osteoporosis, as diagnosed by AWGS/WHO criteria) and frailty in 316 community-dwelling elderly Chinese men and

Table 3: Subject characteristics of women by musculoskeletal diseases classification.

Women $n = 152$	No sarcopenia and no osteoporosis $n = 54$	Sarcopenia and no osteoporosis $n = 28$	Osteoporosis and no sarcopenia $n = 47$	Sarco-osteoporosis $n = 23$	P value
Age (years)	73.2 ± 2.8	77.2 ± 3.1	73.8 ± 3.7	82.5 ± 2.6	0.017
BMI (kg/m^2)	25.4 ± 2.1	21.1 ± 2.6	23.6 ± 3.8	22.3 ± 3.4	0.338
AMI (kg/m^2)	7.2 ± 0.7	5.2 ± 0.3	7.0 ± 0.4	5.1 ± 1.2	0.025
Body fat (%)	28.1 ± 3.7	30.7 ± 2.1	27.9 ± 3.2	32.9 ± 2.3	0.014
Grip strength (kg)	25.9 ± 2.2	13.9 ± 1.6	22.6 ± 1.3	13.1 ± 0.9	0.015
Walking speed (m/s)	1.64 ± 0.17	0.69 ± 0.12	1.46 ± 0.11	0.62 ± 0.09	0.008
BMD (g/cm^2)					
Lumbar spine	0.79 ± 0.07	0.72 ± 0.05	0.63 ± 0.03	0.62 ± 0.05	0.021
Femoral neck	0.67 ± 0.04	0.65 ± 0.03	0.59 ± 0.08	0.60 ± 0.05	0.016
Education (>9 years)	13 (24.1%)	6 (21.4%)	10 (21.3%)	3 (13.0%)	0.156
Supplemental Vitamin D use	16 (29.6%)	14 (50.0%)	7 (14.9%)	4 (17.4%)	0.231
Supplemental calcium use	17 (31.5%)	13 (46.4%)	10 (21.3%)	4 (17.4%)	0.369
Lifestyle related habits					
Drinker	2 (3.7%)	2 (7.1%)	2 (4.3%)	1 (4.3%)	0.023
Smoker	2 (3.7%)	1 (3.6%)	2 (4.3%)	0	0.027
Physically active	17 (31.5%)	8 (28.6%)	10 (21.3%)	6 (26.1%)	0.159
Chronic medical history					
Diabetes	10 (18.5%)	7 (25.0%)	9 (19.1%)	6 (26.1%)	0.137
Hypertension	19 (35.2%)	7 (25.0%)	17 (36.2%)	6 (26.1%)	0.308
CHD	10 (18.5%)	6 (21.4%)	8 (17.0%)	3 (13.0%)	0.289
Dementia	1 (1.9%)	1 (3.6%)	0	1 (4.3%)	0.165
Parkinsonism	2 (3.7%)	1 (3.6%)	1 (2.1%)	1 (4.3%)	0.148
Stroke	1 (1.9%)	1 (3.6%)	0	1 (4.3%)	0.189
Cancer	1 (1.9%)	1 (3.6%)	0	0	0.275
COPD	5 (9.3%)	3 (10.7%)	3 (6.4%)	1 (4.3%)	0.158

Notes. Values are mean ± standard deviation (SD) and number (percentage). BMI: body mass index; AMI: appendicular muscle mass index; BMD: bone mineral density; CHD: coronary heart disease; COPD: chronic obstructive lung disease.

Table 4: Characteristics of men by presence of frailty/prefrailty.

Men $n = 164$	Frailty $n = 19$	Prefrailty $n = 81$	Nonfrailty $n = 64$	P value
Age (years)	83.5 ± 2.9	76.1 ± 3.5	72.1 ± 3.3	0.017
AMI (kg/m^2)	5.4 ± 1.3	6.8 ± 1.6	7.9 ± 1.4	0.014
Body fat (%)	28.6 ± 2.2	27.3 ± 2.0	24.1 ± 2.1	0.036
Grip strength (kg)	22.6 ± 1.9	28.1 ± 2.6	33.6 ± 2.0	0.009
Usual walking speed (m/s)	0.64 ± 0.1	0.83 ± 0.3	1.52 ± 0.4	0.003
BMD (g/cm^2)				
Lumbar spine	0.75 ± 0.09	0.79 ± 0.11	0.82 ± 0.8	0.032
Femoral neck	0.63 ± 0.10	0.67 ± 0.13	0.72 ± 0.11	0.037
Musculoskeletal diseases classification				
Sarcopenia and no osteoporosis ($n = 26$)	4 (21.1%)	20 (24.7%)	2 (3.1%)	0.004
Osteoporosis and no sarcopenia ($n = 31$)	5 (26.3%)	22 (27.2%)	4 (6.3%)	0.015
Sarco-osteoporosis ($n = 17$)	5 (26.3%)	11 (13.6%)	1 (1.6%)	0.003
No sarcopenia and no osteoporosis ($n = 90$)	5 (26.3%)	28 (34.6%)	57 (89.1%)	0.012

Notes. Values are mean ± standard deviation (SD) and number (percentage). AMI: appendicular muscle mass index; BMD: bone mineral density.

Table 5: Characteristics of women by presence of frailty/prefrailty.

Women $n = 152$	Frailty $n = 26$	Prefrailty $n = 74$	Nonfrailty $n = 52$	P value
Age (years)	84.7 ± 2.6	77.1 ± 3.1	74.1 ± 3.9	0.023
AMI (kg/m^2)	5.0 ± 1.6	6.3 ± 1.9	7.2 ± 1.8	0.019
Body fat (%)	33.5 ± 2.7	28.6 ± 2.9	27.4 ± 2.5	0.026
Grip strength (kg)	13.6 ± 1.9	17.1 ± 2.6	24.6 ± 2.0	0.006
Usual walking speed (m/s)	0.65 ± 0.13	0.86 ± 0.09	1.62 ± 0.19	0.003
BMD (g/cm^2)				
Lumbar spine	0.64 ± 0.11	0.69 ± 0.16	0.72 ± 0.07	0.038
Femoral neck	0.61 ± 0.16	0.65 ± 0.08	0.71 ± 0.14	0.031
Musculoskeletal diseases classification				
Sarcopenia and no osteoporosis ($n = 28$)	7 (26.9%)	19 (25.7%)	2 (3.8%)	0.007
Osteoporosis and no sarcopenia ($n = 47$)	8 (30.8%)	26 (35.1%)	13 (25.0%)	0.028
Sarco-osteoporosis ($n = 23$)	10 (38.5%)	12 (16.2%)	1 (1.9%)	0.004
No sarcopenia and no osteoporosis ($n = 54$)	1 (3.8%)	17 (23.0%)	36 (69.2%)	0.013

Notes. Values are mean ± standard deviation (SD) and number (percentage). AMI: appendicular muscle mass index; BMD: bone mineral density.

Table 6: Association between sarco-osteoporosis, sarcopenia, or osteoporosis and frailty/prefrailty in men.

Men	Frailty or prefrailty/nonfrailty		
	Odds ratio	95% Cl	P value
No sarcopenia and no osteoporosis	1		
Sarcopenia	3.11	1.65–6.63	0.018
Osteoporosis	2.07	1.09–13.12	0.037
Sarco-osteoporosis	4.16	2.17–17.65	0.019

Table 7: Association between sarco-osteoporosis, sarcopenia, or osteoporosis and frailty/prefrailty in women.

Women	Frailty or prefrailty/nonfrailty		
	Odds ratio	95% Cl	P value
No sarcopenia and no osteoporosis	1		
Sarcopenia	3.38	1.41–7.62	0.025
Osteoporosis	2.41	1.26–14.15	0.024
Sarco-osteoporosis	4.67	2.42–18.86	0.007

women. We found that, in the frail group, sarco-osteoporosis occurred in 26.3% of men and 38.5% of women, but in lower proportion in the prefrail group (13.6% of men and 16.2% of women) or in the nonfrailty group (1.6% of men and 1.9% of women). In other words, the percentages of sarco-osteoporosis were higher in the frailty/prefrailty groups than in the nonfrailty group in both men and women. Furthermore, the likelihood of being frail/prefrail was substantially higher in the presence of sarco-osteoporosis (OR 4.16; 95% CI, 2.17–17.65; $P = 0.019$ in men; and OR 4.67; 95% CI, 2.42–18.86; $P = 0.007$ in women) than in the presence of sarcopenia or osteoporosis alone (OR 3.11; 95% CI, 1.65–6.63; OR 2.07, 95% CI, 1.09–13.12 in men and OR 3.38; 95% CI, 1.41–7.62; $P = 0.025$; OR 2.41; 95% CI, 1.26–14.15; $P = 0.024$ in women, resp.) compared with neither sarcopenia nor osteoporosis.

Many elderly people have multiorgan problems. Frailty is a practical, unifying notion in the care of elderly adults that directs attention away from single-system illness towards a more holistic viewpoint of the patient. Reduction of the occurrence or severity of frailty is likely to have large benefits for individuals, their families, and society. Some clinical trials have confirmed that complex interventions including exercise, nutrient supplement, and pharmacological agents can increase the likelihood of continuing to live at home, mainly through a reduced need for care-home admission and fewer falls [8, 9]. To identify the subjects with high risk factors for frailty should be an essential part of further complex intervention. The immune system, skeletal muscle, brain, and endocrine system are intrinsically interrelated and are the organ systems that are best studied in the development of frailty [6]. Notably, frailty has also been associated with loss of physiological reserve in the cardiovascular [10], renal [11], respiratory [12], haemopoietic, and clotting systems [13, 14], and nutritional status can also be a mediating factor [1, 15–17]. The results from our study implied that sarcopenia and osteoporosis were predictors of frailty. Importantly, the results suggested that the joint predictive value of sarcopenia and osteoporosis was stronger than that of sarcopenia or osteoporosis alone. This finding supports the idea that, when physiological decline reaches an aggregate crucial level, frailty becomes evident [18].

The results from the Women's Health and Aging Study (WHAS) II showed almost sixteen percent had sarcopenia concomitant to severe osteopenia/osteoporosis in community-dwelling older women according to Baumgartner/WHO criteria [19]. Our present study for the first time showed the epidemiology of sarco-osteoporosis and its associative clinical characteristics in community-dwelling elderly Chinese men and women. Sarco-osteoporosis was prevalent in 10.4%

of men and 15.1% of women among the study population. Sarco-osteoporosis prevalence was lower than that of isolated sarcopenia (15.9% of men and 18.4% of women) or isolated osteoporosis alone (18.9% of men and 30.9% of women). We also found that ≧80 years old or women had an increased likelihood of sarco-osteoporosis compared to <80 years old or men (OR 4.8, 95% CI: 3.05–10.76, $P = 0.027$; OR 2.6, 95% CI: 1.18–2.76, $P = 0.036$, resp.). Moreover, higher level of comorbidity (the number of chronic diseases was more than 3) was associated with sarco-osteoporosis (OR 3.71, 95% CI: 1.61–10.43, $P = 0.021$).

Muscle/bone relationships have recently been noted as a new research field related to the interactions among several organ systems. Muscle/bone relationships include two factors: local control of muscle to bone and systemic humoral interactions between muscle and bone. Genetic, endocrine, and mechanical factors affect both muscle and bone simultaneously. Further progress in understanding the common genetic etiology of osteoporosis and sarcopenia will provide valuable insight into important biological underpinnings for both conditions and may translate into new approaches to reduce the burdens of both conditions through improved diagnosis, prevention, and early targeted treatment. [20]. Osteoporosis and sarcopenia may be affected by genetic polymorphisms of several genes, such as androgen receptor, estrogen receptor, catechol-O-methyltransferase, IGF-I, Vitamin D receptor, and low-density-lipoprotein receptor-related protein 5 [20]. Vitamin D [21], the growth hormone/insulin-like growth factor *I* axis [22], and testosterone [23] are physiologically and pathologically important as endocrine factors. Mechanical stress changes, such as immobilization and lack of gravity, greatly influence both muscle and bone [24]. These findings suggest the presence of interactions between muscle and bone, which might be very important for understanding the physiology and pathophysiology of sarco-osteoporosis. The loss of muscle strength and mass during the aging process causes structural changes in the microarchitecture of the bones and decreases mineral density, resulting in bone quality decline. These factors of skeletal muscle and bone activate a vicious cycle leading to accelerated frailty and ultimately to physical disability.

There are shared factors between sarcopenia and frailty such as slow walking speed and grip strength. If sarcopenia is integrated into the diagnosis of frailty, it would be positively identified and graded for severity. And it would help essential research to gain a deeper insight into the complex mechanisms of frailty and aid the development and evaluation of interventions to improve outcomes.

This study had several advantages over previous studies. First, the subjects were recruited from a community-based elderly population, represented a single Chinese older adults. Second, previous studies used EWGS criteria for the definition of sarcopenia to obtain a sufficient number of subjects within the group for statistical analysis [25]. In contrast, we used the criterion of AWGS for defining sarcopenia, which is known to be more suitable for Chinese [7]. But the present study has three limitations. First, the sample size of the subgroups in the analysis is relatively small and provides limited statistical power, and further investigation of the joint effects of sarcopenia and osteoporosis on frailty is needed. Second, the individuals were originally excluded if unable to walk without the assistance of another person, or their renal function and liver function is abnormal, or their heart function classification is of grades III and IV according to NYHA standard; this may have biased our results towards an underestimation of the risk of frailty associated with sarcoosteopenia. Third, DXA scan presents some limitations in BMD evaluation in elderly people, like aortic calcifications and spine osteoarthritis that may produce an increase, up to 10%, in BMD of the lumbar spine [26], and this could underestimate the real prevalence of sarco-osteoporosis in this population and, consequently, the association with frailty status. Therefore, findings from this study should be interpreted in the context of the complexity of skeletal muscle and bone as well as multifactorial nature of the frailty syndrome. Despite these limitations, our findings are helpful for us to better understand sarco-osteoporosis and provide a basis for making an optimal prediction about frailty among community-dwelling Chinese older people.

5. Conclusion

In conclusion, sarco-osteoporosis defined by AWGS/WHO criteria is present in 10.4% of men and 15.1% of women aged over 65 years, and its prevalence rate is higher in community-dwelling Chinese people aged 80 and over. The joint effect of sarcopenia and osteoporosis may be tightly linked to the risk of frailty. Assessment of both bone and muscle mass/function in older adults could potentially enhance frailty risk prediction. Further prospective study is needed to clarify the roles of sarco-osteoporosis in the occurrence of frailty and frailty-related health outcomes. Although no causal attribution is possible in this analysis, sarco-osteoporosis may explain some of the increases in frailty risk currently related to "age." Therefore, it is appropriate to consider sarcopenia together with osteoporosis in the elderly population.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

Authors' Contribution

Yan-Jiao Wang and Yi Wang contributed equally to this work presented here and should therefore be regarded as equivalent authors.

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